

PROTEASE EFFECTS ON THE STRUCTURE OF ACETYLCHOLINE RECEPTOR MEMBRANES FROM *TORPEDO* *CALIFORNICA*

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ABSTRACT

Protease digestion of acetylcholine receptor-rich membranes derived from *Torpedo californica* electroplaques by homogenization and isopycnic centrifugation results in degradation of all receptor subunits without any significant effect on the appearance in electron micrographs, the toxin binding ability, or the sedimentation value of the receptor molecule. Such treatment does produce dramatic changes in the morphology of the normally 0.5- to 2- μ m-diameter spherical vesicles when observed by either negative-stain or freeze-fracture electron microscopy. Removal of peripheral, apparently nonreceptor polypeptides by alkali stripping (Neubig et al., 1979, *Proc. Natl. Acad. Sci. U. S. A.* 76:690-694) results in increased sensitivity of the acetylcholine receptor membranes to the protease trypsin as indicated by SDS gel electrophoretic patterns and by the extent of morphologic change observed in vesicle structure. Trypsin digestion of alkali-stripped receptor membranes results in a limit degradation pattern of all four receptor subunits, whereupon all the vesicles undergo the morphological transformation to minivesicles. The protein-induced morphological transformation and the limit digestion pattern of receptor membranes are unaffected by whether the membranes are prepared so as to preserve the receptor as a disulfide bridged dimer, or prepared so as to generate monomeric receptor.

Acetylcholine receptor membranes, isolated from the electrogenic tissue of *Torpedo* and *Narcine*, are unique systems ideally suited for the study of the structural organization of nicotinic, neuromuscular-type synapses, as the entire innervated surface of their electrocytes is densely packed with acetylcholine receptors. They offer a more readily accessible system than the limited synaptic endplate regions of muscle for the isolation of specialized postsynaptic membrane. Biochemical analysis and

electron microscopy have shown that the postsynaptic membranes can be separated from the basal lamina and much of the underlying cytoskeleton, uninervated electrocyte, and other cellular membranes by homogenization and isopycnic centrifugation, and that these membranes respond to agonist much like the *in vivo* postsynaptic membrane (11, 16).

Conventionally prepared receptor-enriched membrane fractions have receptor specific activi-

ties (nanomoles α -bungarotoxin [α butx]¹ bound per milligram protein) of at most one-half that of the solubilized, affinity purified receptor, and a more complex sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS PAGE) banding pattern. The complexity of these SDS PAGE patterns can be greatly reduced by alkali treatment (29, 10, and this work) of the receptor membranes which removes a number of peripheral proteins yet leaves the receptor functionally intact. Such alkali-treated preparations contain <10% non-receptor protein by gel electrophoresis (29, 10, and this work).

The work of Heuser and Salpeter (18) on gently sheared membranes in which synaptic morphology is largely preserved reveals a highly structured organization of the *Torpedo* neuro-electrocyte synapse, which includes the receptor molecules within the postsynaptic membrane. Basal lamina and cytoskeletal attachment sites on the postsynaptic membranes are evident. Remnants of the postsynaptic membrane organization are visible in freeze-etch micrographs of non-alkali-treated receptor-rich membranes (4) and may reflect the presence of structural proteins other than receptor.

Receptor-enriched membranes have been characterized structurally by both x-ray diffraction and electron microscope analysis (9, 34, 21, 3, 4, 30, 32, 35). Through immunological labeling, the membrane-bound receptor has been identified as a rivetlike molecule of length 110 Å with a maximum in-plane diameter 85 Å, which extends above the postsynaptic membrane into the synaptic cleft by ~55 Å and corresponds to the 9S, 260,000 mol wt monomer (21).

We report here that proteolytic digestion induces a radical structural transformation in acetylcholine receptor membranes. The degree of susceptibility of the membrane proteins to protease digestion revealed by SDS PAGE is variable from preparation to preparation, but parallels the degree of structural transformation (up to 100% transformation of all the vesicles). Even when proteolytically degraded, such that few polypeptides of mol wt >30,000 remain, the receptor molecule continues to bind α butx specifically and

remains a high molecular weight (9S) aggregate of unaltered appearance in electron micrographs.

MATERIALS AND METHODS

Membrane Preparation and Characterization

Membranes enriched in acetylcholine receptor were prepared from freshly killed and dissected *Torpedo californica* electroplaques (Pacific BioMarine Supply Co., Venice, Calif.) which had been cut into small (10 g) pieces after dissection, immediately frozen in liquid nitrogen, and stored at 90°C until use. Receptor-rich membranes were prepared by a modification of the procedure of Reed et al. (33). Membranes were prepared by homogenization in argon-saturated 1 M NaCl, 50 mM Tris, 10 mM EDTA, 10 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4 (H buffer). Receptor was prepared in the disulfide cross-linked dimeric form by the addition of 10 mM *N*-ethylmaleimide (NEM) to the homogenization buffer (which prevents action of endogenous reducing agents) (5), and in monomeric form by its omission. In certain experiments, dimeric receptor was subsequently converted to the monomeric species by reduction with 5 mM dithiothreitol (5 h, 4°C), followed by 10 mM NEM. Monomeric receptor molecules formed in either way have identical properties.

After a low-speed centrifugation to remove debris (10 min, 5,000 g) the membranes were pelleted (60 min, 100,000 g). The membranes were then resuspended in a Dounce homogenizer (Kontes Co., Vineland, N. J.) into 1 mM EDTA, 0.1 mM PMSF (pH 8.0). The remaining cellular debris was spun out as described above, and the membranes were repelleted. At this point, the membranes were resuspended in 28% wt/wt sucrose and run on a 38% → 29% wt/wt sucrose gradient in 10 mM Na phosphate, 1 mM EDTA, 1 mM EGTA, pH 7.4 (gradient buffer), hereafter referred to as "conventionally prepared" membranes, or alternatively the membranes were base-treated to give "alkali-stripped" membranes (29). These alkali-stripped membranes were collected by centrifugation, resuspended in 28% sucrose gradient buffer, and run on 36% → 29% wt/wt sucrose gradient.

Conventionally prepared membranes were alkali-stripped either by first collecting them by centrifugation and resuspending them in doubly distilled water at 4°C or by dilution directly out of the gradient buffer into ~9 vol of distilled water, and brought at once to pH 11–11.2 with 0.5% NaOH in a stirred cell. Membranes were either pelleted immediately by centrifugation for 30 min at 100,000 g at 4°C, or incubated at room temperature for 1 h before centrifugation and neutralization. Polypeptides released by alkali stripping were recovered from the supernate by trichloroacetic acid precipitation or by vacuum dialysis using colloidion bags (Schleicher & Schuell, Inc., Keene, N. H.) with a 25,000-dalton pore retention size.

Membranes were kept at 4°C or on ice at all times and used for experiments within 1 wk. Membranes were stored in 10 mM Na phosphate, 1 mM EDTA, 0.02% Na₂S₂O₃, pH 7.4. Even though the membranes had been washed extensively and commonly contain >90% receptor by gel electrophoretic analysis, the continued presence of endogenous proteases is revealed in the appearance of new polypeptides over time (22).

Membranes were characterized by assaying for protein, α butx binding, and esterase activity as described previously (21). D-tubocurarine was obtained from CalBiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.); all other reagents were of the highest purity commercially available.

¹ Abbreviations used in this paper: α butx, alpha bungarotoxin, NEM, *N*-ethylmaleimide, PMSF, phenylmethylsulfonyl fluoride, SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, STI, soybean trypsin inhibitor.

Linear SDS PAGE (10%) was performed as described by Laemmli (24) in either a standard slab gel apparatus or a minigel apparatus (26), stained with Coomassie Brilliant Blue R, photographed on Kodak Plus-X Pan Type 4187 film, and scanned on a Syntex AD1 autodensitometer (Syntex Analytical Instruments, Cupertino, Calif.). Samples were dissolved in 125 mM Tris, pH 6.8, 5% SDS, 10% glycerol, and 0.01% bromophenol blue. For standard sized gels, 30 μ g of total protein was loaded per lane, 1–3 μ g per lane for minigels. During characterization of the gel system, all gels were run using bovine serum albumin (66,000 daltons), catalase (57,000 daltons), aldolase (40,000 daltons), and lactate dehydrogenase (35,000 daltons). Later, the positions of the receptor subunits were used to calibrate the gels. Gel scans were integrated and smoothed using computer programs written by M. J. Ross and D. A. Agard.

Glycerol gradients (80% \rightarrow 40% wt/wt) were formed in eleven equal steps, and linear sucrose gradients were formed using a gradient maker. Equilibrium centrifugations were run at 10^5 g for >8 h. Velocity sedimentation of 125 I-toxin-receptor and protease-digested 125 I-toxin-receptor complexes was run on 5–20% wt/wt sucrose gradients, in 0.2% Triton X-100, 100 mM NaCl, 10 mM NaPO₄, and 1 mM CaCl₂, in an SW.50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 50,000 rpm for 5 h at 4°C, using β -galactosidase and catalase (Sigma Chemical Co., St. Louis, Mo.) as sedimentation markers. Glycerol and sucrose concentrations were determined with a Zeiss refractometer.

Electron Microscopy

Negative-stain electron microscopy was carried out as described previously (21) using either 2% sodium phosphotungstate, pH 7.4, or 1% uranyl oxylate, pH 6.0 as negative stain. Both stains were effective in visualizing the membranes, though phosphotungstate gave better high resolution images.

Membrane pellets prepared by centrifugation were quick-frozen by slamming them against a block of copper cooled to 4°K. The design and operation of the apparatus used for freezing have been described previously (17). Frozen samples were stored in liquid nitrogen until transfer into a Balzers freeze-fracture machine (Balzers Corp., Nashua, N. H.). They were then fractured by gently scraping the surface with a new ultrasharp razor blade mounted in the freezing microtome. A specimen was fractured at a vacuum of lower than $2 \cdot 10^{-6}$ torr and a tissue temperature of -120°C . Samples were etched for 3 min at 100°C and rotary-replicated (25) with platinum-carbon using a Balzers rotary-stage attachment. The electron beam gun, mounted at a $16\text{--}24^\circ$ angle relative to the tissue surface, was operated at 2,050 V and 70 μA for 6 s, during which time the tissue rotated through 360° six times. Replicas were backed with carbon deposited by 5–7 s of thermionic evaporation from pointed carbon rods. Replicas were removed from the Balzers machine to a plastic scintillation bottle of frozen methanol and allowed to thaw. Tissues were rinsed once in water and submerged in standard household bleach (sodium hypochlorite). Replicas floated off in a few seconds, and were then broken into grid-sized pieces which were individually transferred through two rinses of water and picked up on 75-mesh carbon-coated Formvar grids.

The replicas were photographed at $\times 50,000$ in a JEM 100C electron microscope operated at 80 kV with a $50\text{-}\mu\text{M}$ objective aperture. Stereo microscopy was performed with a high-resolution top-entry goniometer by tilting the grid $\pm 6^\circ$. Electron microscope negatives were routinely contact-reversed. Negative images in which platinum looks white and the shadows are black

have been used for the freeze-etch micrographs throughout this paper. Particle sizes were measured from $\times 5$ enlarged photographic prints.

Protease Digestion

Protease digestions with *Staphylococcus griseus* protease (Sigma Chemical Co., type V1, lot No. 57C-0152), papain (Sigma Chemical Co., Lot No. 58C-8005), and crystallographically pure bovine α -trypsin (38) (chromatographically purified by L. M. Kay) were carried out at 4°C using final enzyme concentrations of 1–10 μM and membrane protein concentrations of 0.5–1 mg/ml. Some membrane fractions were subjected to three successive freeze-thaw cycles from dry ice/ethanol to a 37°C water bath in the presence of trypsin in order to allow the protease to have access to vesicle interiors. Limit digestion with trypsin was carried out until no further change was apparent in the SDS PAGE pattern either by making the solution 1 μM in trypsin every 24 h for 72 h, for a final concentration of 3 μM trypsin, or by making the solution 5 μM in trypsin, and then adding a second aliquot after 6 h for a final concentration of 10 μM . Standard trypsin digestion was carried out using a single addition of 10 μM trypsin (final concentration) to the receptor membranes (0.5–1 mg/ml) which were incubated overnight at 4°C. Protease activity was determined using the casein-agar clearing assay of Bjerrum et al. (1) (Protease Detection Kit, Bio-Rad Laboratories, Richmond, Calif.).

Most experiments were carried out using the purified α -trypsin to reduce the possibility of contamination by other proteases or lipases. (Similarly prepared trypsin was used in the x-ray crystal structure determination of trypsin [38].) To test the possibility that contaminating lipases could be responsible for the observed morphological effects, the trypsin was inhibited either by a 5- to 10-fold molar excess of soybean trypsin inhibitor (STI) (Cal-Biochem-Behring Corp., lot 300798, B grade, 7,200 benzoylarginine ethyl ester inhibitor units/mg), or by a greater than 100-fold molar excess of either PMSF (Sigma Chemical Co.) or benzamidine HCl (Aldrich Chemical Co., Inc., Milwaukee, Wis.), or a combination of the two. PMSF was diluted from a stock solution in 100% ethanol such that the final ethanol concentration in the membranes was $<10\%$, because concentrations of ethanol $>10\%$ cause the fragmentation of receptor membranes (22).

The commercial STI contained a number of polypeptides of molecular weight greater than that of STI which were removed by column chromatography on Sephadex G-75-120 (Pharmacia Inc., Piscataway, N. J.) run in 1 to 10 diluted H buffer. Failure to remove these high molecular weight contaminants resulted in effects on membrane morphology caused by the crude STI alone. Trypsin and inhibitor were premixed and incubated for from 15 to 30 min at room temperature before addition to the membranes. Leaving trypsin at room temperature (23°C) for this length of time had no effect on the result of subsequent digestion. Trypsin activity was measured by the rate of *N*-carbobenzoxy-L-lysine-*p*-nitrophenyl ester (ZLONpH) hydrolysis as described in Koeppel et al. (23).

RESULTS

Acetylcholine receptor membranes prepared by homogenization and isopycnic centrifugation (33, 37) are between 0.5 and 2 μM in diameter (see Fig. 1a). The receptor particles in negatively stained membranes have been identified as receptor both

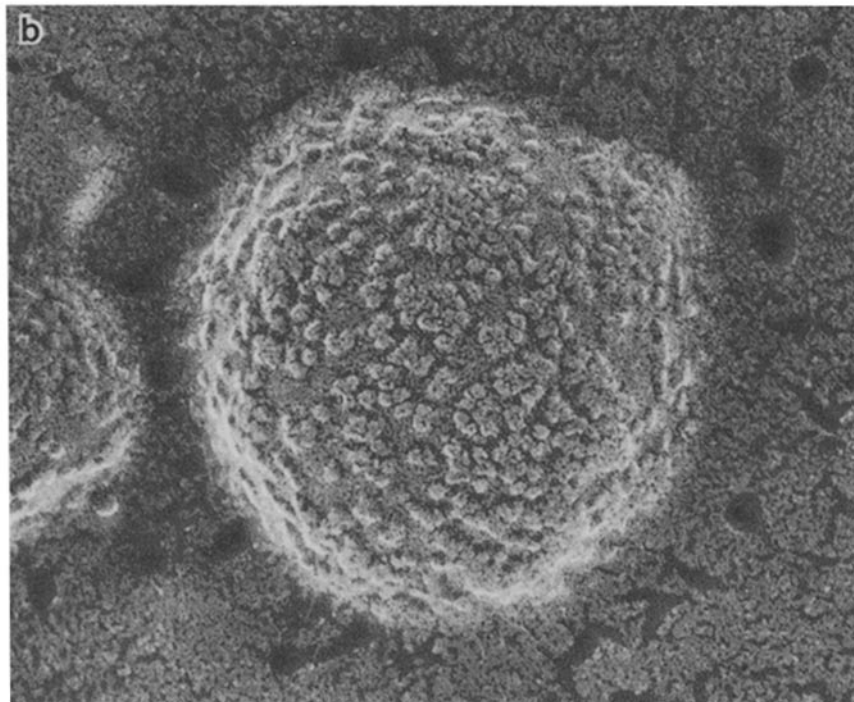
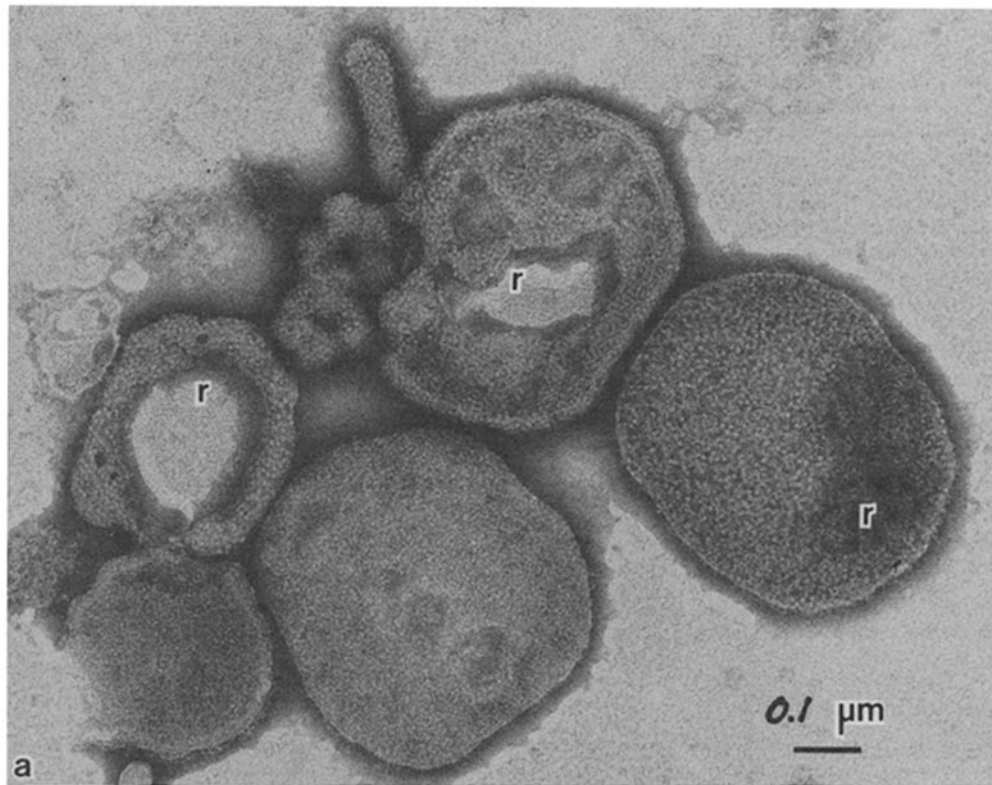


FIGURE 1 (A) Receptor-containing membranes, *r*, show the distorted vesicle shape and homogeneous particle distribution common in negatively stained images. $\times 88,000$. (B) Spherical receptor-filled vesicles are seen in quick-freeze, deep-etch, and rotary-shadowed images. $\times 163,000$.

by their similarities in appearance to detergent-solubilized receptor (27) and by direct and indirect immunospecific labeling (21). Commonly, >90–99% (37) of the membrane vesicles are packed with receptor molecules. Under negative-stain conditions, the membranes collapse and dry down onto the carbon support film with some distortion in membrane shape (22). When the receptor membranes are examined by rapid-freezing, deep-etch, and rotary shadowing techniques (17, 18) (Fig. 1*b*), they appear spherical and exhibit a distinct clustering of particles which is only rarely observed in negative-stain preparations (compare Fig. 1*a* and *b* and reference 4, Figs. 8 and 10). That these frozen and shadowed membranes correspond to the receptor-packed membranes viewed under negative-stain conditions has been demonstrated by their decoration with antireceptor antibodies (J. E. Heuser and M. W. Klymkowsky, unpublished observations).

Conventionally prepared receptor-enriched membrane fractions contain a limited number of polypeptides (Fig. 2) and their specific *abutx* binding activity of 2–4 nmol/mg protein indicates that ~20–50% of the protein present is receptor. The relative proportions of the polypeptides present vary from preparation to preparation, perhaps because of loss of loosely bound components, fortuitous accretion of other components with the membranes, and proteolysis by endogenous proteases during preparation.

The crude homogenate does not clear casein-agar protease detection plates, thus endogenous proteolytic activity must be fairly specific. Froehner and Rafto (12) have noted a proteolytic activity, inhibited by NEM, which preferentially attacks the γ -subunit of receptor. Gel electrophoretic patterns of membrane fractions after sucrose density centrifugation are relatively stable but do decay slowly over a period of 1–2 wk, especially if the membranes are left at room temperature (22). The effects of this endogenous proteolytic activity are minimized by keeping the sample at all times at 4°C.

When receptor membranes are subjected to proteolytic degradation by either papain, *S. griseus* proteases or trypsin, a radical transformation occurs in vesicle morphology (Figs. 3 and 4). Rapid-freeze, deep-etch images indicate that receptor molecules first aggregate into patches, leaving particle-free areas behind (see Fig. 5). Because this technique permits freezing within molecular diffusion times for receptor, it is an ideal technique for analysis of the state in solution. In negative-

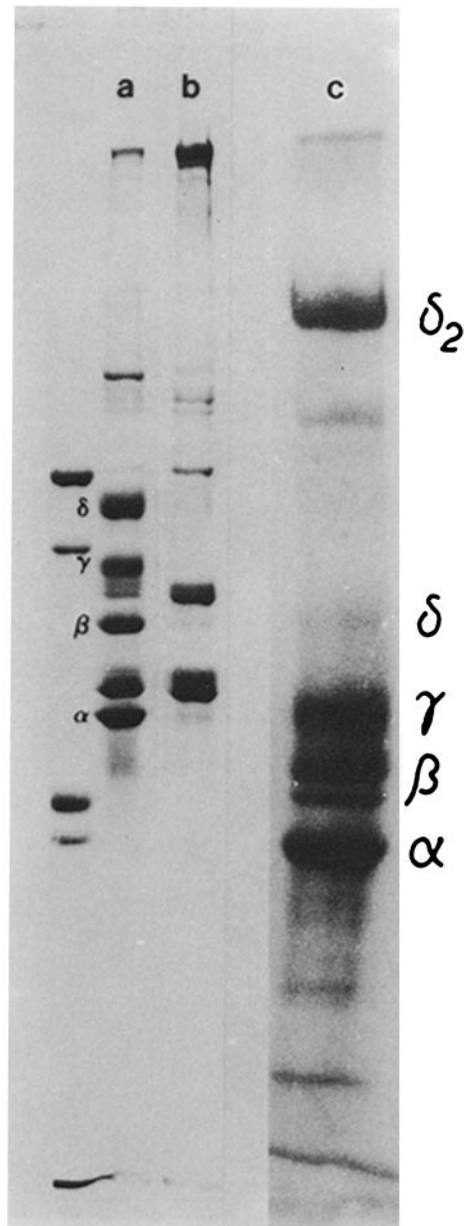


FIGURE 2 Receptor-enriched membrane fractions (*a*) contain only a few polypeptides other than the receptor subunits (labeled α , β , γ , δ) when visualized by SDS PAGE. (*b*) Of these nonreceptor polypeptides, a number can be solubilized by alkali treatment. The track on the left side of track *a* is molecular weight standards. Track *c* is "mini-gel" pattern of alkali-treated receptor membranes under nonreducing conditions.

stain images, this initial aggregation is often not apparent though the vesicles develop gently scalloped edges (see Fig. 3, single arrow). The differ-

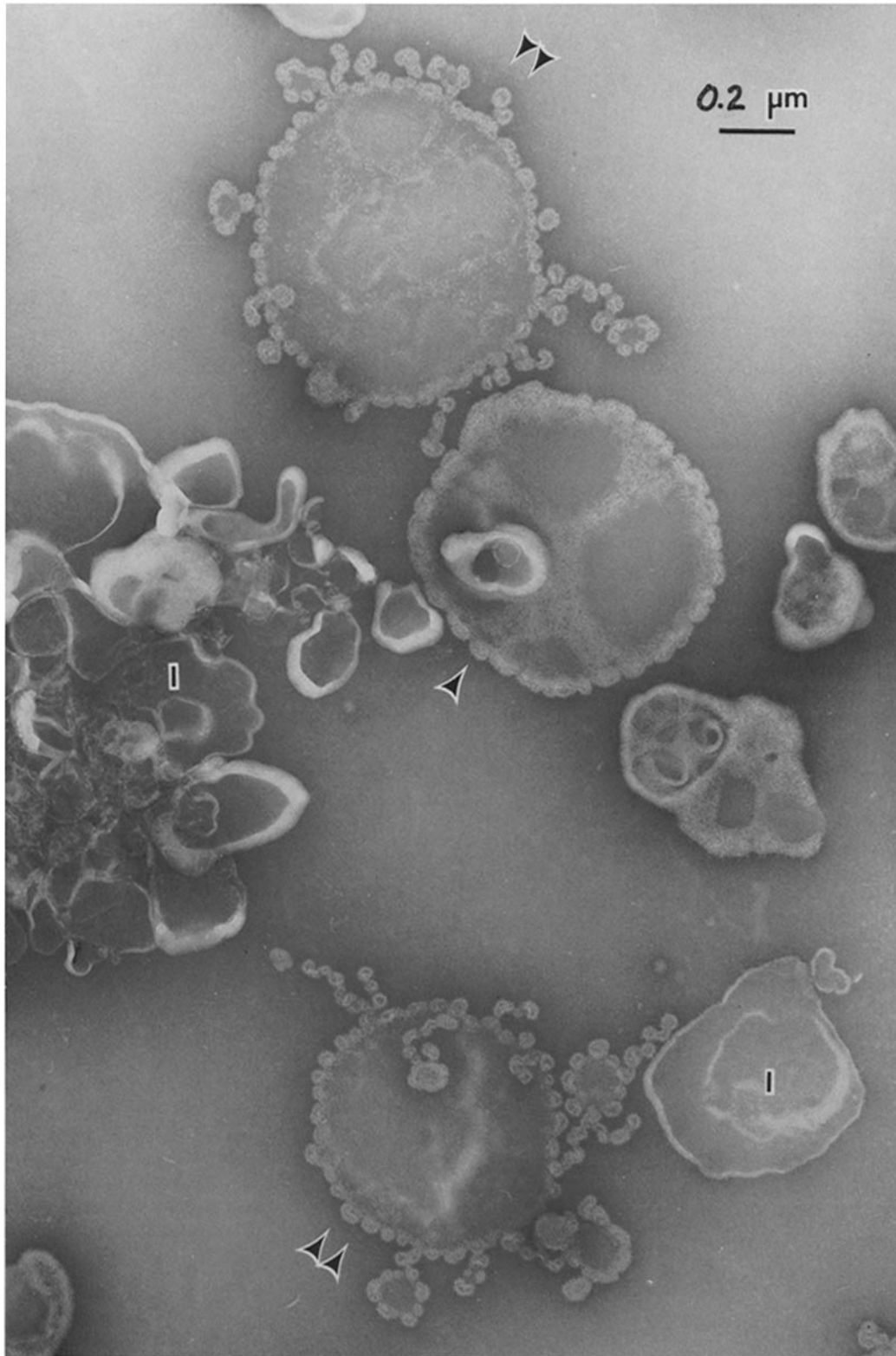


FIGURE 3 After protease digestion, receptor membranes take on a distinctive overall membrane shape. Single arrows mark vesicles at an early stage of the transformation, characterized by a gently scalloped edge structure. At later stages (double arrows) the process has continued and mini-vesicles packed with receptor molecules and connected to the parent vesicle are formed. The central areas of the digested vesicles are free of receptor particles, and vesicles that are entirely free of receptor particles (marked by *I*) may represent the end result of the protease transformation process. $\times 56,000$.

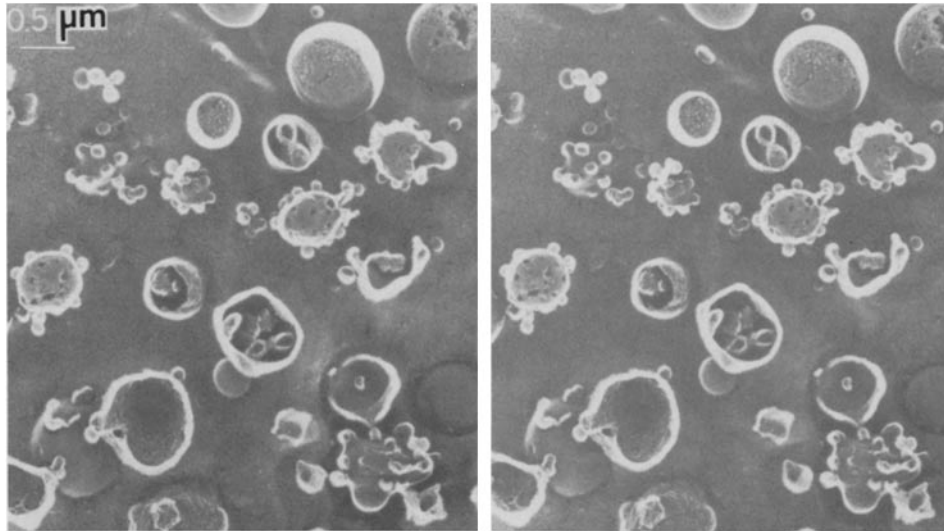


FIGURE 4 A low magnification stereo pair of rapidly frozen, deep-etched, and rotary shadowed, proteolytically digested receptor membranes. Many of the membranes exhibit the blebbing characteristic of the proteolyzed membranes, but without the distortions that arise in the negative stain procedure, the vesicles remain as spheres. Some of the vesicles have been fractured. The image should be viewed with a stereo viewer to have the correct sense of depth. $\times 14,000$.

ence between the two imaging techniques used in Figs. 3 and 4 may arise from the same negative-stain-induced causes that disrupt the clustering which is observed in deep-etch images. Even at the early stage of cluster formation (Fig. 5 *b*), the patches have a positive curvature. These budding processes grow (see Fig. 5 *c*), form a neck, and constrict again, such that blebbed minivesicles of relatively constant size were formed (see Figs. 3, 5 *c*, and 6). The transformation occurs uniformly within any one vesicle (see Figs. 3–6). The end result of the process is the pinching off of the blebs into ~ 100 -nm-diameter mini-vesicles (see Fig. 5 *c*). Thermolysin was also tested but failed to result in membrane morphological change.

Although the trypsin used throughout these experiments had been much further purified than that used by others to clip off extended parts of integral membranes (see, for example, reference 31), the novelty of the observed morphological changes led us to examine the possibility of lipase contamination of the added trypsin. When trypsin was first inhibited by a 10-fold excess of purified STI, the morphological transformation and receptor degradation were blocked. STI alone was without effect on vesicle morphology. The chemical inhibitors PMSF and benzamidine proved much less useful because, even after preincubation of

trypsin with a 500-fold molar excess of PMSF at room temperature for 12 h followed by the addition of a 500-fold molar excess of benzamidine, the trypsin was still active as judged by receptor subunit degradation. Nevertheless, the extent of trypsin activity as judged by both SDS PAGE and ZLONpH hydrolysis parallels the extent of vesicle transformation. In trypsin inhibition experiments in which degradation was not completely inhibited but had not reached the predominantly 27,000-dalton polypeptide stage (Fig. 7), most of the receptor vesicles failed to reach the final transformed morphology and many appeared unaltered (Fig. 8).

About 10–20% of “conventionally prepared” receptor-containing vesicles were transformed after 72 h of trypsin treatment ($1 \mu\text{M}$ trypsin added at 24-h intervals) as determined by negative-stain electron microscopy. Freeze-thawing cycles in the presence of protease, aimed at breaking open the vesicles so as to allow increased protease to have accessibility to the intracellular surface, had no appreciable effect on the extent of transformation. After 72 h of trypsin digestion, the SDS PAGE banding patterns were often relatively unchanged from the predigest pattern (Fig. 9, top), with only 10–20% of the material appearing as new, lower molecular weight bands. Thus, the extent of diges-

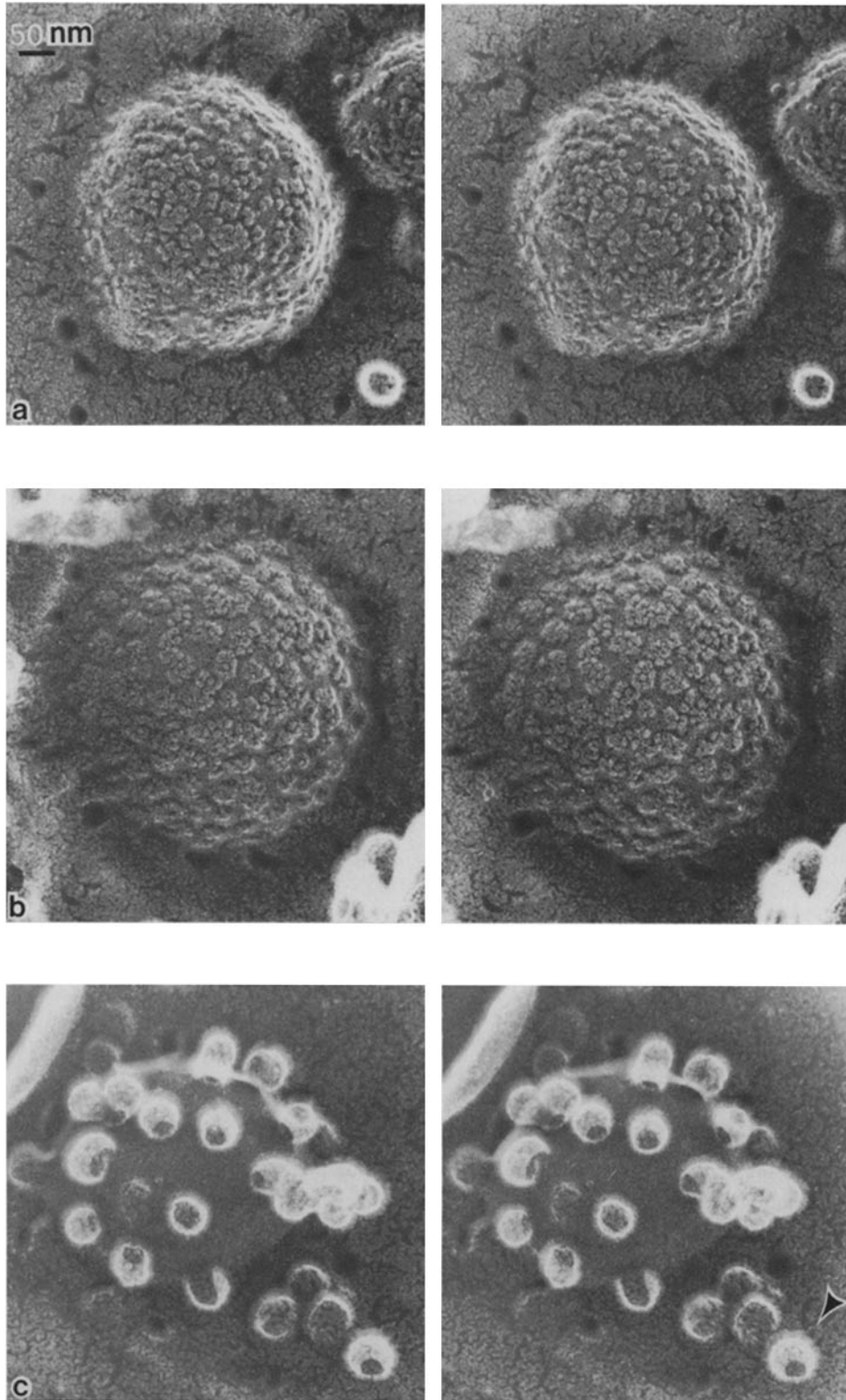


FIGURE 5 At higher magnification the spherical nature of the receptor membranes and the tendency of receptor particles to cluster in the plane of the membrane are apparent (*a*). The effects of trypsin at an early stage can be seen in *b*. Particles in the membrane have clustered into small patches, leaving behind cleared areas. The inherent curvature of these clusters is apparent around the vesicle periphery, even in this early stage. *c* shows a vesicle at a late stage. The entire surface of the residual large vesicle has been cleared of particles, and the aggregated receptors are now budding out of the membrane. Arrow points to what appears to be released mini-vesicles. All, $\times 100,000$.

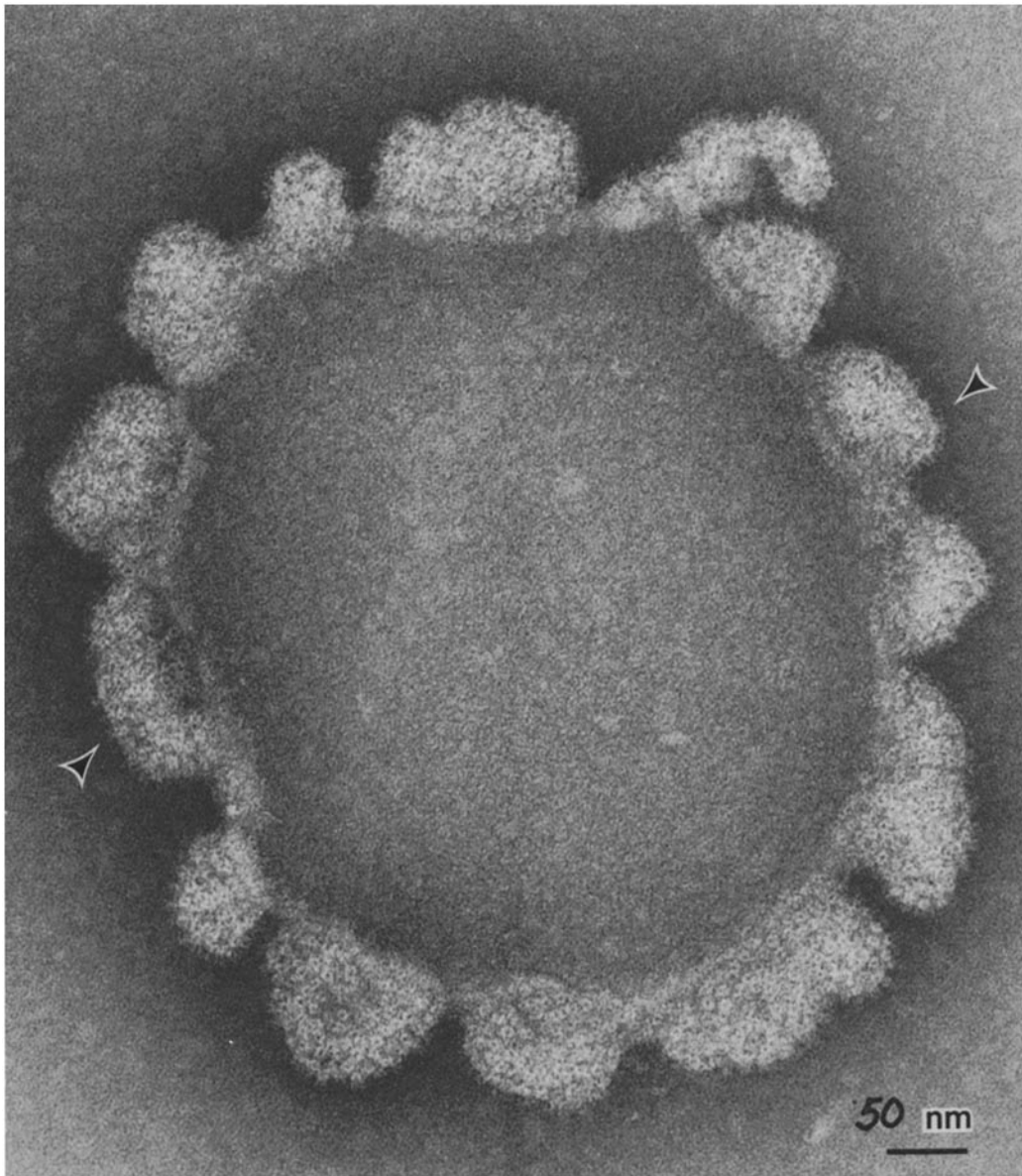


FIGURE 6 A digested receptor vesicle that clearly shows the ring of receptors and the particle-free central area of the membrane. The 55-Å high receptor head can clearly be seen all along the membrane edge (arrows). $\times 215,000$.

tion and the proportion of vesicles transformed are similar in this case.

In alkali-stripped membranes, all receptor subunits can be digested such that no intact subunits remain, whereupon the morphological transformation occurs in all vesicles. Neubig et al. (29) first reported that alkali treatment removes non-

receptor, peripheral membrane proteins from acetylcholine receptor membrane fractions and leaves the receptor membranes intact and functional. With the use of their stripping techniques, a number of major polypeptides were solubilized (Fig. 2*b*). Most notably, a doublet corresponding to the 43,000-dalton polypeptide proposed by Heidmann

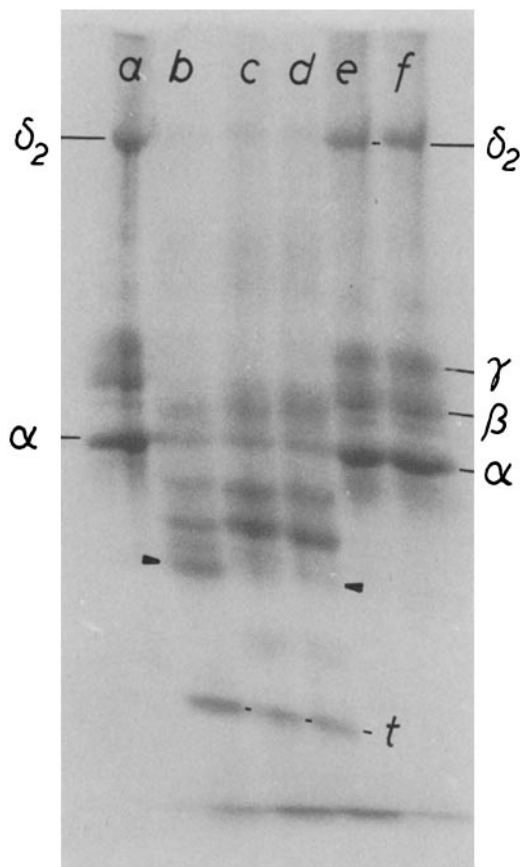


FIGURE 7 (a) The SDS PAGE pattern for undigested dimeric receptor. Digestion of the dimer receptor membranes (b) results in the same pattern as does the digestion of monomeric receptor (see Fig. 9). If trypsin is partially inhibited by incubation with either PMSF (c) or benzamidine (d) (at 100-fold molar excess) trypsin digestion of receptor membranes is incomplete and does not reach the "limiting" 27,000-dalton stage indicated by closed arrows and seen in b. Tracks e and f are for incubation with inhibitor alone (no trypsin) (e, PMSF; f, benzamidine). *t* marks the position of trypsin.

and Changeux to be the receptor ionophore (16) and a second doublet of ~55,000 daltons were removed and released into the supernate. The relative proportions of the receptor subunits sometimes change upon alkali treatment for reasons that are unclear, but none of the subunits is preferentially released into the supernate (Fig. 2b). That the α , β , γ , and δ polypeptides are receptor subunits is supported by both immunological (6) and photoaffinity experiments (28).

Alkali-stripped membranes have higher specific

activities than unstripped membranes have simpler SDS PAGE patterns (Fig. 2c), and appear quite similar to the unstripped membranes in negative-stain electron micrographs. The stripped membranes have significantly lower densities by isopycnic centrifugation in either sucrose (1.14 g/cm³) or glycerol (1.16 g/cm³) (Fig. 10) than do conventionally prepared membranes (1.15 and 1.17 g/cm³, respectively). These observations indicate that some of the solubilizable peripheral proteins are normally contained within the receptor membranes.

The extent to which these polypeptides are removed during purification and alkali treatment appears to greatly affect the sensitivity of the receptor molecule itself to proteolytic degradation (see Fig. 9). The extent of receptor degradation assayed by SDS PAGE parallels the extent of the morphological transformation of the vesicles. After alkali stripping, membranes can be degraded by trypsin so that no distinguishable receptor subunits remain (Fig. 9, bottom) and all the vesicles are morphologically transformed.

The stripped receptor membranes after full limit protease digestion contain particles that appear identical to normal receptor particles (Figs. 6 and 11) and the membranes retain full specific activity. D-tubocurarine is fully competitive with α butx binding (see Fig. 12), indicating that the α butx binding is still specific. Isokinetic sedimentation analysis of Triton-solubilized receptor with catalase ($S = 11.3$) and β -D-galactosidase ($S = 16$) as standards show that the toxin-binding component has an apparent S value of about 9 (Fig. 13), the same as that for undigested receptor.

Membranes prepared by us by the procedure of Chang and Bock (5) contain >80% of their receptor population in a form that sediments at 13S, and have been shown to be a dimeric form, in which receptor monomers are linked by a disulphide bond between δ (65,000 daltons) subunits (5, 13, 39, 40, 19, 14). Such membranes respond to trypsin digestion in the same way as does the monomeric receptor (Fig. 7). The trypsin-digested form of the dimeric molecule sediments at 9S. The decreased S value indicates that the disulphide-bridged δ subunits have been cleaved on both sides of the sulfhydryl in at least one of the cross-linked polypeptide chains.

DISCUSSION

Because ~95% of the receptor-containing vesicles are oriented with their original extracellular sur-

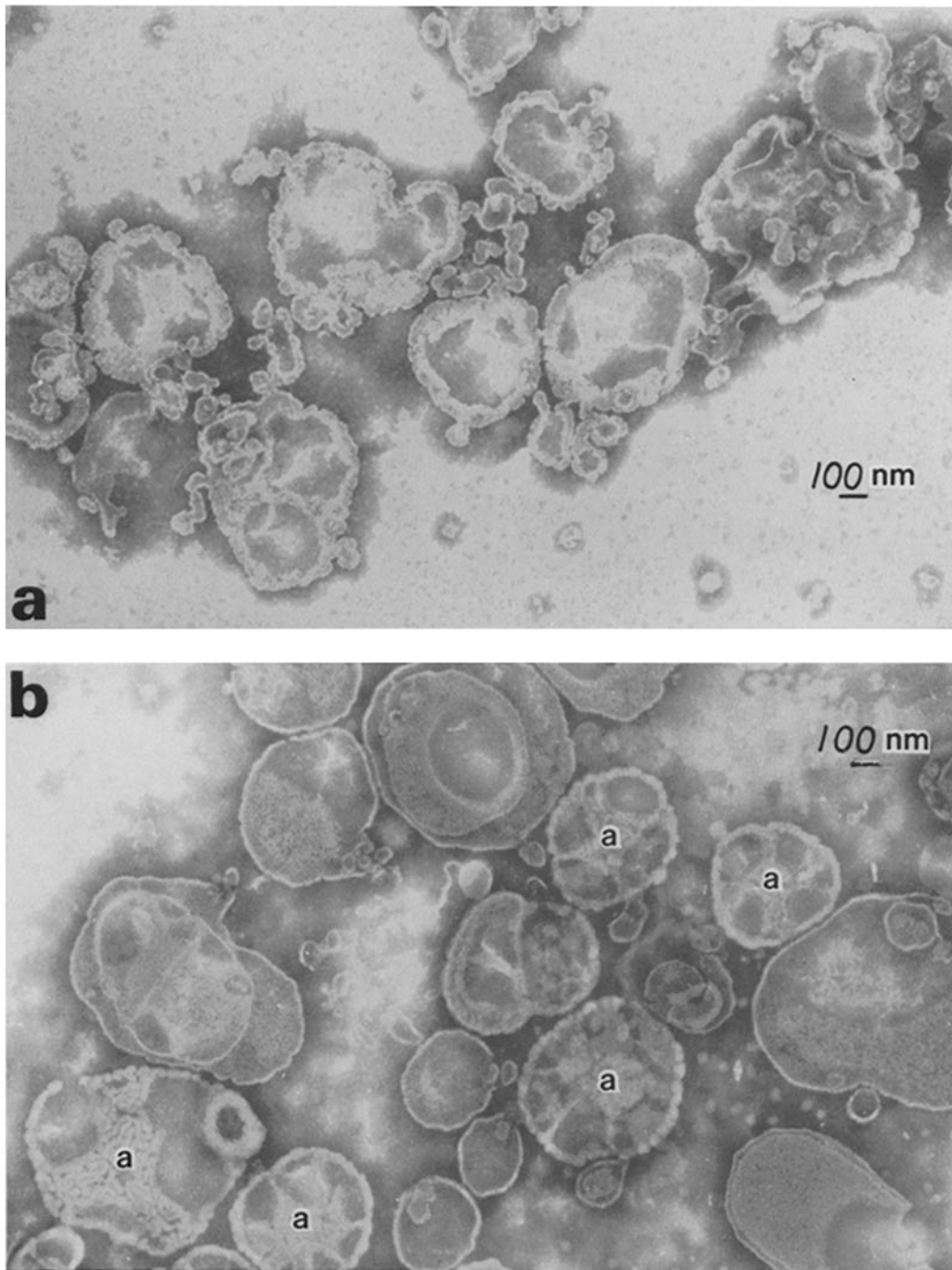


FIGURE 8 Membranes taken from (a) the uninhibited trypsin digestion (Fig. 7, track b) and (b) the PMSF-inhibited trypsin digest (Fig. 7, track c) as visualized by negative-stain (PTA) microscopy. Altered vesicles in b are marked by a. $\times 40,000$.

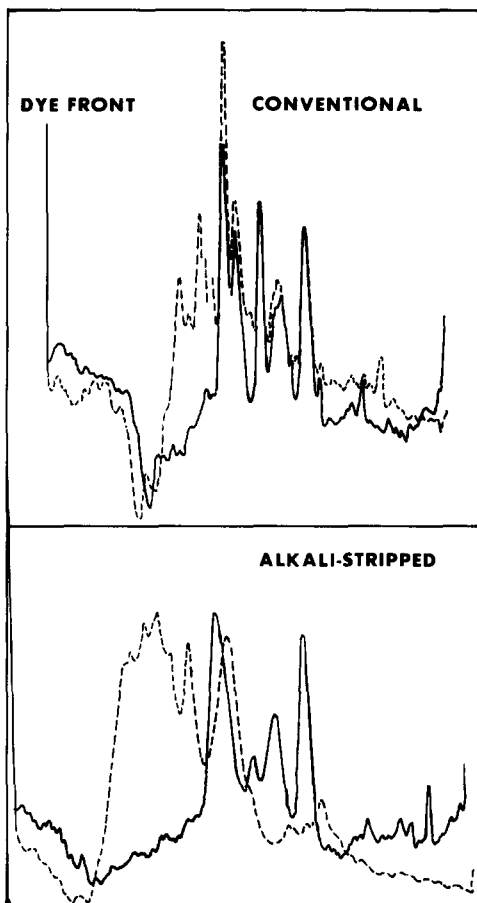


FIGURE 9 The effects of trypsin digestion on the subunit pattern of both conventionally prepared (top) and alkali-stripped membranes (bottom) are shown in these densitometer tracings of SDS PAGE patterns. The top pair of before (solid line) and after (dashed line) limit trypsin digestion reveals that in these conventional membranes little of the receptor subunits are degraded. In alkali-treated membranes, however, all of the receptor subunits have been digested.

faces out (8, 15, 21), and because the receptor molecule projects by $\sim 55 \text{ \AA}$ above the membrane surface (21) (Figs. 6 and 11*b*), it seemed reasonable that protease digestion would either release a soluble, toxin-binding fragment, or result in a preferential digestion of specific subunits giving an indication of their location with respect to the membrane. Kalderon and Silman (20) had reported that autolysis and trypsin digestion would solubilize a toxin-binding receptor fragment without the use of detergents.

After extensive digestion of receptor-rich mem-

branes with trypsin that leaves none of the receptor subunits as intact polypeptides on SDS PAGE (Fig. 9*b*), Triton-solubilized receptor species from membranes containing either monomer or dimer receptor remain as tightly associated aggregates which sediment as single 9S components, the same as for undigested receptor monomer (Fig. 13). In the absence of detergents, the receptor remained

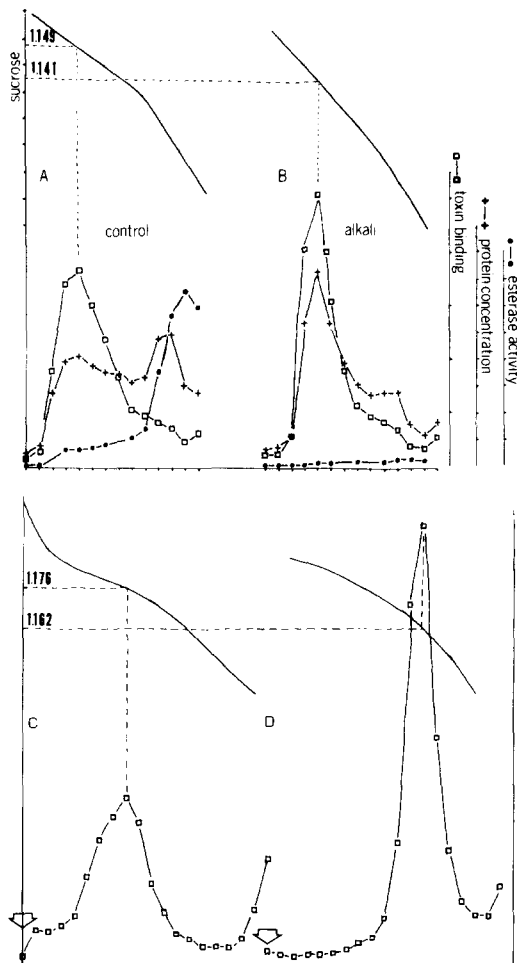


FIGURE 10 Gradient profiles in sucrose (*A* and *B*) and glycerol (*C* and *D*) of ^{125}I - α -btx-receptor membrane complexes show the difference in density between normal (*A* and *C*) and alkali-stripped membranes. The membranes were stripped in base as described in the text before having been run on the sucrose gradient, and then peak fractions were run on glycerol gradient. ^{125}I - α -btx receptor is plotted (\square), in all parts. Protein concentration (+) and acetylcholine esterase (\bullet) activity are plotted in parts *A* and *B*. Esterase activity is totally abolished by the base treatment.

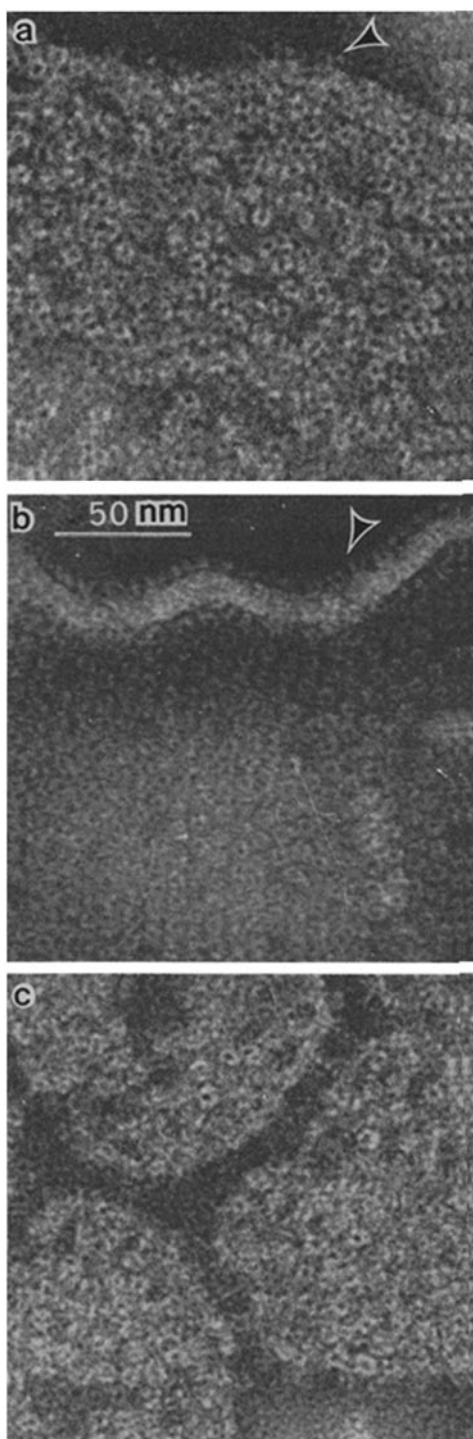


FIGURE 11 Receptor molecules in (a) conventionally prepared, (b) alkali-stripped, and (c) alkali-stripped, trypsin-digested membranes appear quite similar in neg-

ative stain. Arrows in *a* and *b* point out edge structure arising from receptor heads. The edge effect is more prominent in the ordered receptor in *b*. All, $\times 358,000$.

membrane bound (Fig. 6). Trypsin-digested receptor still binds α butx, and the binding can be competed for with the unrelated antagonist D-tubocurarine (Fig. 12), demonstrating that the active site for agonist/antagonist binding is preserved. The receptor particles appear unchanged in negative-stain electron microscopy after trypsin digestion (Figs. 6 and 11), in keeping with the observations of Carroll et al. (2) that the receptor resists denaturation by 6 M guanidinium salts. Shamoo and Eldefrawi (36) in fact reported that a trypsin-digested acetylcholine receptor would, upon reconstitution, respond consistently to acetylcholine and carbamylcholine, producing a monovalent cation flux.

The time-course of trypsin digestion at room temperature (using alkali-treated membranes) indicated a fairly uniform rate of degradation of all the protein components of the membranes, including the receptor subunits (22), and so failed to provide any direct evidence as to which subunits are most exposed within the membrane. All subunits seem to be about equally accessible to the aqueous phase.

However, even though the gross physical characteristics of the receptor molecule were relatively unchanged by trypsin digestion, striking changes did occur in receptor vesicle morphology (Figs. 3–6). Electron microscopy of vesicles in various stages of morphological transformation suggests that the process begins with a critical proteolytic clip in an unidentified “structural” membrane component, which causes the receptor molecules to aggregate (Fig. 5*b*), leaving apparently empty phospholipid bilayer alongside the receptor aggregates (Fig. 5*c*). The scalloped appearance of the membranes in negative stain (Figs. 3 and 6) was caused by the inherent curvature of the receptor aggregates (Fig. 5*b*). The continuation of this process results first in dome formation and then the budding out and beading up of the receptor-rich minivesicles (Fig. 5*c*).

One possible mechanism for the observed protease-induced morphological effect would be a specific aggregation of receptor molecules such that the axes of adjacent molecules perpendicular to the membrane bilayer are no longer nearly parallel as they would be in the membrane of a

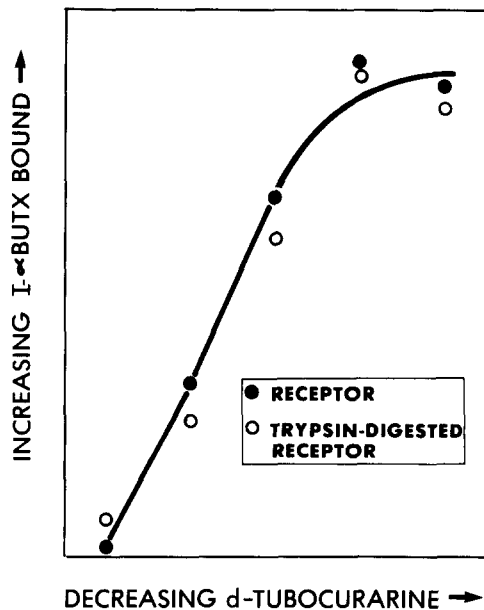


FIGURE 12 Incubation of either normal (solid circle) or digested (open circle) receptor membranes with ^{125}I - α butx and varying amounts of D-tubocurarine (final concentrations ranging from 3 to 0.3 μM) results in identical curves for the binding of the α butx to the receptor.

large vesicle, but in the final budded vesicle form an angle of $\sim 10^\circ$. This would result in the surprisingly uniform curvature of the mini-vesicles observed. This also would explain how the strings of mini-vesicles are formed, the juncture between blebs being weak points where the membranes might split off (Fig. 3).

The development of the blebbed vesicle morphology occurs much more slowly than the initial trypsin cleavages (hours vs. minutes) and seems to correlate more with the completion of trypsin digestion rather than its onset. Such a conclusion is supported by the results of inhibition experiments where inhibition was incomplete. In such cases, where a limit digest pattern was not attained (Fig. 7) most of the vesicles were of normal or only slightly altered morphology when examined by negative-stain electron microscopy (Fig. 8).

Coakely et al. (7) noted a roughly similar blebbing effect on heat-denatured and sheared erythrocytes. In our case, however, where there is no shear stress or obvious lipid effects, an alternative explanation is that the blebbing is caused by the aggregation of the receptor particles within the plane of the membrane and to the geometry of

that aggregation (Fig. 5). The protease is not incorporated into the membranes, and is not seen on gels if the membranes are sedimented before electrophoresis. We have also noted a receptor membrane blebbing effect distinctly different than that produced by proteases caused by ethanol at concentrations $>10\%$ (22). In addition, as receptor vesicle preparations aged (>10 – 14 d), some of the vesicles tended to assume shapes similar to those observed upon proteolysis. While the activity of endogenous protease declines with receptor membrane purity, proteolytic activity was detected even in highly purified preparations ($>90\%$ receptor by SDS PAGE) (22).

The protease-susceptible component(s) that cause or allow receptor to aggregate in the membrane may be either a part of the receptor itself, one of the recognizable nonreceptor polypeptides present in the membranes, or a component present as a minor but as yet unrecognized constituent. This question is somewhat simplified since removal of the peripheral proteins (Fig. 2*b*) leaves membrane shape unchanged and yet enhances susceptibility to the protease-induced effects, so suggesting that a receptor subunit is responsible.

That some of the peripheral proteins are located in receptor-containing vesicles is demonstrated by the decrease in receptor membrane density after their removal upon alkali-stripping, by 0.008 g/cm^3 in sucrose, and by 0.014 g/cm^3 in glycerol as measured by isopycnic centrifugation (Fig. 10). The receptor molecules in the alkali-treated membranes appear equally if not more densely packed (see Fig. 11*a*, and *b*), and very little receptor (Fig. 2) is solubilized, implying that the decrease in membrane density is a direct result of the removal of peripheral proteins.

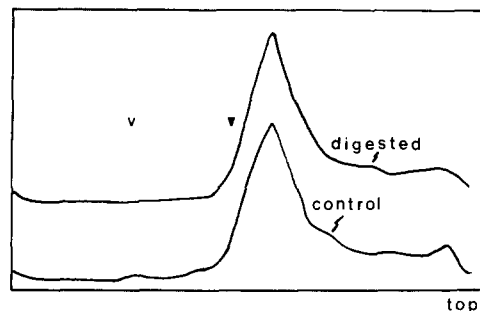


FIGURE 13 Both predigestion control and digested receptor bind toxin and form a complex which sediments with a value of 9S. Open and closed v's mark positions of β -galactosidase and catalase in each of the velocity gradients.

Because alkali-treated membranes contain almost pure receptor (Fig. 2c), and because as visualized by electron microscopy (Fig. 5) the morphological effects appear to be caused by a specific aggregation of receptor molecules, we conclude that the most likely candidate for the target protein is some component of the receptor itself.

The possibility that the vesicle structure change is caused not by the protease itself but rather by another contaminating enzyme such as a lipase is excluded because specific inhibition of trypsin by purified STI completely blocks both subunit degradation (as judged by SDS PAGE) and the morphological changes in the membranes as visualized by electron microscopy.

The results reported here suggest that each of the five receptor subunits is accessible to the aqueous phase on the extracellular side, indicate the complexity of the receptor-rich membranes isolated from *Torpedo californica* electroplaques, illustrate the insensitivity of ligand binding to proteolytic degradation of the receptor, and raise the possibility that integral membrane proteins such as the acetylcholine receptor may have structural roles in determining membrane morphology as well as their more obvious functional ones.

ADDENDUM

After this paper had been submitted, Bartfeld and Fuchs (1979, *Biochem. Biophys. Res. Commun.* **89**:512-519) reported that trypsin digestion of detergent-solubilized acetylcholine receptor produces an 8S species composed of a single 27,000-dalton polypeptide which retains all native immunological and ligand binding properties. Further experiments (M. W. Klymkowsky, Ph.D. Thesis, California Institute of Technology) indicate that detergents do not alter the limit digest pattern but increase the rate and extent of digestion, and that the amount of intact 43,000-dalton peripheral polypeptide remaining after digestion is proportional to the amount of undigested α subunit under limit digest conditions.

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