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Proteolytic Fragments of the Nicotinic Acetylcholine Receptor Identified by Mass Spectrometry: Implications for Receptor Topography†

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ABSTRACT: A triple-state quadrupole or a tandem quadrupole Fourier-transform mass spectrometer was used to detect and sequence the peptides released by proteolytic cleavage of the acetylcholine receptor (AcChR) from *Torpedo californica* electroplax. Fragments in mass range up to 3479 daltons were characterized on the above instrumentation and used to determine proteolytically accessible sites on the receptor. These data were consistent with the cleavage points determined for membrane-bound fragments of the same AcChR samples using gas-phase microsequencing. Each subunit of the receptor is readily cleaved near the C-terminus in the region between the proposed transmembrane hydrophobic α-helices MIII and MIV. This region includes the putative regulatory phosphorylation sites and the amphipathic α-helix. Cleavage is also observed in the N-terminal domain, but occurs much more slowly than in the C-terminal region. No cleavage was detected in the middle third of the receptor, which includes the proposed transmembrane α-helices MI and MII. An evaluation of these data in terms of the transmembrane topography of the AcChR peptides is consistent with a synaptic or extracellular disposition for the region between MIII and MIV.

Membrane ion channels are an extremely important class of membrane proteins that have an essential role in many biological processes. Unfortunately, the structure of ion channels and the events leading to their gating and voltage dependence are not yet understood at a molecular level. The nicotinic acetylcholine receptor (AcChR)[1] is a ligand-gated ion channel that can be purified from the electric organ of *Torpedo californica* [for a review, see Changeux et al. (1984)]. In size, it is similar to other intrinsic ion channels, and it is an ideal system for physical and chemical studies because of the relative ease with which nanomolar quantities of the protein can be purified. The linear structure has been obtained from cDNA sequences for each of the four subunits of *T. californica*

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[1] Abbreviations: AcChR, acetylcholine receptor; BTX, bungarotoxin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PVDF, poly(vinylidene difluoride); egg PC, egg yolk phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPA, palmitoyloleoylphosphatidic acid; DTT, dithiothreitol; CHS, cholesteryl hemisuccinate; FTMS, Fourier-transform mass spectrometry; TX-100, Triton X-100; DEAE, diethylaminoethyl; BSA, bovine serum albumin.

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AcChR (Noda et al., 1982, 1983a; Claudio et al., 1983), as well as for subunits from other species [see Stroud and Finer-Moore (1985) for a review].

As a first step in building models for this complex protein, efforts have been directed at defining the transmembrane orientation (or topography) of the receptor peptide. Several models have been proposed for the folding of the receptor subunits, based in part on information from the linear amino acid sequence. There is homology among the four subunits (α, β, γ, and δ), and each contains four hydrophobic stretches (labeled MI–MIV) that were initially proposed to comprise transmembrane α-helical regions (Noda et al., 1983b). On the basis of this homology, the subunits are believed to have a similar structure and transmembrane topography. The presence of a sequence just before MIV that could form an amphipathic helix lead to the postulate of an additional transmembrane helix that functioned as the interior surface of the AcChR ion channel (Guy, 1984; Finer-Moore & Stroud, 1984). Much of the experimental data on the transmembrane topography of the receptor have been obtained from binding measurements using monoclonal antibodies directed against specific sequences of the receptor. As a result of these measurements, evidence was obtained supporting the original four- and five-helix models as well as additional models that are quite different (Young et al., 1985; Criado et al., 1985; Ratnam et al., 1986). This type of approach, although potentially very powerful, is not always reliable (Guy & Hucho, 1987). For example, some of the data obtained by antibody binding for the C-terminal region of the receptor do not appear to be corroborated by direct chemical evidence (McCrea et al., 1987).

Enzymatic proteolysis is a common method of probing the domain structure of proteins. It is a particularly powerful technique for investigating the transmembrane topography of membrane proteins, since membranes are generally well sealed to macromolecules such as enzymes. The AcChR appears to be well suited for such studies, in part because it remains largely intact upon proteolysis. Its sedimentation characteristics, toxin binding, appearance in transmission EM, and ion channel function are not dramatically affected by proteolysis (Huganir & Racker, 1980; Lindstrom et al., 1980; Conti-Tronconi et al., 1982). In the present work, an examination of the peptides produced by proteolytic digestion of oriented, reconstituted AcChR is described. Both water-soluble and membrane-bound fragments of the AcChR were examined. These peptides are identified by using powerful, new mass spectrometric methods as well as more widely available microsequencing techniques. The findings presented here provide a new level of detail on the enzymatic cleavage points of the AcChR and raise important questions about current models for the transmembrane topography of the AcChR peptides.

Materials and Methods

Materials. Cholic acid was obtained from Sigma (St. Louis, MO) and was recrystallized 4 times from ethanol/water (9:1). Egg phosphatidylcholine (egg PC) was prepared by the method of Singleton et al. (1965) and was stored in $CHCl_3$ at −20 °C under an argon atmosphere. Cholesterol was obtained from Aldrich (Milwaukee, WI) and was recrystallized 3 times before use from ethanol/water (9:1). Palmitoyloleoylphosphatidic acid (POPA) and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids. Sequence-grade trypsin (Boehringer Mannheim, Indianapolis, IN) and *Staphylococcus aureus* V8 protease were used for proteolysis. Bromoacetylcholine was synthesized as described previously (Damle et al., 1978).

Preparation of AcChR-Rich Membranes. Receptor-rich membranes were prepared from liquid nitrogen frozen electroplax from *Torpedo californica* (Pacific Biomarine, Venice, CA) according to the procedure of Jeng et al. (1981) with the following modifications. The tissue was blended with homogenization buffer (5 mM EDTA, 5 mM EGTA, 10 mM MOPS, 0.1 mM PMSF, and 0.1 mM iodoacetamide, pH 7.5) before it had thawed. The crude native membranes from the high-speed centrifugation were homogenized (10% sucrose, 0.1 mM EDTA, 10 mM MOPS, pH 7.5, and 0.02% sodium azide) to a final protein concentration of ≈20 mg/mL. The crude native membranes (≈0.5 nmol of BTX sites/mg of protein) were used directly for further purification either by alkaline treatment or by affinity chromatography, as described below. Alkaline treatment of the AcChR membranes was carried out by treatment between pH 11.03 and 11.08 as described previously (Neubig et al., 1979).

Preparation of the AcChR Affinity Column. Sulfhydryl-derivatized Sepharose was prepared by using a procedure similar to that described by Olsen et al. (1972). Sepharose CL-2B (Sigma Chemical Co., St. Louis, MO), 100 g, was activated with 10 g of cyanogen bromide in 1 M Na_2CO_3, pH 10.0, and was coupled to 20 g of diaminooctane at 4 °C overnight in 0.1 M Na_2CO_3, pH 10.0 (Lowe & Dean, 1974). After being extensively washed, the aminooctyl-Sepharose was reacted with 20 g of *N*-acetylhomocysteine thiolactone at 4 °C in 0.1 M Na_2CO_3, pH 10.0, for 72 h before use. The gel was washed and derivatized with bromoacetylcholine according to a procedure described previously (Ellena et al., 1983), except that 20 mM Tris, pH 8.0, was used throughout. The sulfhydryl concentration of the gel was assayed by using Ellman's reagent and dithiothreitol (DTT) as a standard, and was reproducibly 1 μmol of SH/mL of Sepharose.

Affinity Purification of AcChR. Crude, native membranes were extracted with sodium cholate according to a procedure described by Ellena et al. (1983). The membranes were diluted to a protein concentration of 1 mg/mL in buffer A (100 mM NaCl, 0.1 mM EDTA, 10 mM MOPS, and 0.02% sodium azide, pH 7.5) containing 1% cholate, and stirred at 4 °C for at least 1 h, before pelleting nonsolubilized material by centrifugation (30 min at 95000*g*).

The AcChR affinity column was washed extensively with buffer A and equilibrated with buffer A containing 1% cholate and 1 mg/mL egg PC before use. The supernatant containing the solubilized AcChR was applied to the column at a flow rate of 1–2 mL/min. The column was then washed with 7 column volumes of a buffer A solution containing 1 mg/mL egg PC and 1% cholate, followed by 2 column volumes of PC/PA/cholesterol, 56:19:25 (1 mg/mL in 1% cholate). The AcChR was eluted with 10 mM carbamoylcholine in the latter lipid/detergent solution. The yield of AcChR was determined from the absorbance at 280 nm (Jones et al., 1988). The final ratio of AcChR to lipid was adjusted to 20% (w/w) (Anholt et al., 1981), yielding a molar ratio of 1:2000. Reconstitution was accomplished by dialysis at 4 °C for 60 h (8 × 1 L of buffer A).

Characterization of AcChR-Containing Membranes. The total protein content of native and reconstituted membranes was determined by the method of Lowry et al. (1951) in the presence of 0.1% SDS, using BSA as a standard. Lipid concentrations were obtained by phosphate assay using a modification of the procedure described by Bartlett (1959). The AcChR concentration was determined from the concentration of α-bungarotoxin (BTX) binding sites using [^{3}H]BTX (Amersham, Arlington Heights, IL) with a specific activity of 2

Ci/mmol, as described previously (Hartsel et al., 1987). The reconstituted, affinity-purified AcChR used here had a specific activity of 7.6 nmol of BTX sites/mg of protein.

The transmembrane orientation of the reconstituted AcChR was determined from the ratio of BTX binding sites in the presence and absence of 0.1% Triton X-100 (TX-100). A procedure similar to the DEAE filter disk assay described by Hartig and Raftery (1979) was used with the following modifications. A series of reconstituted AcChR membrane samples from 0.05 to 2.5 pmol of receptor was incubated with 1 pmol of [^{3}H]BTX in 0.1 mL of buffer A containing 1 mg/mL BSA. Incubations were carried out for 1 h at room temperature both in the absence and in the presence of 0.1% TX-100. The [^{3}H]BTX had a specific activity of 83 Ci/mmol and was used at a concentration of 20 nM. The mixture was absorbed onto DEAE filters (DE-81, Whatman) for 1 min, and the filters were washed with 10 mL of 50 mM NaCl, 5 mM MOPS, 0.1% TX-100, and 0.05 mM EDTA, pH 7.5. The filters were placed in scintillation vials, and 0.5 mL of 2 M NaCl was added to desorb the bound toxin–receptor complexes. Scintillation fluid was added, and the samples were counted. Plots of the receptor concentration versus the number of bound counts were made to determine the number of accessible BTX binding sites in each sample (Olsen et al., 1972).

The integrity of native alkaline-extracted membranes was determined from the entrapment of cytochrome *c*. Cytochrome *c*, which is a substrate for trypsin, was entrapped by adding it to alkaline-treated membranes before the membranes were adjusted to neutrality. Approximately 30–40 mg of cytochrome *c* was added to the membrane pellet following alkaline extraction (this pellet was obtained from crude membranes containing a total of 80 mg of protein). The pH was adjusted to neutrality by the addition of buffer A, and the membranes were allowed to equilibrate with cytochrome *c* for 4 h at 4 °C. The membranes were separated on a density gradient of 0.5–1.25 M sucrose, and the AcChR membrane fraction was washed in buffer A to remove external cytochrome *c*. A major AcChR-containing membrane fraction at 1.13 M sucrose was chosen for protease and cytochrome *c* permeability measurements.

The leakage rate of cytochrome *c* was determined by removing aliquots of the membrane suspension, pelleting the membranes in a Beckman Airfuge, and measuring the absorbance of cytochrome *c* at 412 nm in the supernatant. The release of cytochrome *c* was followed for 3 h as a function of temperature, and in the presence and absence of proteases.

The trapped volume of reconstituted AcChR-containing membranes was determined by dispersing AcChR/PC/PA/cholesterol mixtures (lipid:protein ratio of 5:1 w/w) with 1% cholate and [^{14}C]sucrose on ice. Approximately 1 mL of the detergent/lipid/protein suspension (at 1 mg of protein/mL) containing 1 μCi of [^{14}C]sucrose (specific activity of 1 Ci/mol) was applied to a Sephadex G-50 column (approximately 1 × 45 cm) equilibrated with buffer A at 4 °C. Vesicles that eluted in the void volume were well separated from the untrapped sucrose, which travled in the included volume of the column. The quantity of sucrose entrapped in these vesicles was determined by measuring the radioactivity associated with the vesicle fraction.

Negative-stain electron microscopy was used to characterize the morphology of the reconstituted vesicle suspensions and employed a procedure similar to one used previously (Castle & Hubbell, 1976). Vesicles formed from cholate dialysis had diameters of approximately 600 ± 240 Å and exhibited no dramatic changes in appearance following extensive proteolysis.

Proteolysis of Reconstituted AcChR. The AcChR (2.5 nmol) in PC/PA/cholesterol was freeze–thawed twice and treated with sequencing-grade trypsin, or V8 protease. Trypsin was used at concentrations of 0.01, 0.1, 1.0, and 5.0% (w/w), and the digest was terminated by the addition of 1 mM PMSF. V8 protease was used at 2% (w/w), without PMSF treatment. The membranes were pelleted for 15 min in a Beckman Airfuge, and the pellets were washed twice with buffer A. The supernatants were pooled and lyophilized. The soluble peptides were desalted by using Sep-Pak preparative cartridges (Waters Associates, Milford, MA) before HPLC. The pellets were dissolved in gel sample buffer, which inhibits any residual trypsin activity. The final SDS concentration was 4%.

Gel Electrophoresis and Electroblotting. Gel electrophoresis was performed by using the discontinuous procedure of Laemmli (1970), as described by Jones (1980), and the Tricine buffer system described by Schagger and von Jagow (1987). Electroblotting onto Immobilon poly(vinylidene difluoride) (PVDF) transfer membranes (Millipore, Bedford, MA), as well as staining of the blots, was performed according to the procedures described by Pluskal et al. (1986).

Protein Sequencing. Soluble peptides were separated by reversed-phase HPLC and then analyzed by either triple-stage quadrupole or tandem quadrupole Fourier-transform mass spectrometry using methods described previously (Hunt et al., 1986, 1987). Membrane-bound fragments were separated by SDS–PAGE and electroblotted onto PVDF membranes. Bands of interest on the PVDF blots were excised and subjected to amino-terminal sequencing using an Applied Biosystems (Foster City, CA) gas-phase sequenator. Analyses were performed by the Commonwealth of Virginia Protein and Nucleic Acid Research Facility at the University of Virginia.

Results

Proteolytic Fragments of the AcChR Can Be Characterized Directly in Partially Fractionated Mixtures by Mass Spectrometry. Shown in Figure 1 is a mass spectrum recorded on an aliquot of one HPLC fraction of the peptides released by treatment of reconstituted AcChR with trypsin for 15 min. Signals at m/z 1117.7, 1290.8, 1508.9, and 2301.3 are those expected for $(M + H)^+$ ions corresponding to the tryptic peptides DLANFAPEIK (γ, 405–414), LVANLLENYNK (α, 7–17), IFADDIDISDISGK (α, 341–354), and IGFGNNNENIAASDQLHDEIK (δ, 400–420), respectively. Evidence to support these assignments was obtained by one of two methods. In the first approach, aliquots from each HPLC fraction were lyophilized and treated with methanolic HCl in order to convert all oligopeptides to the corresponding methyl esters. This process causes the mass of each peptide to increase by 14 units/carboxyl group present in the molecule. Conversion of the peptides in the above HPLC fraction to the corresponding methyl esters shifts the observed $(M + H)^+$ ions to higher mass by 42, 28, 70, and 70 units, respectively. These results are those expected for peptides containing two, one, four, and four acidic residues, respectively, plus the carboxyl group at the C-terminus of each molecule.

In the second approach used to confirm peptide assignments, the process of collision-activated dissociation on the triple-quadrupole mass spectrometer was employed to determine the amino acid sequence present in each $(M + H)^+$ ion. In this experiment, the first set of quadrupoles is employed to select a particular peptide $(M + H)^+$ ion from the mixture and to transport it to a chamber containing a second set of quadrupoles. There the $(M + H)^+$ ions are caused to suffer as many as 10 collisions with argon atoms. Kinetic energy is converted to vibrational energy, and the $(M + H)^+$ ions undergo more

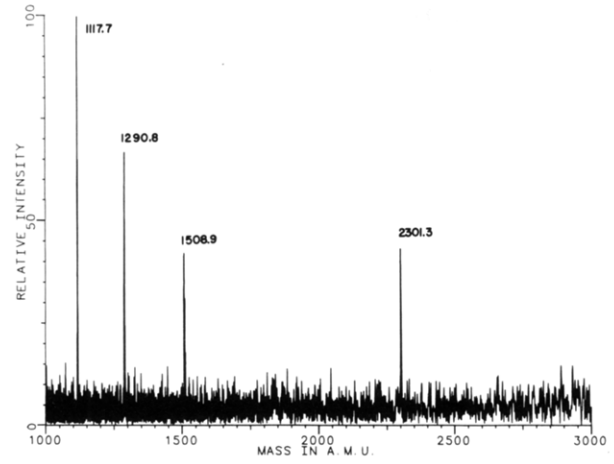

FIGURE 1: Fourier-transform mass spectrum recorded on an aliquot of a single HPLC fraction of the peptides released by treatment of reconstituted AcChR with trypsin for 15 min. Signals at m/z 1117.7, 1290.8, 1508.9, and 2301.3 are those expected for $(M + H)^+$ ions corresponding to the tryptic peptides DLANFAPEIK (γ, 405–414), LVANLLENYNK (α, 7–17), IFADDIDISDISGK (α, 341–354), and IGFGNNNENIAASDQLHDEIK (δ, 400–420), respectively. Conversion of these peptides to the corresponding methyl esters shifts the observed $(M + H)^+$ ions to higher mass by 42, 28, 70, and 70 units, respectively. These results are those expected for peptides containing two, one, four, and four acidic residues plus the carboxyl group at the C-terminus of each molecule.

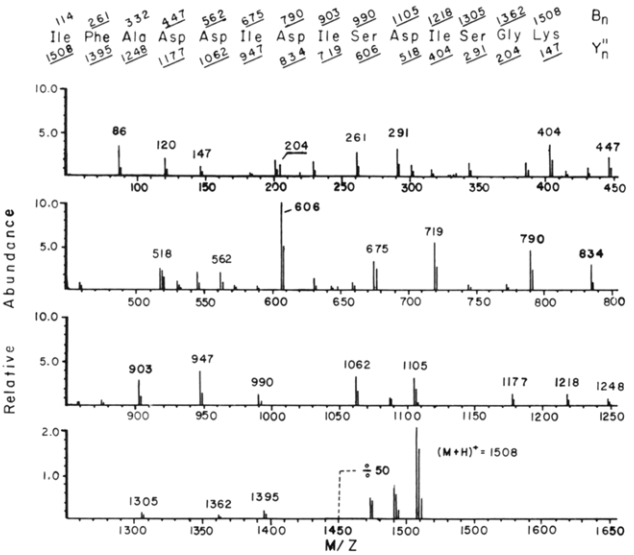

FIGURE 2: Collision-activated dissociation mass spectrum recorded on the $(M + H)^+$ ion at m/z 1508 in Figure 1. The amino acid sequence IFADDIDISDISGK (α, 341–354) was deduced from this spectrum. Fragment ions of types B and Y″ predicted for this peptide are shown above and below the structure at the top of the figure, respectively. Those observed in the spectrum are underlined.

or less random fragmentation at the various amide bonds along the backbones of the peptide molecules. The resulting collection of charged fragments is then transmitted to a third set of quadrupoles that separates the charged fragments according to mass. The result is a mass spectrum which defines the sequence of amino acids in the selected peptide. Since our present triple-quadrupole instrument has an upper mass range limit of 1800 daltons, the above approach was only applied to those $(M + H)^+$ that occurred below this ceiling.

Shown in Figure 2 is the collision-activated dissociation mass spectrum recorded on the $(M + H)^+$ ion at m/z 1508 in Figure 1. The amino acid sequence, IFADDIDISDISGK (α, 341–354), was deduced from this spectrum. Fragment ions of types B (containing the N-terminus) and Y″ (containing

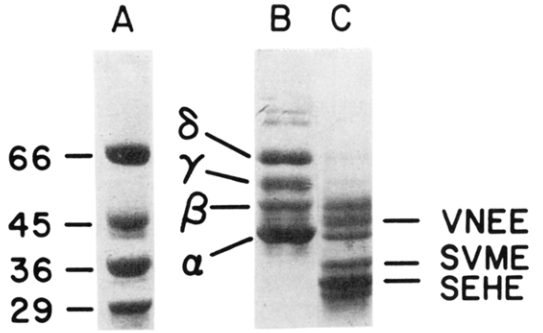

FIGURE 3: SDS–PAGE (10% acrylamide) of reconstituted AcChR. Lane A, molecular mass standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and carbonic anhydrase (29 kDa). Lane B, untreated reconstituted AcChR, intact subunits are labeled α, β, γ, and δ. Lane C, AcChR membranes after 30 min of digestion with 1% (w/w) trypsin at 37 °C. The N-terminal sequences obtained from the electroblots are indicated next to the fragmented subunits.

the C-terminus) predicted for this sequence of amino acids are shown above and below the structure at the top of Figure 2, respectively. Those observed in the spectrum are underlined.

Summarized in Table I are the peptides released from the reconstituted AcChR of *T. californica* by treatment with either trypsin or V8 protease. Peptide sequences shown without brackets were determined directly by the process of collision-activated dissociation. Those in brackets were characterized by molecular weight measurements made on both the free peptide and the corresponding methyl ester. All experiments were performed on receptor that had been affinity purified and reconstituted into PC/PA/cholesterol, a mixture which has been shown to preserve the native conformation of the protein (Ochoa et al., 1983). Peptides listed in Table I as being detected following a "short" time were observed in digests performed at low protease concentrations, or when digestion time was <30 min. Peptides designated as "late" were detected following several hours of digestion.

To summarize these data, of the peptides in the mass range of the triple-stage quadrupole mass spectrometer ($450 < m/z < 1800$), 22 out of 66 total predicted trypsin fragments were sequenced. Of these, 73% were from the proposed cytoplasmic and amphipathic helix regions. Fourier-transform mass spectrometry detected an additional 21 fragments, many of which contained smaller peptides previously identified. From these data, trypsin fragmentation appears to rapidly release oligopeptides from the C-terminal region of each subunit (particularly in the region between the proposed membrane α-helices MIII and MIV) and from a sequence at the N-terminus of α. The peptide sequences from the amino-terminal third of the protein are cleaved much less quickly and efficiently. Cleavage near the N-terminus is observed with increasing time or protease concentration, but there was no region where a high density of cleavage sites was observed as was the case for the C-terminal domain. No peptide fragments were released from the middle third of the receptor (which includes the putative transmembrane α-helices MI and MII).

Microsequencing of Electroblotted AcChR Fragments. The mass spectrometric data shown here suggest that cleavage of the receptor occurs with high efficiency at several points near the C-terminus. In order to confirm these results, the digest pattern of the reconstituted AcChR was examined by SDS–PAGE. The fragments were electroblotted onto PVDF membranes and then subjected to microsequencing using the gas-phase sequenator. The results are shown in Figure 3. Trypsinization of the reconstituted AcChR produces fragments

Table I: AcChR Oligopeptides Identified by Mass Spectrometry

subunit	amino acid residues	RCOOH $(M + H)^+$ m/z	RCOOMe $(M + H)^+$ m/z	sequence[a]	digestion time S[b]	digestion time L[c]
α	5–13	1029		TRLVANLLE		
	7–17	1291		LVANLLENYNK	X	
	58–64	945		QQWIDVR		X
	108–115	923		LLLDYTGK		X
	108–125	2110	2138	[LLLDYTGKIMWTPPAIFK]		X
	116–125	1204		IMWTPPAIFK		
	315–330	1905	1933	[IFIDTIPNVMFFSTMK]		X
	339–359	2267	2351	[NKIFADDIDISDISGKQVTGE]		
	341–354	1509		IFADDIDISDISGK	X	
	355–368	1573		QVTGEVIFQTPLIK	X	
	355–373	2127	2169	[QVTGEVIFQTPLIKNPDVK]	X	
	360–367	2013	2055	[VIFQTPLIKNPDVKSAIE]		
	374–380	703		SAIEGVK	X	
	381–387	892		YIAEHMK		X
β	1–18	2087		[SVMEDTLLSVLFETYNPK]		X
	5–13	1037	1079	[DTLLSVLFE]		
	33–46	1542	1570	[VGLTLTNLLILNEK]		X
	65–76	1391	1433	[LQWDPAAYEGIK]		
	148–165	2043	2099	[SYTYDTSEVTLQHALDAK]		
	156–167	1282	1324	VTLQHALDAKGE		
	172–177	716		EIVINK	X	
	173–182	1150	1192	[IVINKDAFTE]		
	351–358	1028	1070	ANDEYFIR	X	
	355–378	2693		[YFIRKPAGDFVCPVDNARVAVQPE]		
	359–372	1490	1532	[KPAGDFVCPVDNAR]	X	
	373–379	798		VAVQPER	X	
	379–383	652		RLFSE		
	380–385	755		LFSEMK	X	
	386–402	1961	1989	[WHLNGLTQPVTLPQDLK]		X
	403–409	759		EAVEAIK		X
γ	24–34	1268	1310	[TLDHIIDVTLK]		X
	323–331	1072	1086	[HLFLGFLPK]		X
	324–331	934		LFLGFLPK		X
	332–350	2230	2300	[YLGMQLEPSEETPEKPQPR]		X
	353–360	883		SSFGIMIK	X	
	361–367	866		AEEYILK	X	
	364–372	1134	1162	[YILKKPRSE]		
	371–379	1141	1197	[SELMFEEQK]	X	
	390–404	1673	1729	[MTSDIDIGTTVDLYK]		X
	405–414	1118		DLANFAPEIK	X	
δ	7–16	1155	1183	LINDLLIVNK		X
	148–170	2594	2678	[FTALNYDANEITMDLMTDTIDGK]		X
	328–333	778	806	[QIFLEK]	X	
	337–342	756		ILHMSR	X	
	343–356	1676	1760	[ADESEEQPDWQNDLK]		X
	369–376	1013		AQEYFNIK	X	
	372–380	1144		YFNIKSRSE		
	379–385	884		SELMFEK	X	
	390–395	678		HGLVPR	X	
	400–420	2300	2370	[IGFGNNNENIAASDQLHDEIK]	X	
	400–431	3479	3567	[IGFGNNNENIAASDQLHDEIKSGIDSTNYIVK]	X	
	419–435		1979	[IKSGIDSTNYIVKQIKE]		
	421–431	1197		SGIDSTNYIVK	X	
	488–501	1568	1624	[GDPFDYSSDHPRCA] (C-terminus)		

[a] Amino acid sequence determined by the process of collision-activated dissociation on the triple-quadrupole mass spectrometer. Sequences in brackets were identified by the m/z value of the $(M + H)^+$ ions determined for the peptide and its corresponding methyl ester on the tandem quadrupole Fourier-transform instrument. [b] Short digestion time, <30 min. [c] Long digestion time, 5 h.

of 34, 36, and 47 kDa from the α, β, and δ subunits, respectively. Each of these peptides retained the original amino terminus, indicating that the cleavage occurred near the carboxyl-terminal end of the subunits. The fragmentation appeared to be nearly quantitative, which is consistent with the 75% right-side-out orientation of the protein in reconstituted membranes. The same pattern of cleavage was obtained using alkaline-treated native membranes, as well as other reconstituted systems (e.g., egg PC, PE/PS/CHS).

With increased digestion time, the large fragments were further degraded, either decreasing in staining intensity or, in the case of α, shifting to lower molecular weight (data not shown). This suggests that the N-terminal portions of the subunits are more resistant to proteolysis. The primary cleavage sites predicted from the change in molecular weight of the subunits are consistent with the cleavage points observed using mass spectrometry. Low molecular weight, membrane-bound fragments of 10–15 kDa were also observed by electrophoresis and were shown by microsequencing to have sequences that are predicted from the mass spectrometry data. For example, a 12-kDa product from the δ subunit having an N-terminal sequence of IGFG was found along with a 9–10-kDa product from the α subunit having the N-terminal sequence QVTG.

Integrity of Native and Reconstituted AcChR-Containing Membranes. Shown in Figure 4 is a current popular folding

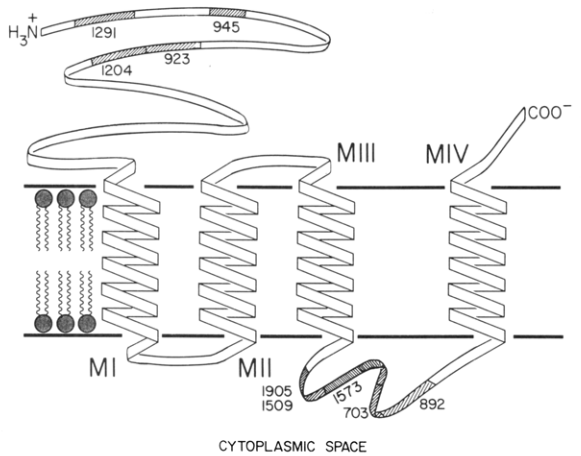

FIGURE 4: One popular model for the transmembrane topography of the receptor subunits of the AcChR [see Noda et al. (1983b)]. In this model, both the N-terminus and C-terminus are positioned in the synaptic space. Peptides that are released from the α subunit of the reconstituted AcChR are indicated by shaded areas on this folding model along with their detected m/z values.

model for each AcChR peptide subunit, where the shaded areas indicate regions that are cleaved and detected for the α subunit using mass spectrometry (data from Table I). Fragmentation in this region of the peptide is also indicated from sequences of electroblots and fragments shown in Figure 3. Given this model, efficient cleavage of peptide near the C-terminus (between MIII and MIV) is not expected in sided, well-sealed vesicles. Cleavage in this area could occur if the vesicles were not well sealed or became leaky upon proteolysis.

Two experiments were carried out to determine whether the region between MIII and MIV could be cleaved in sealed vesicle systems. Cytochrome *c* was trapped within native alkaline-extracted AcChR vesicles as a control for intactness and trypsin leakage into the vesicle interior. Approximately 0.4–0.5 mg of cytochrome *c* per milligram of AcChR was trapped within alkaline-extracted membranes. On the basis of the measured diameters of these vesicles (Klymkowsky et al., 1980), this represents 48–55% of the expected trapping by these vesicles if they were spherical. At 37 °C, approximately half of the cytochrome was released within ca. 30 min. The remainder of the cytochrome *c* remained sealed in the alkaline-extracted vesicles over a period of at least 3 h. However, at 0 °C, these membranes did not appear to be leaky to cytochrome *c*, and less than 10% of the protein leaked out over a period of 3 h. Treatment with detergents (e.g., 1% SDS) immediately released the trapped cytochrome. At the higher temperature, cytochrome *c* could be degraded by externally added trypsin. At the low temperature, cytochrome *c* was not cleaved by trypsin (except when exposure times exceeded 2 h). Under these conditions, initial cleavage of the AcChR in the C-terminal region was complete within 15 min as judged by SDS gel electrophoresis. When detergent was added to disrupt the membranes, the cytochrome *c* was degraded as well. These results indicate that the trapped volumes of these alkaline-treated suspensions are not accessible to proteases under conditions that produce cleavage of the receptor in the domain between MIII and MIV.

Vesicles containing the AcChR were also reconstituted by cholate dialysis down Sephadex G-50 under conditions that trapped [^{14}C]sucrose. When reconstituted from 1 mL of a detergent–protein–lipid mixture that contained approximately 1 mg of purified AcChR and 5 mg of PC/PA/cholesterol, the vesicles trapped 1% of the available [^{14}C]sucrose. This corresponded to a trapping rate of 2.2 mL/g of lipid. This value is comparable to or higher than the trapping volumes reported previously for these lipid mixtures (Ochoa et al., 1983). For 600-Å-diameter spherical vesicles, a maximum trapping rate of approximately 4.5 mL/g of lipid is expected. Treatment of these vesicles with trypsin cleaved the AcChR in a manner identical with that shown in Figure 3, or with that obtained from alkaline-extracted membranes.

These experiments indicate that efficient cleavage can occur in the region between MIII and MIV under conditions where the AcChR is predominantly right-side-out and incorporated into vesicles that are apparently well sealed.

Discussion

Through a combination of mass spectrometry and microsequencing, it is possible to identify both the high and low molecular weight cleavage products that result from enzymatic digestion of the AcChR. The fragments that are detected by mass spectrometry appear to be major proteolysis products. The detection of minor cleavage products by mass spectrometry was a concern, since the ability to detect peptides by mass spectrometry is dependent on the partitioning of the peptide in the thioglycerol matrix. This partitioning tends to enhance the detection of the more hydrophobic peptides (Naylor et al., 1986). Gel electrophoresis allowed the major fragments to be characterized by gas-phase sequencing. As shown above, the molecular weights of the fragments in Figure 3 and their corresponding amino-terminal sequences are in excellent agreement with the predictions of the mass spectrometry data. This result is important because it indicates that cleavage products detected by mass spectrometry are not the result of a small population of receptor in a denatured or in an unusual configuration. Previous proteolysis work is in good agreement with the results presented here. The digest patterns reported for trypsin digestion of alkaline-treated native AcChR membranes are similar to the pattern shown in Figure 3 using purified and reconstituted AcChR (Wennogle & Changeux, 1980; Klymkowsky et al., 1980; Strader & Raftery, 1980; Conti-Tronconi et al., 1982).

Proteolytic cleavage of AcChR-containing membranes yields abundant peptide fragments from the regions composed of the putative regulatory phosphorylation sites and the proposed transmembrane amphipathic helix.[2] All four subunits yielded several peptides from this region (the segment between MIII and MIV). In fact, this is the first part of the receptor to yield fragments when treated with trypsin. Because peptides are rapidly released from the putative amphipathic helix, this segment is unlikely to form a stable transmembrane helix. This conclusion is independent of the sidedness or leakiness of the AcChR-containing vesicles.

One popular folding model for the receptor peptide (see Figure 4) places the region between MIII and MIV in the cytoplasmid domain. Other models that have been proposed also place this large region in the cytoplasmic domain (Ratnam et al., 1986; Criado et al., 1985). As noted above, efficient fragmentation in this region is somewhat surprising, especially since the vesicles used here are expected to be well sealed. There are a number of ways to account for this result. If the bulk of the vesicles were leaky, or if the membranes became permeable upon proteolysis, cleavage in the MIII–MIV domain

[2] In the receptor preparations investigated here, the oligopeptides βANDEYFIR γSSFGIMIK, γAEEYILK, δAQEYFNIK, and δYFNIKSRSE were sequenced and identified. These are oligopeptides that contain proposed regulatory phosphorylation sites (Huganir & Greengard, 1987). As isolated here, no evidence for phosphorylation of these sites was found using mass spectrometry.

might be observed even if it was cytoplasmic (the population of inside-out receptor in the reconstituted system alone could not account for this result since it represents only a minor population of the total AcChR).

Independent experiments were carried out on an alkaline-treated system and on a reconstituted system to address this question. The alkaline-treated system studied here was not leaky to proteases at 0 °C, and the bulk of the membranes appeared to be sealed. Under these conditions, the region between MIII and MIV was cleaved rapidly and in high yield. Proteolysis was also performed on a reconstituted system that efficiently trapped sucrose. The trapping rates of these vesicles were high and indicated that a major fraction of the lipid vesicles was sealed. Again, the receptor could be cleaved in the region between MIII and MIV.

The mass spectrometry was carried out on purified AcChR reconstituted into PC/PA/cholesterol because this mixture is believed to produce well-characterized membranes having functional receptor (Ochoa et al., 1983). This was not the only reconstituted vesicle system we examined. Proteolysis of the AcChR reconstituted into egg phosphatidylcholine alone, PE/PS–cholesteryl hemisuccinate, or crude soybean phospholipids also produced fragments consistent with the data obtained for the PC/PA/cholesterol system (Moore et al., 1987).

These results provide a strong indication that the region between MIII and MIV is synaptic. However, other possibilities that can be imagined have not yet been ruled out.[3] For example, proteolysis could produce a dramatic unfolding of the receptor in the C-terminal domain, making the region between MIII and MIV accessible to proteases. This possibility is currently being addressed by enzymatically derivatizing the AcChR in well-sealed vesicles, combined with mass spectrometric identification of the labeled fragments.

The data presented here are not the only data consistent with a synaptic disposition for this region of the sequence. The amphipathic helix region of the α and β subunits can be labeled equally well in native membranes and in detergent solution by pyridoxal phosphate and NaB^3H_4 (Dwyer, 1988), consistent with a synaptic disposition for the region between MIII and MIV. Furthermore, Roth et al. (1987) recently demonstrated that when the AcChR is trypsinized in a native state and purified by affinity chromatography, it loses the ability to bind monoclonal antibodies directed against the amphipathic helical region. These data indicate that this region was exposed to the extracellular medium before solubilization. The region between MIII and MIV represents about 20% of the AcChR peptide. Recent electron diffraction data indicate that there is almost no cytoplasmic density associated with the receptor (Toyoshima & Unwin, 1988), also consistent with a noncytoplasmic location for this region.

At the present time, an unambiguous model for the folding of the receptor is difficult to construct. From previous work, there is good evidence that MI and MII are transmembrane, involved in the ion conduction pathway of the receptor, and arranged as shown in Figure 4 [see Imoto et al. (1988)]. There is also good evidence that the C-terminus of δ is synaptic (McCrea et al., 1987). The data shown here are consistent with these conclusions (in fact, the C-terminus of δ is detected by mass spectrometry). If the region between MIII and MIV indicated in Table I is synaptic, the topography of the C-terminal region may be significantly different than depicted in Figure 4 (or any other proposed model).

[3] It is possible that efficient fragmentation in a cytoplasmic domain could occur if the receptor was incorporated into a population of vesicles that was leaky. Conceivably, a significant trapped volume might still be measured if another sealed population of vesicles that lacked the receptor was present. In fact, at higher lipid to protein ratios than those used here, reconstitution can produce a population of vesicles lacking the AcChR (Anholt et al., 1981). To test this possibility, the density of the reconstituted vesicles used here was measured on sucrose gradients, and yielded vesicles of uniform density. This suggests that the reconstituted systems used here were not highly heterogeneous in their distribution of receptor.

Summary

A combination of mass spectrometic and microsequencing methodologies was used to characterize the cleavage of intact, reconstituted acetylcholine receptor from *Torpedo* electroplax. The receptor is cleaved by externally added protease most readily in the C-terminal domain of the receptor subunits. Except for the N-terminus of α, the amino third of the receptor appears to be cleaved much more slowly. The middle third of the receptor appears to be resistant to the proteases used here. It is difficult to reconcile this data with current folding patterns for the receptor, since it provides a strong indication that the region between the proposed transmembrane helices MIII and MIV is synaptic.

The use of mass spectrometric sequencing to identify cleavage sites of membrane proteins appears to be a very powerful tool. A large number of fragmentation points can readily be identified in a single experiment. The sensitivity of the methodology is excellent (for example, a Fourier-transform mass spectrum can be obtained with as little as 10 pmol), and peptides can be identified without purification. This should be a valuable tool for examining membrane protein topography, particularly when combined with site- and region-specific covalent labeling techniques. This methodology should also allow the localization of fluorescent and spin probes that can be used for the physical characterization of membrane proteins.

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