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Functional Stability of *Torpedo* Acetylcholine Receptor. Effects of Protease Treatment†

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ABSTRACT: The effect of tryptic degradation on structural and functional properties of the membrane-bound acetylcholine receptor from *Torpedo californica* has been investigated. Under conditions of proteolysis which resulted in extensive degradation of receptor subunits, the membrane preparations retained their full capability of mediating agonist-induced cation flux as measured in rapid kinetic experiments. Low concentrations of trypsin also cleaved receptor dimers to monomers, and this effect was paralleled by degradation of the M_r 65 000 subunits which are known to contain sulfhydryl group(s) involved in receptor dimerization through an interchain disulfide bond(s). This conversion to monomers occurred at lower trypsin concentrations when the enzyme was added to the outside of the vesicles compared with the effects observed when the enzyme was present inside the vesicles. Similarly M_r 43 000 protein consistently found in preparations of the membrane-bound acetylcholine receptor, which can readily be removed without apparent effect on receptor function, displayed greater susceptibility to proteolysis when trypsin was added to the exterior medium rather than inside the vesicles. The results emphasize the full functionality of the monomeric form of the acetylcholine receptor comprised of four polypeptides.

The nicotinic acetylcholine receptor (AcChR)[1] from the electric organ of *Torpedo californica* is a pseudosymmetric complex of four homologous subunits of molecular weight 40 000, 50 000, 60 000, and 65 000 in the stoichiometry of 2:1:1:1 (Raftery et al., 1980). Following detergent extraction the receptor sediments on sucrose gradients as both a 9S

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[1] Abbreviations: AcChR, acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; α-BuTx, α-bungarotoxin; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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monomer and a 13.7S dimer (Raftery et al., 1972), and it has been shown (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978) that dimerization arises from disulfide bond(s) cross-linking between the 65 000 molecular weight subunits.

The AcChR has been shown to be susceptible to attack by exogenous proteases. Extensive tryptic degradation of the detergent solubilized (Bartfeld & Fuchs, 1979) or the membrane-bound (Klymkowsky et al., 1980) receptor led to complete degradation of the four subunits as determined by polyacrylamide gel electrophoresis of the receptor after denaturation in $NaDodSO_4$. These preparations did, however, retain their ability to bind both specific antibodies and cholinergic ligands. The sedimentation coefficient of the trypsin-treated receptor was only slightly reduced from 9.3 to 8.1 S (Bartfeld & Fuchs, 1979). Lindstrom et al. (1980a,b) have recently shown that papain treatment also led to dramatic alterations in $NaDodSO_4$ gel patterns of AcChR subunits without significantly affecting either the morphology of the preparations or their sedimentation behavior. Therefore, many of the fundamental properties of the receptor seem to be unaltered by proteolytic degradation.

The subunit composition of the AcChR has been widely debated due to reports of preparations from *Torpedo marmorata* (Sobel et al., 1977) which, upon $NaDodSO_4$ electrophoresis, gave only a single major subunit of about 40 000 daltons. This difference from the observed subunit composition of *T. californica* may be due to the different susceptibilities of the subunits to degradation by endogenous proteases during purification since the higher molecular weight subunits are more readily degraded by endogenous Ca^{2+}-activated proteases (Vandlen et al., 1979), trypsin (Strader & Raftery, 1980) and papain (Lindstrom et al., 1980a,b).

In the membrane-bound state the predominant form of the *Torpedo* AcChR is the dimer (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978). The functional significance of the dimeric form is not yet clear since monomers and dimers have a similar capability of mediating cation flux (Wu & Raftery, 1981). Dimers have not yet been identified in all AcChR preparations, such as those from *Electrophorus*. Some proteases like trypsin and chymotrypsin are able to cleave the dimers (Lindstrom, 1976), but others, like papain, are not (Lindstrom et al., 1980a,b).

Lindstrom et al. (1980a,b) have recently reported that, after treatment with papain, AcChR reconstituted into lipid vesicles retained the $^{22}Na^+$ flux response to the addition of carbamoylcholine. This technique for determination of cation transport (Kasai & Changeux, 1971), because of its limited time resolution, gives only a qualitative measure of receptor function, and even if only a single channel per vesicle is capable of activation after proteolysis, a full flux amplitude may be observed (Wu & Raftery, 1980).

The recent development of a rapid kinetic technique based on agonist-mediated Tl^+ influx leading to its quenching of the fluorescence of a fluorophore trapped within the vesicles (Moore & Raftery, 1980) allows a quantitative measure of the rate of ion transport. Since the rate of ion flux is directly proportional to the number of open channels, this method is sensitive to relatively small changes in receptor function and thus permits a quantitative and high-resolution analysis of functional perturbations.

In this paper, we describe the effects of tryptic degradation of the interior and exterior vesicular surfaces on structural and functional properties of the membrane-bound acetylcholine receptor from *Torpedo californica*. Proteolytic effects on the Tl^+ flux rate and the receptor monomer–dimer ratio have been examined, and in addition, the relative sensitivities of the inside and outside of the vesicles to trypsin have given information on structural organization within the membrane.

Materials and Methods

Membrane Preparations. AcChR-enriched membrane preparations were prepared from *Torpedo californica* electric organs as described by Elliott et al. (1980) and were routinely submitted to one cycle of extraction to remove peripheral proteins (Neubig et al., 1979; Elliott et al., 1979, 1980). The concentration of α-BuTx binding sites was measured by the DEAE disc assay of Schmidt & Raftery (1973) using [^{125}I]-α-BuTx obtained from New England Nuclear and calibrated by the procedures of Blanchard et al. (1979). Protein concentrations were determined by the method of Lowry et al. (1951), and the specific activities of the membrane preparations lay in the range 2–4 nmol of α-BuTx sites/mg of protein. In each experiment the buffer used was 10 mM Hepes and 35 mM $NaNO_3$, pH 7.4.

Tryptic Degradation of Vesicles. The methods used for tryptic degradation of membrane vesicles were modified from those of Strader & Raftery (1980) by using bovine pancreatic trypsin (type 1) and soybean trypsin inhibitor (type 1-S) from Sigma Chemical Co. In each experiment the membrane fragments were diluted so that for each trypsin concentration used the trypsin–membrane protein ratio (w/w) was constant.

Trypsin was loaded inside the vesicles by freezing the preparations in N_2 immediately after addition of the chosen trypsin concentration and then thawing slowly at 4 °C. In most experiments a second freeze–thaw procedure was carried out immediately after the first, although this was found to have no apparent effect on the experimental results. In order to permit adequate resealing, the thawed vesicles were allowed to warm rapidly to about 8 °C, and then the trypsin inhibitor was immediately added in 600-fold molar excess over trypsin to inhibit degradation on the outside of the vesicles. After 60-min incubation at room temperature, the trypsin inhibitor was equilibrated with the vesicle interior by a further freeze–thaw cycle, thus terminating the proteolytic reaction.

Tryptic degradation on the outside of the vesicles was achieved by first subjecting the membrane suspension to one or two freeze–thaw cycles in the absence of enzyme and then incubating with trypsin for 60 min at room temperature. Excess trypsin inhibitor was added and a further freeze–thaw cycle was carried out.

Thallium Flux Kinetics. The ability of the AcChR-enriched membrane fragments to mediate agonist-induced cation flux after trypsin treatment was measured by the Tl^+ fluorescence quenching method of Moore & Raftery (1980). Membrane vesicles were loaded with the fluorescent probe ANTS, and the fast kinetics of Tl^+ flux across the membrane were measured by using the stopped-flow instrumentation previously described (Moore & Raftery, 1980). The effect of trypsin degradation on the outside of the vesicles was measured by incubating the ANTS-loaded vesicles with trypsin for 20–60 min at room temperature prior to addition of excess trypsin inhibitor. These preparations were used in the Tl^+ flux experiments without further treatment. Degradation on the inside of the vesicles was achieved by first loading trypsin inside by one freeze–thaw cycle and inhibiting the external trypsin with trypsin inhibitor as described above. After incubation for 60 min at room temperature, 2 volumes of 25 mM ANTS and 10 mM Hepes, pH 7.4, was added, and the mixture was subjected to two further freeze–thaw cycles to load the ANTS

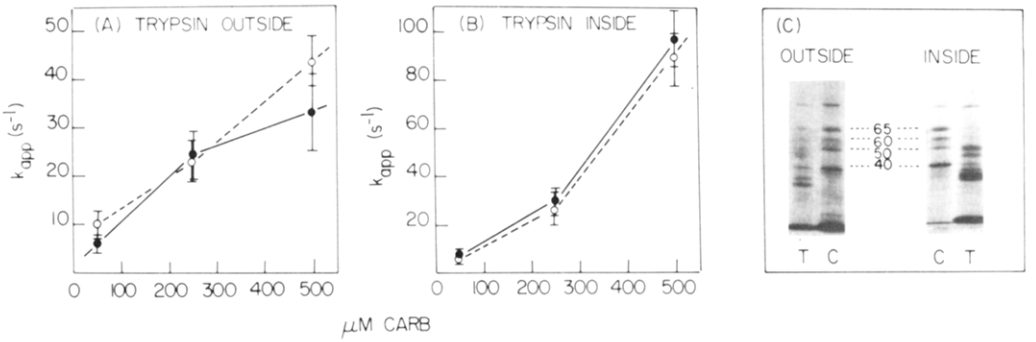

FIGURE 1: Effect of trypsin on the ability of AcChR-enriched membrane fragments to mediate agonist-induced cation transport. The rate of Tl^+ flux was measured at three Carb concentrations for control membranes and for those which had been treated with 50 nM trypsin [0.27% (w/w)] on the outside (A) or inside (B) of the vesicles. Data were analyzed as described in the text, and the results are the averages of at least four kinetic traces recorded at each Carb concentration. The error bars are ±standard deviation. Experiments were carried out at 25 °C. (C) Acrylamide gel electrophoresis of the control (C) and trypsin-degraded (T) membrane fragments which were used in the Tl^+ flux experiments.

and the trypsin inhibitor within the vesicles. Following removal of external ANTS by gel filtration, the membranes were used in the stopped-flow experiments without further dilution.

Kinetic Data Analysis. Thallium flux data were analyzed by a nonlinear regression method using Fortran programs adapted from those of Dunn et al. (1980). The data were fitted to

$$F(t) = \frac{F_0}{1 + KT_\infty[1 - \exp(-k_1 t)]} + A_1 + k_2 t$$

where $F(t)$ and F_0 are the fluorescence levels at time t and equilibrium, respectively, k_1 is the apparent rate of Tl^+ flux, A_1 is the data base line, and k_2 is the slope of this base line. The term KT_∞ was fixed as a constant and was calculated from the known final Tl^+ concentration, T_∞, of 17 mM and the Stern–Volmer constant, K, of 96 M^{-1} (Moore & Raftery, 1980).

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by using the system of Laemmli (1970) and 8.75% acrylamide gels.

Gel electrophoresis in sodium cholate was carried out by using gels of 4% acrylamide, 0.14% bis(acrylamide), and 0.1% sodium cholate in the buffer system of Fairbanks et al. (1971) at 80% strength as described by Deutsch & Raftery (1979). Samples for these gels were prepared by incubating the membranes (~2.5 mg of protein/mL) with 2% sodium cholate for 5 min at room temperature followed by removal of insoluble material by centrifugation for 10 min in a Beckman airfuge. Gels were stained with Coomassie Brilliant Blue and were scanned at 550 nm by using a Gilford Model 240 spectrophotometer equipped with a linear transport unit.

Sucrose Gradient Centrifugation. Linear gradients of 5–20% sucrose in 0.1% Triton, 10 mM sodium phosphate, and 50 mM NaCl, pH 7.4, were layered with 100-μL samples prepared by solubilizing membrane fragments with 1% Triton X-100 followed by incubation with [^{125}I]-α-BuTx. Gradients were centrifuged for 16–18 h at 40 000 rpm in a Beckman SW41 rotor, and fractions were collected from the bottom of the tube.

Results

Incubation of AcChR-enriched membrane vesicles with 50 nM trypsin [0.27% (w/w) trypsin/AcChR] led to extensive changes in the receptor subunit pattern visualized by $NaDodSO_4$–acrylamide gel electrophoresis (Figure 1). Untreated membranes gave a pattern characteristic of the subunits of the purified receptor having molecular weights of 40 000, 50 000, 60 000, and 65 000. In some preparations the peptide of M_r 43 000 was still present after alkali treatment (see Figure 5 and Materials and Methods). Exposure of either the interior or exterior surface of the vesicles to trypsin resulted in significant degradation of each of these subunits to peptides of lower molecular weight. Under the two sets of conditions the gel patterns were, however, quite different (Figure 1C), and the relative susceptibilities of each subunit to degradation from the interior or exterior medium were dependent on the trypsin concentration used.

The effect of proteolysis on the ability of the AcChR to mediate Tl^+ flux in response to the binding of carbamoylcholine was examined in stopped-flow experiments. When membrane vesicles loaded with ANTS were rapidly mixed with buffer containing Tl^+ (10 mM Hepes and 17 mM $TlNO_3$, pH 7.4; final concentration after mixing), the signal change observed was a slow quench (k_{app} ~0.1 s^{-1}) characteristic of the leakage of Tl^+ across the membrane. This leak rate was unchanged after 30-min incubation of the loaded vesicles with 50 nM trypsin [0.27% (w/w)], and in addition, the signal amplitude remained constant, indicating that proteolysis did not cause a significant leakage of the fluorophore from the vesicles. A further significance of this result is the likelihood that if the vesicles remain sealed to ANTS they will also remain sealed to trypsin and trypsin inhibitor during the incubation period, an assumption which is critical for estimation of the relative effects of tryptic digestion from the interior or exterior of the vesicles.

When carbamoylcholine was included in the buffer with $TlNO_3$, a marked increase in the rate of Tl^+ transport was observed. This agonist-induced stimulation of the rate of Tl^+ flux was also displayed by membrane vesicles which had been incubated with trypsin. An analysis of the flux rates at Carb concentrations of 50, 250, and 500 μM has been made, and the data are shown in Figure 1A,B. It is clear that treatment of the vesicles with 50 nM trypsin on either the exterior or interior surface had no effect on the measured flux rate. The AcChR therefore retained its full functional capacity for ion translocation even after extensive proteolytic degradation of its subunits, as evidenced by the gel patterns in Figure 1C.

Subjection of the exterior surface of the vesicles to the more extreme trypsin concentrations of 0.5 and 1.5 μM also caused no reduction in the rate of Tl^+ transport. Under these conditions the intact subunits characteristic of the purified receptor were almost completely degraded. These results emphasize the remarkable resistance of the flux response to tryptic degradation.

The sedimentation behavior of the trypsin-treated receptor was investigated by sucrose gradient centrifugation. Mem-

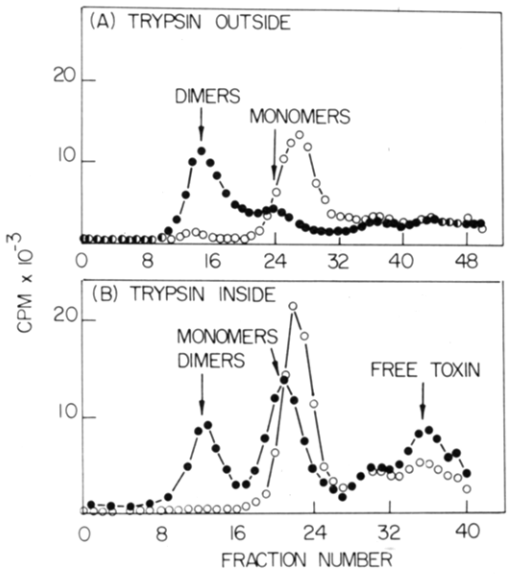

FIGURE 2: Sedimentation behavior of AcChR before (●) and after (○) treatment with 50 nM trypsin [0.27% (w/w)] on the outside (A) or inside (B) of the vesicles. Samples werre labeled with [^{125}I]-α-BuTx after solubilization in 1% Triton. After centrifugation on 5–20% linear sucrose gradients, fractions were collected from the bottom of of the tube and counted for ^{125}I.

Table I: Sedimentation Behavior of AcChR before and after Tryptic Degradation[a]

treatment	app sedimentation coefficient(s) monomers	dimers
control	9.0[b] ± 0.1	13.7[b] ± 1.0
trypsin outside	8.4 ± 0.2	14.0 ± 0.5
trypsin inside	8.4 ± 0.1	13.6 ± 0.3

[a] The results are the averages of three experiments in which the trypsin concentration was varied from 38 [0.2% (w/w)] to 500 nM [2.7% (w/w)]. [b] Values were normalized to 9.0 and 13.7 for the controls.

brane fragments were incubated with trypsin on the inside or outside of the vesicles and then labeled with [^{125}I]-α-BuTx. Figure 2 shows profiles obtained for control preparations and those after degradation by 50 nM trypsin, i.e., conditions identical with those used in the kinetic experiments. Proteolysis from either side of the membrane led to an almost complete conversion of receptor dimers to monomers (from 95% to 100%). The profiles shown in Figure 2 suggest that the monomers obtained by trypsin treatment may be slightly lighter than those of the control preparation. The relative mobilities of the degraded species were therefore compared with those of the untreated receptor, and the apparent sedimentation coefficients averaged from three such experiments are listed in Table I. A slight reduction in the apparent size of the monomers was found after treatment.

The sidedness of the vesicles was investigated by comparing the [^{125}I]-α-BuTx binding activity of membranes solubilized in 1% Triton before being labeled with excess toxin with that of vesicles which had only their exterior sites labeled by incubation with radiolabeled toxin followed by addition of excess cold BuTx before solubilization. In each case the total amount of radioactive toxin bound was identical, suggesting that almost all of the vesicles were oriented with their α-BuTx sites exposed to the outside medium, in agreement with previous results from similar preparations (Hartig & Raftery, 1979; Strader & Raftery, 1980).

At the concentration of trypsin employed for treatment of the membranes used in the thallium flux experiments, an

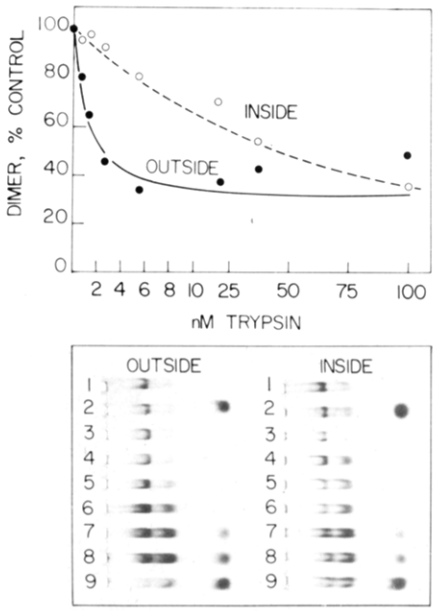

FIGURE 3: Effect of trypsin concentration on the receptor monomer–dimer equilibrium. Membrane vesicles were incubated with trypsin for 60 min as follows: (1) 0; (2) control, 100 nM trypsin + 60 μM trypsin inhibitor added simultaneously; (3) 0.69 nM; (4) 1.38 nM; (5) 2.75 nM; (6) 5.5 nM; (7) 22 nM; (8) 38 nM; (9) 100 nM. Samples were run under nondenaturing conditions on 4% acrylamide gels in 0.1% sodium cholate. Gels were stained with Coomassie Brilliant Blue, and the relative contributions of monomers and dimers were estimated from their staining intensities at 550 nM.

almost complete conversion to receptor monomers occurred. The dependence of the monomer–dimer equilibrium on trypsin concentration was therefore examined in experiments in which the relative concentration of each species was estimated by gel electrophoresis of the proteolyzed membrane samples in 0.1% sodium cholate, i.e., nondenaturing conditions. The gel patterns shown in Figure 3 illustrate clearly the conversion of dimers to monomers as the trypsin concentration was raised from 0 to 100 nM. Scanning of these gels allowed a quantitative estimate of this conversion, and Figure 2 shows that the disappearance of dimers occurred at a lower trypsin concentration when the enzyme was added to the outside of the vesicles.

Dimerization of the AcChR results from a disulfide linkage(s) between the 65 000 molecular weight subunits (Chang & Bock, 1977; Suarez-Isla & Hucho, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978). It was therefore of interest to investigate whether the disappearance of dimers as a consequence of tryptic degradation was paralleled by a loss of this 65 000-dalton protein. Membrane samples which had been treated in a manner identical with those shown in Figure 3 were therefore electrophoresed on $NaDodSO_4$–polyacrylamide gels, and the results are shown in Figure 4. Examination of these gel patterns indicates that, while very low concentrations of trypsin had little apparent effect on the subunit composition, when the concentration of trypsin was raised, the 65 000-dalton subunit had a greater susceptibility to proteolytic degradation on the outside of the vesicles. The staining intensity of this band was used to quantitate the relative amount of the subunit remaining at each trypsin concentration, and the results are illustrated in Figure 4. The disappearance of the M_r 65 000 subunit therefore had a qualitatively similar trypsin dependence to the loss of dimers (Figure 2).

A protein of M_r 43 000 is consistently found in preparations of AcChR-enriched membrane fragments (Sobel et al., 1977).

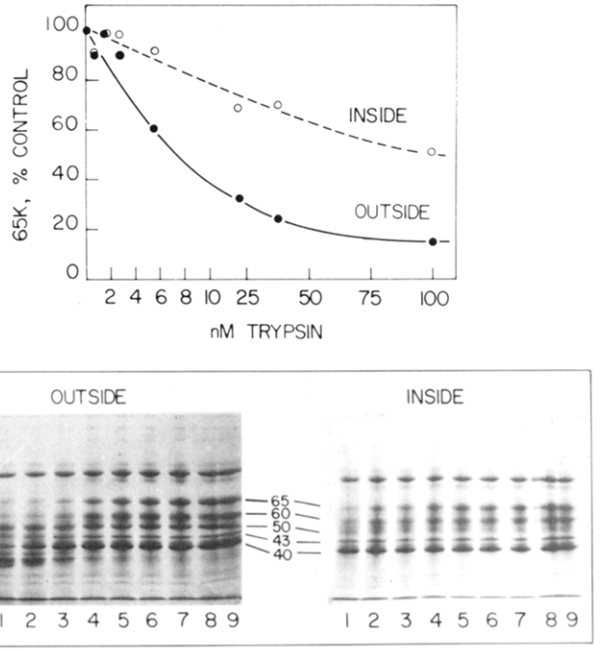

FIGURE 4: Polyacrylamide gel electrophoresis of membrane fragments after treatment with trypsin added on the outside or inside of the vesicles. Trypsin concentrations were the following: (1) 100 nM; (2) 38 nM; (3) 22 nM; (4) 5.5 nM; (5) 2.75 nM; (6) 1.38 nM; (7) 0.69 nM; (8) control, 100 nM trypsin added at same time as trypsin inhibitor; (9) no trypsin. Gels were stained with Coomassie Brilliant Blue and scanned at 550 nm. The amount of the M_r 65 000 subunit was estimated from its staining intensity relative to the total staining intensity of each sample.

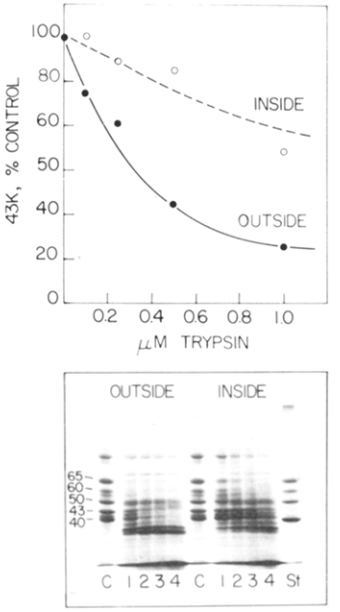

FIGURE 5: Effect of trypsin added on the outside or inside of the vesicles on the degradation of the M_r 43 000 protein. Trypsin concentrations used were (C) no trypsin control, (1) 100 nM, (2) 250 nM, (3) 500 nM, and (4) 1 μM. Track St was a standard preparation of solubilized AcChR showing the characteristic receptor subunits at M_r 40 000, 50 000, 60 000, and 65 000.

This component is a peripheral protein which can be removed by alkali treatment with no obvious detrimental effect on receptor function (Neubig et al., 1979; Elliott et al., 1979, 1980), and its significance is therefore unknown. Recently it has been reported that the 43 000-dalton chain is exposed only on the interior surface of vesicles derived from *T. marmorata* electroplax and as such is susceptible to degradation by proteolytic enzymes only when they are present during sonication of the membranes (Wennogle & Changeux, 1980). The effect of trypsin on the M_r 43 000 protein has therefore been investigated by using membrane preparations which, although they had been alkali extracted, retained a significant concentration of this protein. Figure 5 shows the results of these experiments. The protein was found to be relatively resistant to proteolysis, but with increasing concentrations of trypsin (0–1 μM), increased degradation occurred. In these experiments the extent of proteolysis was significantly greater when trypsin was added to the outside of the vesicles rather than to the inside.

Discussion

The experimental evidence presented here allows the conclusion that the nicotinic AcChR from *Torpedo californica* has extreme functional stability to proteolysis. As shown in Figure 1, even when no intact subunits of M_r 40 000, 50 000, 60 000, and 65 000 remained, as a result of proteolysis either from the inside or from the outside of the vesicles, extensively degraded preparations remained fully functional in terms of cation translocation. It could be argued that full functionality in the face of extensive degradation precludes major conformational changes of large segments of the molecule during the cation gating process. The finding that AcChR retains its function even after extensive proteolytic cleavage lends further credence to the validity of results obtained in electrophysiological experiments in which nerve terminals were stripped away following proteolytic treatment which probably caused extensive degradation of AcChR (Betz & Sakmann, 1973).

In the absence of any intact subunits, the extensively nicked monomers remained associated to a large extent, as indicated by the sedimentation coefficient (Figure 2). The only effect of trypsin on the sedimentation behavior, observed also at extremely low trypsin concentrations (Figure 3), was the conversion of dimers to monomers. Since this conversion of AcChR dimers to monomers had no effect on the rate of cation transport, the AcChR ion gating function does not require the organization of AcChR molecules as covalently bound dimers. Although we cannot exclude the possibility that in our experiments the monomers were still noncovalently associated as dimers, these results are consistent with the idea that the intact AcChR dimer is not necessary for normal function (Wu & Raftery, 1981).

After extensive tryptic degradation, vesicles containing AcChR undergo gross morphological changes with complete disruption of the normal receptor distribution (Klymkowsky et al., 1980). Since, after complete tryptic degradation, the cation transport properties of AcChR-rich vesicles are unaffected, no particular organization of receptors in this postsynaptic membrane is required for ion transport.

Comparison of the results shown in Figures 2 and 3 indicates some discrepancy in the values obtained with the two methods used for quantitation of the monomer–dimer equilibrium after trypsin treatment. The conversion to monomers appeared to be essentially complete from both the inside and the outside only when it was evaluated by density gradient centrifugation in the presence of Triton X-100. In cholate gels about 30% residual dimers were consistently present. This discrepancy may be explained if some trypsin-cleaved dimers still remained noncovalently associated in the presence of cholate.

The degradation of the 65 000-dalton subunit (Figure 4) seems to have a shallower trypsin concentration dependency than the splitting of dimers (Figure 3). This is expected, since whereas a single tryptic cleavage (i.e., of one of the two subunits) is sufficient for loss of dimer, one of the two M_r 65 000

subunits will remain intact. The susceptibility of AcChR dimers to proteolytic cleavage and the fact that some intact M_r 65 000 chain may still be left when all the dimers are cleaved may explain why in several other species like *Electrophorus* and various mammals, dimeric forms of AcChR are frequently absent. In this respect the lability of the chain corresponding to the *Torpedo* M_r 65 000 chain in *Electrophorus* AcChR preparations should be noted (Lindstrom et al., 1980). Thus, it cannot be excluded that the existence of dimeric forms is a common feature of AcChR from most creatures.

The monomers produced by tryptic cleavage were consistently slightly lighter than untreated preparations when sedimented on a density gradient; this slight shift in the sedimentation properties was particularly evident when the cleavage was carried out from the outside and was independent of the extent to which the AcChR subunits were degraded. This indicates that even after extensive nicking most segments of the degraded subunits remained structurally associated. However, a few of them, including the segments linking the M_r 65 000 subunits, are lost even at very low trypsin concentrations. The loss of only discrete pieces of the AcChR molecule after trypsin treatment is also indicated by the loss of specific antigenic determinants, all belonging to the "linear" type, i.e., formed by a linear sequence of amino acids in the primary structure rather than by the juxtaposition of different stretches in the secondary and tertiary folding (Bartfeld & Fuchs, 1979).

AcChR dimers can be converted to monomers by trypsin acting from either side of the membrane. The simplest explanation for this is that the intact M_r 65 000 subunit contains multiple trypsin-sensitive sites and that these occur on both sides of the postsynaptic membrane. Indeed, it has recently been shown that in detergent solution the 66 000-dalton subunit of *Torpedo marmorata* AcChR contains more than a single trypsin-sensitive bond(s) (Wennogle et al., 1981).

The interior and the exterior surfaces of AcChR-rich vesicles have different sensitivities to tryptic degradation. The sidedness of the effects of trypsin can be studied only if the membrane preparation used is formed by sealed vesicles. In our system the large majority of AcChR vesicles must have been sealed as indicated by the following observations:

(1) The gel pattern of the degradation products of AcChR was different, depending on whether trypsin was present inside or outside the vesicles. (2) When trypsin was added on either the outside or inside of the vesicles, there was a conversion of receptor dimers to monomers. Although the outside surface showed greater sensitivity at low enzyme levels, at high trypsin concentration the extent of conversion was virtually the same from either side. If the vesicles had been leaky when trypsin was added inside, leaky vesicles would be protected from degradation by the huge excess of trypsin inhibitor added to protect the outside, and a reduced final level of cleavage would be expected compared to the level observed when enzyme was added to the outside, where in the absence of any inhibitor, all the possible dimers would be split. (3) The sedimentation coefficient of the monomers produced by tryptic action from outside or from inside the vesicles was consistently slightly different. (4) In kinetic experiments the amplitude of the fluorescence change due to Tl^+ leakage, which is a measure of the concentration of ANTS within the vesicles, did not change after the addition of high concentrations of trypsin. This means that the vesicles remained sealed to small molecules after trypsin treatment.

In most AcChR-enriched membrane preparations, a protein of M_r 43 000 is consistently found. This protein is an extrinsic membrane constituent, since it is stripped away by alkali treatment at low ionic strength. This M_r 43 000 protein has been suggested as a major component of the microfilamentous network covering the cytoplasmic surface of *Torpedo* postsynaptic membranes (Sealock, 1980, 1981). Further support for this hypothesis has come from a report that this protein was susceptible to proteolytic degradation only when the protease was added inside the vesicles derived from postsynaptic regions (Wennogle & Changeux, 1980). Contrary to this finding, using alkali-treated membrane preparations where a significant amount of M_r 43 000 protein was left, at trypsin concentrations ranging between 0.1 and 0.1 μM, we obtained significant degradation of this protein from both sides, and particularly when trypsin was present outside the vesicles. As this M_r 43 000 protein is an extrinsic membrane component, our results indicate either that alkali treatment induced a redistribution of this protein on both surfaces or more likely that it was originally present on both surfaces. The precise location and functional significance of this entity on the postsynaptic membrane still remain obscure.

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Elongation Factor T_u·Ribosome Dependent Guanosine 5′-Triphosphate Hydrolysis: Elucidation of the Role of the Aminoacyl Transfer Ribonucleic Acid 3′ Terminus and Site(s) Involved in the Inducing of the Guanosinetriphosphatase Reaction†

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ABSTRACT: We have studied the interaction between *Escherichia coli* $tRNA^{Phe}$·poly(U)·70S ribosome·EF-T_u·GTP complex and 2′(3′)-*O*-(aminoacyl)nucleosides and -oligonucleotides (analogues of the AA-tRNA 3′ terminus) which triggers GTP hydrolysis. The results show that the binding of effectors (3′-terminal fragments of AA-tRNA) to an EF-T_u site, in the presence of ribosomes, triggers the GTPase. The affinity of effectors for the enzymatic complex depends greatly on their structure, e.g., K_a decreasing as the length of the oligonucleotide chain increases. Thus, K_a for Phe-$tRNA^{Phe}$ is approximately 1000-fold lower than that of C-C-A-Phe. On the other hand, the K_m for GTP is much less affected by the chain length, with the K^{GTP}_m in the presence of C-C-A-Phe being only 5-fold higher than that in the presence of Phe-tRNA. It follows that the aminoacylated C-C-A sequence of AA-tRNA is the most critical domain of tRNA for promotion of EF-T_u-dependent GTPase. The EF-T_u site that binds the 3′ terminus of AA-tRNA has the following requirements for interaction with the effectors: (i) it binds the side chain of the aminoacyl residue; (ii) it recognizes the entire C-C-A sequence of the AA-tRNA 3′ terminus [with the first (Ado) and third (Cyt) residues being most critical]; (iii) it is stereospecific but displays a surprising degree of flexibility, since it can functionally accommodate a substituent in lieu of the α hydrogen of the aminoacyl residue; (iv) it has stringent requirements for the recognition of the 3′-terminal ribose moiety of the effector. The GTP hydrolysis triggered by A-Gly, C-A-Gly, and C-C-A-Gly was strongly stimulated by thiostrepton, which is known to bind to the 50S ribosomal subunit. Since thiostrepton enhances the binding of fragments to EF-T_u and since it is also known to inhibit the EF-T_u-dependent binding of AA-tRNA to the 70S ribosome and the associated GTP hydrolysis, it follows that the antibiotic probably interferes with ribosomal binding of some AA-tRNA domain other than the 3′ terminus or anticodon. Thus, this unidentified portion of AA-tRNA, by virtue of its binding to the ribosome, plays a role in the promotion of EF-T_u GTPase in addition to the crucial role of the AA-tRNA 3′ terminus. Collectively, these results provide an insight into the highly coordinated chain of events leading to GTP hydrolysis by EF-T_u with active participation of AA-tRNA and ribosomes.

Elongation factor T_u (EF-T_u)[1] promotes the mRNA directed binding of AA-tRNA to the ribosome via a ternary AA-tRNA·EF-T_u·GTP complex. In the course of this binding reaction, one molecule of GTP is hydrolyzed, EF-T_u dissociates from the ribosome in the form of a binary EF-T_u·GDP complex, whereupon AA-tRNA enters the ribosomal acceptor site. At this site, the AA-tRNA molecule is able to participate in the peptide bond formation step as an acceptor (Miller & Weissbach, 1977). Thus, GTP hydrolysis appears to be directly involved in releasing EF-T_u from the ribosome, thereby allowing AA-tRNA to attain a reactive A site configuration. It has been proposed (Thompson & Stone, 1977) that GTP hydrolysis is also implicated in the proofreading process, which is required to maintain a high fidelity of translation. It is

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[1] Abbreviations: AA-tRNA, aminoacyl transfer ribonucleic acid; EF-T_u, elongation factor T_u; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; DTT, dithiothreitol; Me_2Gly, α-aminoisobutyric acid; cyclo-Leu, cycloleucine (1-amino-1-carboxycyclopentane); A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; similar abbreviations are used for other nucleoside and oligonucleotide derivatives; 3′-dA-3′-NH-Phe, 3′-deoxy-3′-L-phenylalanylamidoadenosine; 2′-dA-2′-NH-Phe, 2′-deoxy-2′-L-phenylalanylamidoadenosine; A(2′Me)Phe, 2′-*O*-methyl-3′-*O*-L-phenylalanyladenosine; A(3′Me)Phe, 3′-*O*-methyl-2′-*O*-L-phenylalanyladenosine; C-2′-dA-Lys, cytidylyl-(3′–5′)-2′-deoxy-3′-*O*-L-lysyladenosine; C-3′-dA-Lys, cytidylyl-(3′–5′)-3′-deoxy-2′-*O*-L-lysyladenosine; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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