Oligomeric Forms of the Membrane-bound Acetylcholine Receptor Disclosed upon Extraction of the M_r 43,000 Nonreceptor Peptide

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ABSTRACT Oligomeric forms of the acetylcholine receptor are directly visualized by electron microscopy in receptor-rich membranes from *Torpedo marmorata*. The receptor structures are quantitatively correlated with the molecular species so far identified only after detergent solubilization, and further related to the polypeptide composition of the membranes and changes thereof. The structural identification is made possible by the increased fragility of the membranes after extraction of nonreceptor peptides and their subsequent disruption upon drying onto hydrophilic carbon supports.

Receptor particles in native membranes depleted of nonreceptor peptides appear as single units of 7–8 nm, and double and multiple aggregates thereof. Particle doublets having a main-axis diameter of 19 ± 3 nm predominate in these membranes. Linear aggregates of particles similar to those observed in rotary replicas of quick-frozen fresh electrocytes (Heuser, J. E. and S. R. Salpeter. 1979, *J. Cell Biol. 82:* 150–173) are also present in the alkaline-extracted membranes. Chemical modifications of the thiol groups shift the distribution of structural species. Dithiothreitol reduction, which renders almost exclusively the 9S, monomeric receptor form, results in the observation of the 7–8 nm particle in isolated form. The proportion of doublets increases in membranes alkylated with *N*-ethylmaleimide. Treatment with 5,5′-dithio-*bis*-(nitrobenzoic acid) increases the proportion of higher oligomeric species, and particle aggregates (n = oligo) predominate.

The nonreceptor ν-peptide (doublet of M_r 43,000) appears to play a role in the receptor monomer-polymer equilibria. Receptor protein and ν-peptide co-aggregate upon reduction and reoxidation of native membranes. In membranes protected *ab initio* with *N*-ethylmaleimide, only the receptor appears to self-aggregate. The ν-peptide cannot be extracted from these alkylated membranes, though it is easily released from normal, subsequently alkylated or reduced membranes. A stabilization of the dimeric species by the nonreceptor ν-peptide is suggested by these experiments.

Monospecific antibodies against the ν-peptide are used in conjunction with rhodamine-labeled anti-antibodies in an indirect immunofluorescence assay to map the vectorial exposure of the ν-peptide. When intact membranes, ν-peptide depleted and "holey" native membranes (treated with 0.3% saponin) are compared, maximal labeling is obtained with the latter type of membranes, suggesting a predominantly cytoplasmic exposure of the antigenic determinants of the ν-peptide in the membrane.

The influence of the ν-peptide in the thiol-dependent interconversions of the receptor protein and the putative topography of the peptide are analyzed in the light of the present results.

Membrane fractions enriched in the acetylcholine receptor can now be obtained with a high degree of purity from the electric organs of *Torpedinidae* and other convenient sources of this protein. They contain few protein components aside from the one carrying the recognition site for acetylcholine and α-neurotoxins (14) and presumably the molecular machinery for the

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gating of agonist-dependent ionic channels (9, 20, 25). Among the nonreceptor proteins, a polypeptide component having an apparent molecular weight of ~43,000 (the 43,000 mol wt protein of references (30, 31), the ν-peptide of reference 12, or the ν-doublet of reference 4 has received considerable attention. Interest in this peptide originated after the publication of Sobel et al. (30) reporting a membrane preparation consisting exclusively of one receptor subunit, the M_r 40,000 (the α subunit), and the ν-peptide. The latter was further said to possess the recognition site for local anesthetics and, by inference, to constitute the ionic channel (30). It was subsequently shown in various laboratories that elimination of the ν-peptide altered neither the local anesthetic binding nor the ion flux properties of the receptor membranes (9, 20, 25). The reduction of the receptor polypeptide composition to only the α-subunit has also been shown to result from proteolytic degradation of the other peptide chains, the pentameric nature of the receptor protein now being more firmly established (see e.g. references 9, 16, and 20–22).

The structural counterpart of the acetylcholine receptor protein has been identified by electron microscopy with a 7- to 8-nm rosette-shaped particle (reviewed in references 2 and 16). This unit is integrated normally in very densely packed assemblies having densities of >10,000 particles per square micrometer (5, 27, 37). Extraction of the ν-peptide results in alterations of the receptor packing habit (4). These observations were related to an increased freedom of motion of the receptor molecules (4), and it was proposed that the ν-peptide plays a role in processes like synapse formation during ontogenesis, receptor clustering, and stabilization of the adult synapse. Enhanced rotational freedom of the receptor in membranes depleted from the ν-peptide has been found by using phosphorescence spectroscopy (23) and electron spin resonance (28).

Most recently Cartaud et al. (6) have described single and double particles compatible with the masses of receptor monomer and dimer respectively, after detergent solubilization and reincorporation of the protein into artificial lipid vesicles. In the present work it is shown that direct observation of the oligomeric organization of acetylcholine receptor particles can be undertaken without resorting to detergent solubilization of the membranes. This is due to the increased fragility of the receptor-rich membranes upon extraction of the ν-peptide. The structural counterparts of the native oligomeric receptor states and those observed after chemical modification of the thiol groups are identified by electron microscopy and subjected to statistical analysis. The structures are then further correlated with the molecular species made apparent by equilibrium gradient centrifugation and interpreted in the light of corresponding changes in polypeptide patterns observed in SDS-gel electrophoresis. The monomer:dimer relationship obtained here in native membrane remnants is in agreement with that reported by Cartaud et al. (6) in reconstituted systems at high lipid-to-protein ratios. The latter authors neglected the higher oligomeric forms present in their preparations (6), which are shown here to constitute a significant proportion of the receptor species. The association of the acetylcholine receptor protein with the ν-peptide, the putative topography of the peptide with respect to the receptor and the membrane, and the influence of the ν-peptide on the monomer-polymer equilibrium of the receptor are further analyzed.

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), 5,5′-dithio-*bis*-(nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), aprotinin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., Munich, W. Germany. Sodium cholate and Triton X-100 were from Serva, Heidelberg, W. Germany. Tritium-labeled bungarotoxin (sp act 48 Ci/mmol) was purchased from Amersham Buchler, Braunschweig, W. Germany. [^{125}I]-protein A (sp act 60–100 μCi/μg) was from New England Nuclear, Boston, Mass. Rhodamine-labeled antirabbit IgG was purchased from Miles Laboratories, Munich, W. Germany. Electrophoretically pure molecular weight standards were from LKB Products, Bromma, Sweden. All other reagents were analytically pure and obtained from Merk, Darmstadt, W. Germany.

Methods

Torpedo marmorata young specimens of <40 cm in length were obtained from the Marine Biological Station at Arcachon, France, and kept in an aquarium for at least 2 d before killing by pithing. The preparation of the membrane fractions rich in acetylcholine receptor is to be reported elsewhere (Barrantes, manuscript in preparation). Of the various media tested, use is made in the present work of the one having the following composition: 0.4 M NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulphonylfluoride (PMSF) and 10 mM sodium phosphate buffer, pH 7.4 (reference 13). Thiol reagents like NEM at a final concentration of 10 mM, or aprotinin (5 U/ml) were also prepared in the same medium for fractionation.

Protein determinations were made by the method of Lowry et al. (24) using BSA as standard. Toxin binding activity was determined as in (3) using [^{3}H]α-bungarotoxin and the DEAE-cellulose filter assay. The sidedness of the membrane vesicles was determined as described by Hartig and Raftery (13).

Extraction of Peripheral Proteins from Receptor Membranes

The procedures used followed the alkaline stripping introduced by Neubig et al. (25), in which concentrated stock suspensions of the receptor membranes (2–5 mg protein/ml) were diluted in ice-cold water (2°C), brought to pH 11 by dropwise addition of 1 N NaOH and kept under gentle stirring in a Reactivial (Pierce Chemical Co., Rockford, Ill.) for 1 h. The membranes were then diluted fortyfold in water and centrifuged at 2°C for 60 min in a Sorvall SS34 rotor (DuPont Instruments, Newtown, Conn.) at 19,500 rpm. The resulting soft pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.4. Resuspension in double distilled water and repetition of the alkaline extraction constituted the "double alkaline extraction".

Thiol Group Modification of the Purified Membranes

Stock solutions of DTT (0.2 M), DTNB (0.1 M), or NEM (0.2 M) were freshly prepared before use. Conditions for reduction, alkylation, and reoxidation of the membranes have been reported (3). Normally, reagent concentrations were chosen that were found to accomplish saturation of the corresponding reaction and to lead to identified functional states of the receptor (3). Various combinations of these reactions were performed on the membrane-bound and the detergent-solubilized receptor, as detailed in the text and figure legends.

Gradient Centrifugation Experiments

Standard membranes or those obtained in NEM-containing medium, with or without additional thiol treatments, were sedimented in a Beckman Airfuge (Beckman Instruments, Fullerton, Calif.) at 100,000 rpm (27 psi) for 5 min and resuspended in 100 mM NaCl, 1 mM $CaCl_2$, 5 mM Na Pi buffer, pH 7.1 containing 10 mM NEM and 1% Triton or 1% Na cholate. Detergent solubilization was allowed to proceed for 1 h at 20 C. [^{3}H]α-bungarotoxin was then added to give final concentrations of about 1:1 toxin:receptor sites and the labeling was carried out overnight at 4°C. Aliquots of 100 μl of the samples were layered atop of 5.2 ml of a 5–20% sucrose gradient prepared in the above buffer but with a detergent concentration of 0.2%. Centrifugation was carried out in a Beckman Instruments SW 50.1 rotor for 5 h at 50,000 rpm (2°C). Samples of 120–150 μl were collected, applied to Whatman GF/C glass filters (Whatman, Inc., Clifton, N. J.), dried, and counted in POPOP-toluene mixtures with efficiencies of 35–40%.

Polyacrylamide Gel Electrophoresis

SDS/10% polyacrylamide running gels with 5% acrylamide stacking overlay were prepared according to Laemmli (19) with the modifications indicated in the figure legends. Molecular weight standards included gelatine monomer and polymers (M_r 12,000–290,000), catalase, aldolase, BSA, ovalbumin, chymotrypsin, purified *Torpedo* actin (gift from Dr. K. Zechel), and purified acetylcholine receptor protein.

Preparation of Monospecific Antibodies Against the ν-peptide

Preparative SDS polyacrylamide gels of samples like those shown in Fig. 4 *e* were used to purify the ν-peptide. A horizontal strip containing the peptide was excised from the 3-mm gel after brief immersion in 0.25 M KCl for identification (11). A vertical gel strip was also stained with Coomassie Blue as a check. The peptide was eluted from the gel in 0.1% SDS as in reference 11, precipitated with acetone at −20°C and lyophilized.

Antisera against the ν-peptide were raised in rabbits by subcutaneous injections, at biweekly intervals, of the antigen emulsions made by suspending the lyophilized material in 0.5 ml of 0.9% NaCl and emulsified in 0.5 ml of Freund's complete adjuvant (first injection) or incomplete adjuvant (all subsequent three injections). Usually 300 μg protein were used per injection into the back of Grey Giant rabbits. After four boosts blood was collected by bleeding the animals through the carotid artery. The immune sera were subjected to affinity chromatography using the purified antigen covalently coupled to CNBr-activated Sepharose 4B following the technique of Weber et al. (34).

Testing the Specificity of the Purified Monospecific IgGs

Specificity testing of purified monospecific IgGs was done by the immune blotting technique using slight modification to the technique reported by Towlin et al. (33). Unstained, analytical SDS-polyacrylamide gels were sandwiched between two sheets of nitrocellulose (Schleicher & Schuell) wetted with 150 mM Tris/glycine buffer, pH 8.6, and kept overnight in a chamber saturated with the same buffer. The nitrocellulose films were subsequently washed in 1% BSA in 10 mM Tris buffer, pH 7.4, containing 0.9% NaCl for 24 h. Three additional washes with the same buffer for 20 min each followed. The nitrocellulose sheets were then sealed in polyethylene bags together with the purified IgG against the ν-peptide at a concentration of 10–20 μg/ml dissolved in Tris/NaCl buffer containing 2% BSA. After 1-h shaking at room temperature the nitrocellulose films were removed and washed three times with Tris/NaCl. They were then incubated with ^{125}I-labeled protein A (5 μg, sp act 70–100 μCi/μg, New England Nuclear) for 1 h in a rotary device. The sheets were then washed three times for 10 min each in Tris/NaCl buffer containing 0.05% Triton X-100. After drying at 60°C the sheets were submitted to autoradiography using Kodak R x-ray film. Exposure was for 6–60 h at −70°C.

Fluorescence Experiments

Normal membranes, those depleted of the ν-peptide as above, and normal membranes treated with 0.1% saponin (Sigma Chemical Co.) for 30 min as reported by Cohen et al. (8) were incubated with 10 μg/ml purified IgGs in 50 mM Tris/HCl buffer, pH 7.4 for 30–60 min, washed twice by centrifugation in an Airfuge (10 min, 27 psi) and incubated with rhodamine-labeled antirabbit IgG (10 μg/ml) for 1 h. The membranes were washed twice by centrifugation as above and finally resuspended in Tris buffer as above. Aliquots were taken into 0.5 × 0.5 cm quartz cuvettes. Fluorescence measurements were done as previously reported (1) using excitation at 540 nm and collecting the fluorescence emitted between 590 and 620 nm.

Electron Microscopy

Membrane samples at a concentration of 50–100 μg protein/ml were deposited on a Teflon surface (10–20 μl). Copper grids reinforced with freshly prepared carbon films (2–4 mm thick) were floated on the drops for 1–2 min. The grids were then passed through a series of double-distilled water drops and finally stained with 1% uranyl acetate for 30 s. Specimens were examined with a Phillips 300 electron microscope at an acceleration potential of 80 kV and micrographs were taken at instrumental magnifications of 59,000–103,000 times.

Data Processing

Measurement of particle size, surface density, interparticle distance and subsequent statistical treatment of the data were carried out in a PDP 11/40 minicomputer after digitizing the tenfold magnified electron micrographs by means of a Leitz A. S. M. instrument.

RESULTS

Polymorphism of Receptor Oligomeric States

One of the salient features of the acetylcholine receptor protein, whose exact physiological significance is still to be assessed, is its extremely high packing density in the adult synaptic region (2, 14, 16). This property is normally preserved in the receptor-rich membrane fragments isolated from electric organs (5, 9, 17, 27, 31, 37). The particles attributed to the receptor protein occur in such membranes at a mean density of 10,000 ± 1500/μm^2, the mean nearest-neighbor interparticle distance being 8.5 nm. This characteristic suffices to explain why it has not been possible as yet to determine the oligomeric organization of the receptor in its native membrane-bound form by direct observation.

Treatment with pH 11 for 1 h at 2°C (9, 25) releases loosely associated peptides from the membrane. The integrity of the vesicular structure of the receptor membranes after alkaline extraction can be inferred from the maintenance of the ion flux properties (9, 20, 25). Alkaline-extracted membranes are, however, more fragile than the corresponding controls. Similar observations have been made by Sobel et al. (31). In the present work the inherent fragility of the alkali-stripped membranes is turned to advantage: (*a*) the fragility results in the disruption of a large proportion (~70%) of the vesicles upon drying onto hydrophilic carbon supports, (*b*) the partially disrupted vesicles exhibit holey areas where only one membrane layer is observed, and the "released" material (presumably originating from such areas) lies in the immediate vicinity of the vesicles (Fig. 1). It is then possible to directly observe the receptor arrangement within and outside the membrane remnants, without resorting to solubilization by detergents. (*c*) The increased particle dispersion facilitates the statistical processing of particle arrangement.

The double-alkaline extraction at 2°C accomplishes a more complete depletion of the ν-peptide (data not shown). Morphologically, the membrane remnants consist almost exclusively of single-layer sheets (Fig. 1 *b*). A tendency to linear arrangement of particles is observed in some areas (Fig. 1 *b*, arrows), resembling the particle packing reported by Heuser and Salpeter (15) for rotary replicas of quick-frozen fresh electrocytes. Within the boundaries of the vesicle remnants, a slight increase in interparticle distances can be appreciated, together with the diminution of the particle density. Statistical treatment of the interparticle distance data obtained from a large number of measured particles in such vesicle areas shows a peak at 8.7 ± 1 nm (mean 10.2 nm, n = 230) that declines sharply towards larger interparticle distances as seen with native membranes (4). This stands out from the flatter, wider nearest-neighbor spacings averaged over total surface in alkali-stripped membranes (8–18 nm, reference 4). The interpretation of this peak becomes apparent when the particle aggregational forms observed in the immediate vicinity of the membrane remnants are subjected to statistical processing. Released particles occur as single units, and aggregates of two, four, or more particles (Fig. 1).

The distribution profile of particle mean diameters is shown in Fig. 2 *a* together with those obtained after modification of the thiol groups in the receptor membranes. The peak inter particle distance within vesicle remnants reported above increases with the relative increase of a component, measured outside the membrane remnants, having the same mean interparticle distance and a particle size of 19 ± 3 nm (mean largest diameter). This latter component is the predominant one (50 ± 20%) in normal membranes, augmenting to >75% in NEM-membranes (those prepared throughout in *N*-ethylmaleimide) (Fig. 2 *d*).

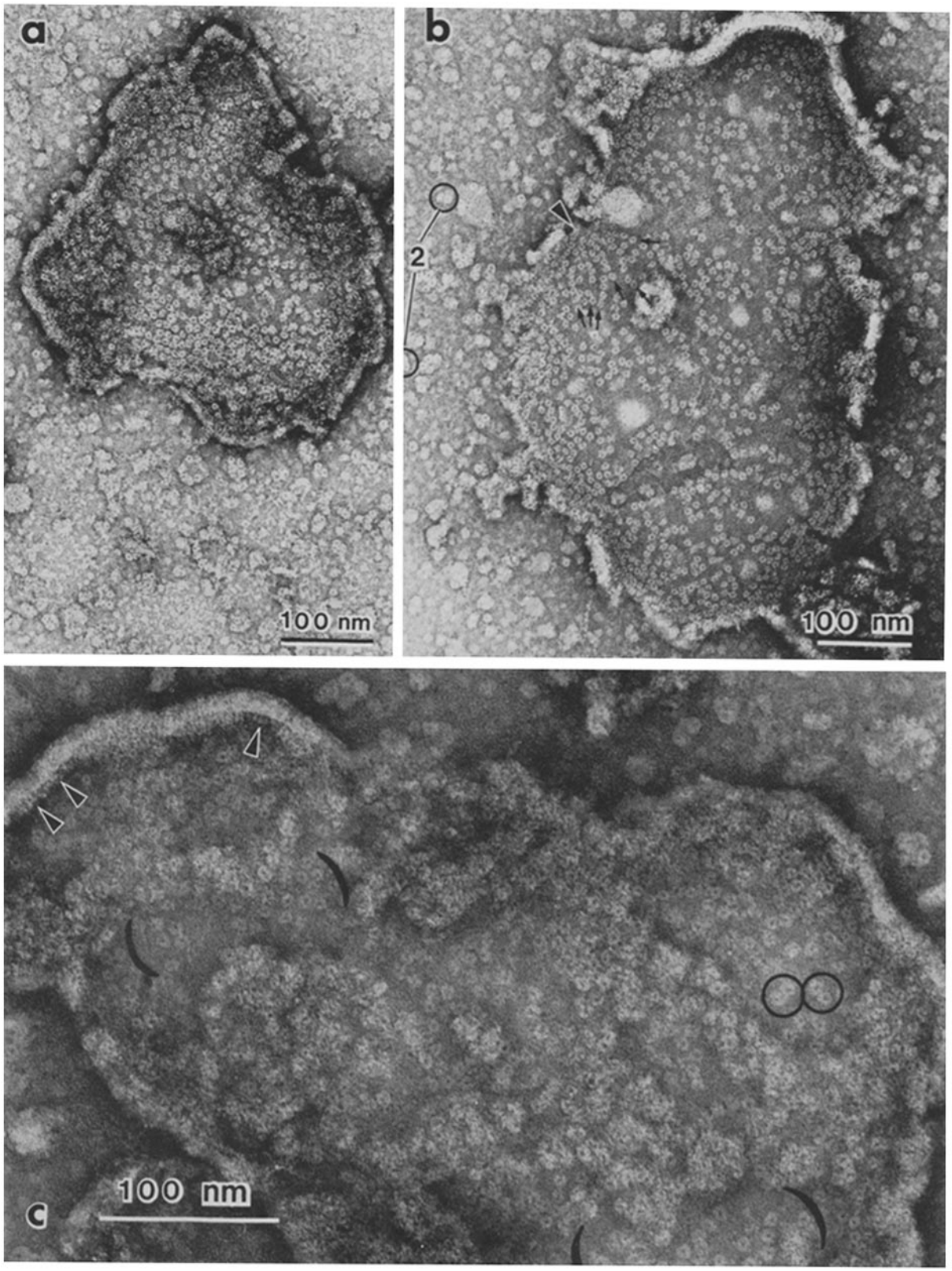

FIGURE 1 Acetylcholine receptor-rich membranes used for the identification of the receptor oligomeric species. The morphology of the membranes after 1 h treatment with 1 N NaOH at 2°C is shown in (*a*). (*b*) Double alkaline stripping (1 h × 2, pH 10.85, 2°C). Notice diminished particle density. The thin arrows point to rows of AChR particles, converging towards the membrane edge at the point indicated by the arrowhead. The circled areas depict particle doublets. Protein concentration: 200 μg/ml. × 150,000. In *c*, at higher magnification the array of aligned acetylcholine receptor protrusions (compare with Fig. 4 of reference 17) can be appreciated. The notable feature of these protrusions is that they are lying towards the inner, "cytoplasmic" side of the vesicle. Toxin binding experiments established that >95% of the membranes were originally (i.e. before processing for electron microscopy) sealed and oriented right side-out. Notice the presence of isolated particles within and outside the membrane boundaries and the predominance of paired circular profiles (*circles*). Negative contrast: 1% uranyl acetate. × 294,000.

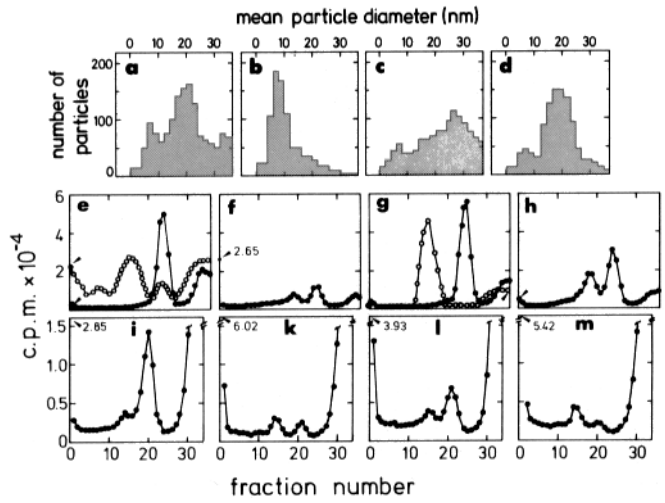

FIGURE 2 Correlation between particle distribution observed in the immediate vicinity of the alkaline-treated membranes and receptor molecular forms after detergent solubilization. *a*) Control membranes. *b*) Membranes reduced with 5 mM DTT before alkali stripping. *c*) Membranes treated with 200 μM DTNB for 30 min before alkaline treatment. *d*) NEM-membranes.

Sucrose gradient centrifugation of [^{3}H]α-bungarotoxin labeled receptor after solubilization in 1% Triton X-100 (*e–h*) or 1% sodium cholate (*i–m*) for 1 h before or after thiol modifications as indicated below. All samples except (*e*) were finally alkylated with 10 mM NEM before centrifugation. *e*) Alkali-treated native (O- - -O) or DTT-reduced (●- - -●) membranes. *f*) DTNB on native membranes followed by detergent solubilization. *g*) DTT on native membranes, detergent solubilization, DTNB, and final NEM alkylation (●) or NEM membranes (O) *h*) DTNB treatment after detergent solubilization. *i*) NEM alkylation, DTT reduction, solubilization in cholate, DTNB reoxidation. *k*) same as (*f*), but in cholate. *l*) same as (*g*), but in cholate. *m*) NEM alkylation, detergent solubilization and DTNB reoxidation. All reactions were performed at 20°C for 30 min with intermediate washings (10 min, 105,000 *g* in the Airfuge) at final reagent concentrations of 10 mM DTT, 1 mM DTNB and 10 mM NEM. Labeling with the radioactive toxin was carried out overnight at 4°C using a 1:1 ratio (per mole) of receptor sites:toxin. Numbers in *f–m* refer to the radioactivity found in the pelleted material (arrowheads).

To verify whether the statistics of particle distribution bear any relation to the reported receptor oligomeric states (7, 12, 16), gradient centrifugation experiments were carried out on the material solubilized with two different detergents from the membranes studied by electron microscopy. As shown in Fig. 2 *e*, alkaline-extracted membranes subjected to detergent solubilization after blockage of free sulphydryl groups by *N*-ethylmaleimide (thus avoiding artificial cross-linkage via disulphide bond formation) yield sedimentation profiles consisting of ~50% dimer, 15% monomer, and ~35% higher oligomeric forms. The correspondence with the statistics of the particle distribution (Fig. 2 *a*) is apparent. Membranes prepared throughout in NEM are almost exclusively made up of the 13S, dimeric receptor form (Fig. 2 *g*), also in agreement with the observed size distribution (Fig. 2 *d*).

Changes in Receptor Oligomeric State by Thiol Modification of Native Membranes

Dithiothreitol reduction (Fig. 2 *a*) results in the complete conversion of all the receptor forms into the monomeric, 9S form. DTNB, one of the most specific thiol reagents (10), fails to reoxidize DTT-reduced receptor in Triton X-100 (Fig. 2 *g*), but generates higher oligomeric forms in cholate (Fig. 2 *l*), probably at the expense of the dimeric species (compare with Fig. 2 *e*). When acting on the unreduced membrane-bound receptor DTNB slightly increases the proportion of higher S-value components (Fig. 2 *f* and *k*), also diminishing the percentage of dimeric species. DTNB does not generate higher oligomeric receptor forms when reacted with the Triton-solubilized, unreduced receptor (Fig. 2 *h*). Fig. 2 also makes apparent that (*A*) DTNB is unable to cross-link receptors via cleaved disulphides when NEM alkylation of free sulphydryl groups in the membrane precedes the reduction (compare Fig. 2 *i* and *l*); (*B*) DTNB reoxidation in cholate via total, nonprotected -SH groups occurs (Fig. 2 *l–m*), in contrast to the effects of DTNB in Triton X-100 (Fig. 2 *g–h*); (*C*) The increase in polymeric forms after DTNB reoxidation presumably originates from further associations of dimers (Fig. 2 *l–m*) and from monomers in the case of DTNB reaction not preceeded by reduction (Fig. 2 *m*).

The structural counterpart of the monomeric, 9S receptor form is seen in Fig. 3 *a*. After detergent solubilization of DTT-reduced, NEM-alkylated, alkaline-extracted membranes the distribution of particle size coincides with that of particles measured outside the boundaries of the membrane remnants (Fig. 2 *b*), with a peak at ~8.5 nm. The number of particles released from the membranes is noticeably lower than that of controls. This may be related to the tendency of receptor particles to maintain a relatively high density packing in these reduced, alkali-stripped membranes, a property that is not observed with reduced and alkylated, detergent-solubilized receptor even at much higher protein concentrations (Fig. 3 *a*).

Samples of NEM-membranes, almost exclusively made up of the 13S, dimeric receptor form (Fig. 2 *c*) show predominantly the paired-particle structure of ~20 nm largest diameter (Fig. 3 *b*). Isolated single particles and larger particle aggregates are also observed. In contrast to DTT-reduced membranes, DTNB generates receptor oligomeric forms that are readily released from the membrane after alkaline treatment (Fig. 3 *c*). The receptor forms consist of single, double, and multiple particles, as is observed with unmodified and NEM-membranes, but after DTNB treatment there is a clear tendency in size distribution towards the higher polymeric forms (see Fig. 2 *c*). The size of the aggregates, however, does not increase indefinitely, and the distribution of particle aggregates is independent of protein concentration.

The participation of the ν-peptide in the thiol-dependent receptor interconversions is made apparent by the experiments shown in Fig. 4. DTNB reoxidation of NEM-alkylated membranes hinders the penetration of the receptor peptides into acrylamide gels during electrophoresis in the absence of reducing agents, but the ν-peptide migrates to its normal position (Fig. 4 *a*). Reoxidation of normal (i.e. not protected by NEM) membranes totally abolishes the penetration of the ν-peptide and the receptor polypeptides (Fig. 4 *c*). This is suggestive of extensive cross-linking, via mixed disulphide formation, between receptor and ν-peptide in native membranes.

The differences between native membranes and those prepared throughout in NEM ("NEM-membranes") can be further extended to the observation of significant modifications in the electrophoretic properties and extractability of the ν-peptide upon NEM alkylation (Fig. 5). Firstly, NEM alkylation of native membranes retards the mobility of the ν-peptide with respect to the receptor subunits, facilitating the observation of the ν-doublet (Fig. 5 *a–b*). However, NEM-alkylation of DTT-

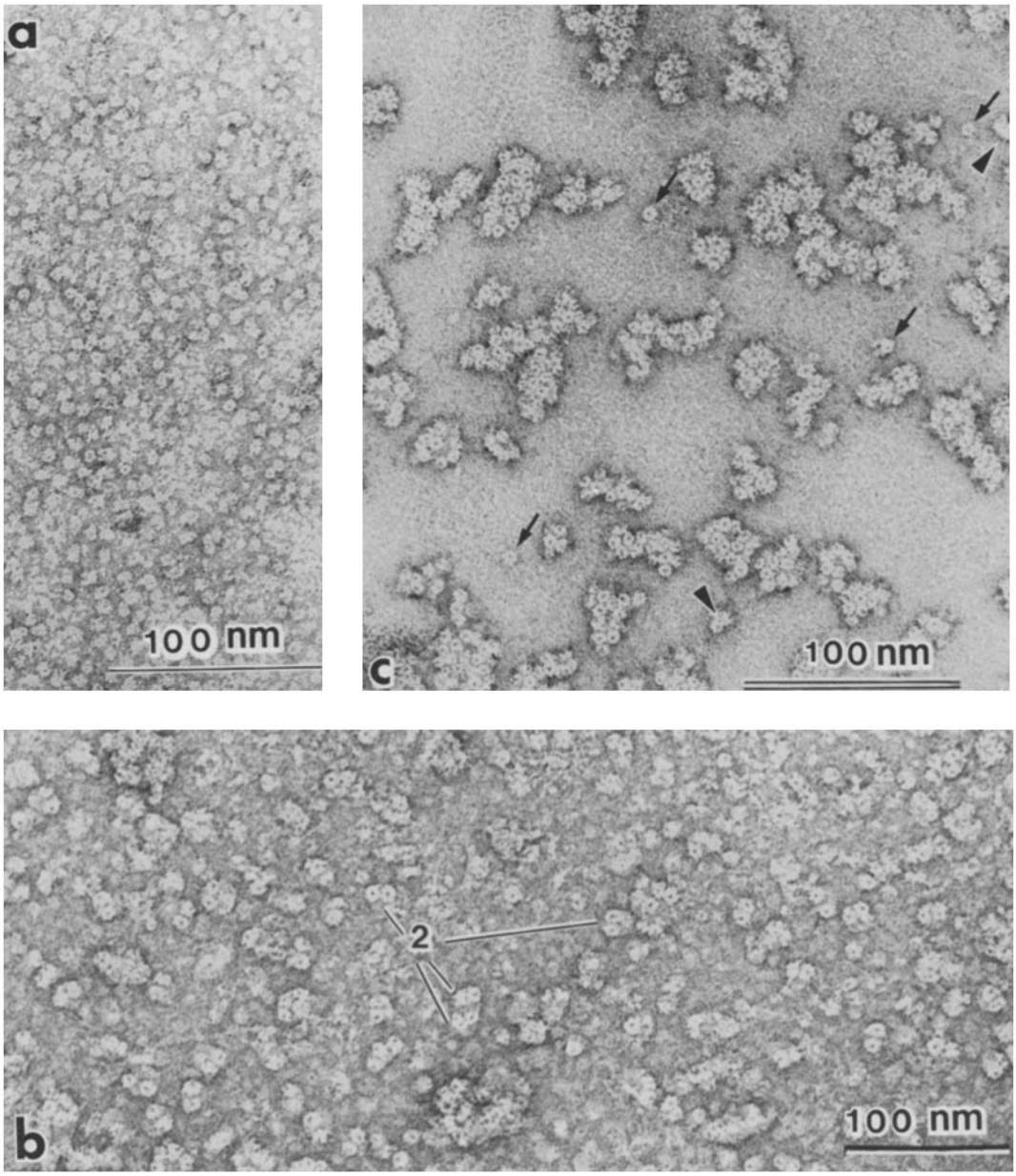

FIGURE 3 *a*) The 9S receptor monomer after solubilization in Triton X-100. Protein concentration: 200 μg/ml. Even at such high concentrations the reduced and alkylated receptor (as in Fig. 2 *a*) is seen as discrete single rosettes. (×294,000). Reduced membranes differ from the controls only in that very few receptor particles are seen in the vicinity of the vesicle remnants (not shown). *b*) Membranes prepared throughout in NEM are made up of the dimeric, 13S receptor species. The predominant structure of these samples is, in typical end-on views, a particle doublet (indicated by the *2* and the arrows) of ~19 nm main diameter (see Fig. 2 *d*). (×225,000). *c*) DTNB-treated receptor membranes. (×294,000). Negative contrast (1% uranyl acetate) was used throughout.

reduced membranes shows only a ν-"singlet" (Fig. 5 *e*), apparently the slow-moving component of the ν-doublet (compare Fig. 5 *e* and *b*). Secondly, it is well known that alkaline treatment extracts loosely associated peptides from native membranes (4, 9, 18, 25, 28), conspicuously the ν-peptide. Here it is shown that alkaline stripping readily solubilizes the ν-peptide, actin (32) and the presumptive ATPase heavy chain from native (Fig. 5 *c*) and DTT-reduced (Fig. 5 *d*) membranes, but that alkaline treatment is ineffective in extracting the peptide from NEM-membranes, either without (Fig. 5 *g*) or with (Fig. 5 *h*) prior DTT-reduction. The increased proportion of dimeric species, which is maintained even after extraction in nondenaturing detergents when NEM alkylation is performed early enough in the fractionation (7, 20), is found here to be correlated to the lack of extraction of the ν-peptide.

The Predominately Cytoplasmic Exposure of Antigenic Determinants of the ν-peptide

The reduction-reoxidation treatment of the receptor membranes proved to be a convenient purification step for the obtention of the ν-peptide essentially free of the adjacent α-subunit of the receptor and actin (see Fig. 4 *e*). Immune sera raised against the electrophoretically pure ν-peptide (Fig. 6 *b*) were purified by affinity chromatography as given in Materials and Methods. As shown in Fig. 6 *d'*) the resulting IgG reacts specifically with the ν-peptide, as tested by the very sensitive immune blotting technique (33).

The obtention of monospecific anti-ν-peptide IgG enabled the use of indirect immunofluorescence techniques. As shown in Table I, the rhodamine-labeled goat antirabbit IgG is bound

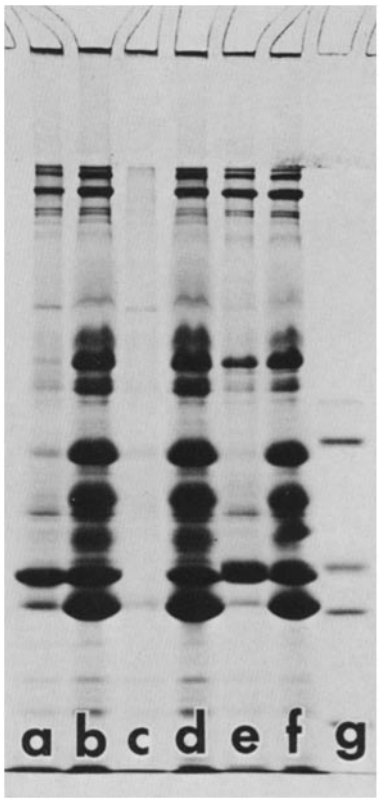

FIGURE 4 PAGE of receptor membrane subjected to thiol modifications analogous to those used in the electron microscope and ultracentrifugation experiments. Tracks *a–d* correspond to standard membranes; *e–f* to membranes prepared in 10 mM NEM throughout. *a*) NEM alkylation, DTT reduction, DTNB reoxidation, NEM alkylation. *b*) NEM alkylation, DTT reduction, NEM alkylation. *c*) DTT reduction, DTNB reoxidation, NEM alkylation. *d*) DTT reduction, NEM alkylation. *e*) DTT reduction, DTNB reoxidation, NEM alkylation. *f*) DTT reduction, NEM alkylation. All samples (185 μg protein) were first pelleted in an Airfuge at 105,000 *g* for 7 min and resuspended in 100 μl water (*c–f*) or 100 μl of 10 mM NEM (*a*, *b*). After 30 min at 2°C the centrifugation was repeated, the pellets resuspended and washed twice, and all samples reduced with 10 mM DTT in 50 mM Tris-HCl buffer, pH 8.0 for 30 min. The centrifugation-washing steps were repeated twice, and samples *a*, *c*, and *e* reoxidized with 1 mM DTNB (30 min, 2°C); samples *b*, *d*, and *f* were maintained in water at 2°C. After repeating the washings and centrifugations, all samples were treated with 10 mM NEM as above and the concentrated pellets were solubilized in 2% Triton X-100 containing 2 mM NEM for 30 min. Protein was precipitated with acetone at 2°C and dried in vacuo. SDS-PAGE was performed in the absence of reducing agents.

to a larger extent to membranes treated with 0.1% saponin, a procedure reported to permeabilize the AChR vesicles to molecules larger than M_r 450,000 without altering binding properties or the morphology of the vesicles (8). Correspondingly, membranes depleted of the ν-peptide bind significantly lower amounts of IgG. Normal, intact membranes bind intermediate amounts of IgG (Table I).

DISCUSSION

Some of the possible relationships between the acetylcholine receptor oligomeric organization, its aggregational states, and the ν-peptide can now be analyzed in the light of the above experimental findings. Firstly, it is clear that depletion of the ν-peptide does not hinder the thiol-dependent interconversion of receptor. However, the presence or absence of the ν-peptide seems to affect the native redox equilibrium: ν-peptide-depleted membranes show a slight shift to higher oligomeric states upon reoxidation as compared to standard membranes. Conversely, membranes in which the dimeric species predominate (the "NEM-membranes") are refractive to depletion of the ν-peptide by alkaline extraction. It has already been stressed that the redox balance of the excitable membranes is likely to depend not only on the redox state of the receptor itself but on the multiple contributions from all other components (3). Taking into consideration (*a*) the richness in thiol groups of the ν-peptide (30), (*b*) the observed changes in particle packing habits upon its extraction from normal membranes, (*c*) the particle tendency to be closely packed in reduced, alkaline-extracted membranes, (*d*) the inability of alkaline treatment to extract the ν-peptide from NEM-membranes, and (*e*) the dependence of receptor solubility (or aggregational) properties indicated by the experiments in Fig. 4, the putative, unknown protein of Chang and Bock (7), having "unusually low redox potential and a close proximity to the receptor" seems to materialize in the ν-peptide. The rationale behind this interpretation is straightforward: the ν-peptide could be envisaged as a "redox buffering system" normally impairing the high-order associations of the receptor monomers. These constraints seem to be absent after depletion of the ν-peptide, and the receptor units are able to associate into forms that may or may not spontaneously interconvert in the living cell, but which nevertheless seem to occur at the membrane-bound level of receptor organization and are limited to a discrete distribution of (n = oligo)

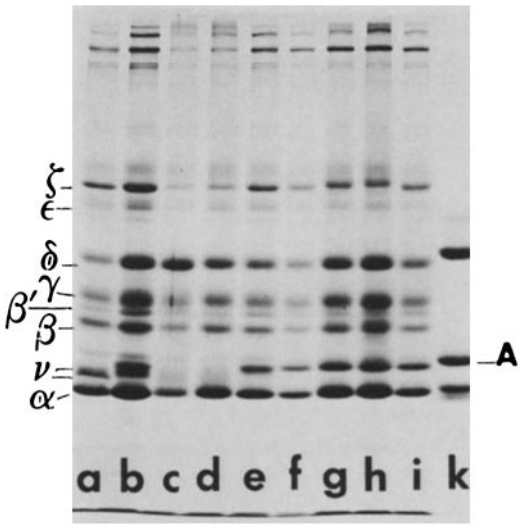

FIGURE 5 Thiol-dependent changes in the mobility of the ν-peptide and its lack of extractibility from NEM-membranes. *a–e* are standard membranes; *f–i* are NEM-membranes. *a*) control. *b*) the same, after NEM alkylation (10 mM, 30 min, 20°C) before PAGE. *c*) Same as (*a*), *double extraction with 1 N NaOH (2 × 1 h, 2°C). d) 10 mM DTT-reduction (30 min, 20°C) followed by double alkaline extraction as in* (*c*), and NEM alkylation as in (*b*). *e*) DTT-reduction and NEM-alkylation. *f*) NEM-membranes; no additional treatment. *g*) the same after alkaline extraction as above. *h*) NEM-membranes reduced and alkali-stripped as above. *i*) Reduced NEM-membranes. *k*) Standards, from bottom to top: aldolase, *Torpedo* actin, and bovine serum albumin. All samples are reduced with 100 mM DTT (20°C) before PAGE. Samples *b–k* include additional alkylation with NEM (30 min, 20°C).

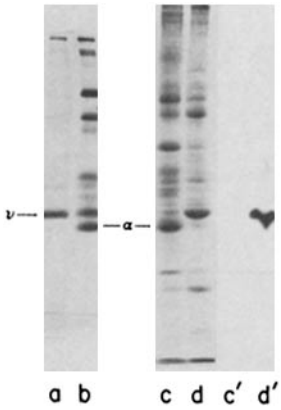

FIGURE 6 Specificity of the antibody against the ν-peptide. Immune sera were raised using samples like that of Fig. 4 *e* (also see Materials and Methods), which show a single band in the M_r 43,000 region (*a*). (Track *b* shows the pattern of a standard membrane). Tracks *c–d* correspond to the supernatant and pellet respectively of a membrane fraction processed as in Fig. 4 *e*, and further solubilized in 2% Triton X-100 for 1 h, and centrifuged in an Airfuge for 10 min (27 psi). Notice that the ν-peptide is found in the pellet. Tracks *c'–d'* are the same two fractions, after transferring to nitrocellulose sheets and submission to immune blotting as in reference 33 using the purified anti ν-peptide IgGs and ^{125}I-protein A. The immune IgGs are only specific for the ν-peptide; no cross-reactivity with receptor or nonreceptor peptides is observed. Samples were not reduced with DTT after denaturation.

TABLE I

Availability of Antigenic Determinants of the ν-peptide in Acetylcholine Receptor Membranes *

Sample	Relative fluorescence (arbitrary units)		
	Total	Background‡	Specific
	A	*B*	*A* − *B*
Normal membranes	46	16 ± 4	30
Alkaline-extracted membranes	32	10 ± 5	22
0.1% Saponin-treated membranes§	68	10 ± 2	58

* Fluorescence emission arising from rhodamine-labeled goat antirabbit IgG, and associated with the membranes subjected to the treatments given above. Fluorescence measurements performed using lock-in amplification; integration times 2–8 sec. Duplicate measurements performed in all cases.

‡ Background fluorescence is defined as that arising from the combination of (*a*) free rhodamine-labeled IgG (~0.1%); *b*) unspecifically trapped fluorescent-labeled goat IgG or rabbit anti-ν-peptide IgG or both; *c*) bound or free nonspecific antibodies (<1%). Contribution from *b*) dominated the unspecific background fluorescence, subtracted in all cases.

§ See reference (8) and text.

states. These states have previously been characterized in terms of their equilibrium and kinetic ligand binding parameters (3) and the retention of functionality of alkaline-treated membranes is also documented in the literature (9, 20, 25).

A further point worth discussing here is the topographical localization of the ν-peptide and its relationship to receptor structure. The first indirect evidence towards a putative localization can be deduced from the physicochemical criteria outlined for the categorization of membrane proteins (see reference 2 and references therein) and which have successfully been applied to other components of the membrane in question (2, 26). The discriminant function (2, 26) calculated here for the ν-peptide on the basis of published amino acid data (30) is $Z = 0.124$, in excellent agreement with the mean value of typical peripheral proteins (2). Additional qualifiers to this tentative classification stem from independent biochemical and histochemical data. For instance, the sensitivity of the ν-peptide to proteolytic degradation under various conditions (35) and recent histochemical evidence (29) suggest a "cytoplasmic-facing", peripheral location of the peptide. The immunofluorescence experiments using the monospecific anti-ν-peptide antibodies (Table I) also point in this direction, showing that the determinants of the ν-peptide in the membrane are mainly accessible from the cytoplasmic side of the receptor vesicle. The observation of a class of receptor particles after alkaline extraction with a different (i.e. lower) electron density profile (Fig. 1), and a slightly larger diameter (D. C. Neugebauer and H. P. Zingsheim, manuscript submitted for publication) than those of the controls could then be attributed to the "unmasking" of a normally occluded portion of the receptor protein by exclusion of the ν-peptide. X-ray (5, 27), neutron scattering (36) and electron microscope (4, 5, 15, 27, 31, 37) studies all suggest that the acetylcholine receptor is an asymmetric body of ~10 nm in length and 7- to 8-nm in-plane diameter (reviewed in references 2 and 16). The peculiar low electron density, in-plane end-on view of the particles observed in single sheets of membrane remnants might thus correspond to the cytoplasmic-facing part of the prolate ellipsoid proposed by Wise et al. (36), made apparent after removal of the mass normally obliterating this view. Further studies using high resolution, minimal electron dose scanning transmission electron microscopy and image reconstruction techniques are now in progress with the aim of establishing the precise localization of the ν-peptide and its topographical relationship with respect to the receptor protein. These studies, together with previous (4) and the present one from our and other laboratories (23, 28) are converging towards the establishment of a definite role of the ν-peptide in the architecture of the postsynaptic membrane and of its influence in the dynamics of the receptor macromolecule.

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Note Added in Proof: Froehner (*Biochemistry*, 1981, 20:4905–4915) has applied two of the procedures used in the present work to identify latent antigenic determinants of the receptor protein in *Torpedo* membranes. Alkaline extraction uncovers determinants in the α, γ, and δ subunits, and saponin treatment makes latent sites on the α and δ accessible to the antibodies. Froehner suggests that the category of sites on the β, γ, and δ subunits represent intracellular sites normally masked by receptor-peripheral protein interactions. The class of receptor particles revealed after alkaline extraction in the present paper, and interpreted as the resultant of "unmasking" of a normally occluded portion of the receptor, might well constitute the morphological counterpart of Froehner's findings.

Froehner, Guldbransen, Hyman, Yeng, Neubig, and Cohen (*Proc. Natl. Acad. Sci. U. S. A.*, 1981, 78:5230–5234) raised antisera against the total alkaline-extractable material from *Torpedo* membranes. The total antisera reacted with the ν-peptide and other nonreceptor peptides, but not with the receptor subunits. The synaptic localization of

the antigens was demonstrated in *Torpedo* electrocytes and mammalian muscle. This morphological study complements the present one, in which the predominantly cytoplasmic exposure of the ν-peptide was revealed with monospecific anti-ν-peptide IgG's.

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