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Epitope Mapping of Antibodies to Acetylcholine Receptor α Subunits Using Peptides Synthesized on Polypropylene Pegs†

Manoj K. Das and Jon Lindstrom*
Receptor Biology Laboratory, The Salk Institute for Biological Studies, San Diego, California 92138
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ABSTRACT: Concurrent synthesis of overlapping octameric peptides corresponding to the sequence of the *Torpedo* acetylcholine receptor (AChR) α subunit has been carried out on polypropylene supports functionalized with primary amino groups according to a method developed by M. Geysen [(1987) *J. Immunol. Methods 102*, 259-274]. The peptides on the solid supports have been used in an enzyme-linked immunosorbent assay. Interactions of the synthetic peptides with antibodies are then detected without removing them from the solid support. By this procedure, epitopes of both antisera and monoclonal antibodies to the *Torpedo* acetylcholine receptor, its subunits, and synthetic peptide fragments have been mapped. Both rat and rabbit antisera to the α subunit show major epitopes spanning the residues 150-165, 338-345, and 355-366 on the *Torpedo* AChR α subunit. Epitopes of monoclonal antibodies to these major epitopes and to others have been rather precisely mapped by using this technique with peptides of varying lengths. The specificity of several of these mAbs are of interest because they have been used in mapping the transmembrane orientation of the AChR α-subunit polypeptide chain.

Mapping epitopes on the primary sequence of subunits of nicotinic acetylcholine receptors (AChRs)[1] is important because monoclonal antibodies (mAbs) have proven to be useful probes for AChR structure and because autoantibodies to muscle AChRs are responsible for causing the muscular weakness characteristics of myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG).

AChRs of fish electric organs and mammalian muscle are composed of four types of homologous subunits arranged like

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* To whom correspondence and reprint requests should be addressed at 503 Clinical Research Building, 422 Curie Boulevard, University of Pennsylvania Medical School, Philadelphia, PA 19104-6140.

[1] Abbreviations: αBgt, α-bungarotoxin; ABTS, 2,2′-azinobis[3-ethylbenzothiazolinesulfonate] diammonium salt; AChR, acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; ELISA, enzyme-linked immunosorbent assay; Fmoc-aa-OPfp, (fluorenylmethyloxycarbonyl)amino acid pentafluorophenyl ester; mAb, monoclonal antibody; MAR, mouse anti-rat IgG; MG, myasthenia gravis; MIR, main immunogenic region; PBS, phosphate-buffered saline.

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barrel staves around a central cation channel in the stoichiometry $\alpha_2\beta\gamma\delta$ [reviewed in Stroud and Finer-Moore (1985), Karlin et al. (1986), Maelicke (1987), Lindstrom et al. (1987a,b), and Changeux (1990)]. All of the subunits have basic similarities in amino acid sequences: Much or all of the N-terminal ~200 amino acids are throught to form a domain that is largely (Criado et al., 1985a; Pedersen et al., 1990) or completely extracellular; after this, three hydrophobic, putative transmembrane domains comprise the next ~100 amino acids; then a highly variable sequence of ~100–150 amino acids basically unique to each subunit is thought to form a domain on the cytoplasmic surface; next there is a fourth hydrophobic, putative transmembrane domain of ~20 amino acids followed by another 10–25 amino acids, which may be on the extracellular surface. In all of the subunits, the second hydrophobic sequence is thought to be the barrel stave contributed by each subunit to the lining of the channel [reviewed in Dani (1989)]. All of the subunits, when used as denatured immunogens, provoke a similar pattern of subunit-specific mAbs directed primarily at epitopes in the large cytoplasmic domain (Ratnam et al., 1986a,b). The large cytoplasmic domain seems to be loosely structured, because it is recognized by many mAbs that recognize both native AChR and denatured subunits. Most mAbs to prominent features on the extracellular surface appear to depend strongly on the native conformation of the AChR for their ability to bind. α subunits are especially interesting because they form two important extracellular domains: the acetylcholine binding site and the main immunogenic region (MIR). The acetylcholine binding site, which regulates opening of the cation channel, includes amino acids near α190 and α192 (Kao et al., 1984; Dennis et al., 1988; Abramson et al., 1989). The MIR, against which more than half of serum antibodies to native AChR and autoantibodies to AChR are directed, includes amino acids within α68–76 (Barkas et al., 1988; Bellone et al., 1989; Tzartos et al., 1990; Das & Lindstrom, 1989; Saedi et al., 1990).

We have concentrated our efforts to precisely map epitopes on α subunits for some of the anti-AChR mAbs in our extensive library for these reasons: (1) because α subunits are typical in sequences of other AChR subunits and of subunits of other receptors in the ligand-gated ion channel gene superfamily (Barnard et al., 1987), (2) because α subunits play a special role in binding ACh and gating the cation channel, and (3) because the MIR on α subunits dominates the pathologically significant autoimmune response to AChRs in MG and EAMG.

In this report we precisely map epitopes using the Geysen technique in which short overlapping peptides are synthesized on polypropylene pegs and then antibodies bound to the pegs are detected by ELISA (Geysen et al., 1987). Initially, the entire sequence of *Torpedo* α subunits was synthesized as a series of overlapping octamers in which the first peg had the peptide α1–8, the second 2–9, etc. After determining the pattern of epitopes on antisera to native AChR and denatured α subunits by this method, the epitopes for several mAbs were mapped. Some of these mAb epitopes were even more precisely mapped by using the Geysen technique with peptides of varying lengths. Previously, we mapped the epitopes of mAbs to AChR with less precision by using subunit peptide fragments or larger synthetic peptides (Ratnam et al., 1986a,b). Some of our preliminary results using the Geysen technique have been reported (Lindstrom et al., 1989), and we have used this technique with longer peptides as well as with soluble peptides and in vitro mutagenesis to map the MIR (Das & Lindstrom, 1989; Saedi et al., 1990).

Materials and Methods

Reagent Antibodies. Mouse anti-rat (MAR) 18.5, a κ-chain-specific mAb (Lanier et al., 1982), was used as the second antibody in most epitope mapping studies on mAbs. MAR 18.5 was conjugated to horseradish peroxidase according to the procedure of Hurn and Chantler (1980). The same procedure was used for coupling HRP to goat anti-rat IgG.

Antisera and mAbs against the Receptor and Its Subunits. All antisera and mAbs used were prepared in rats. The preparation of mAb 13 to eel and *Torpedo* AChR is described in Tzartos and Lindstrom (1980). mAbs 236, 254, and 255 to the synthetic peptides *Torpedo* α152–167 and α235–242 are described in Criado et al. (1985a,b). The preparation of the remaining mAbs to AChR from the *Torpedo* electric organ is described in Tzartos et al. (1986).

Peptide Synthesis on Polypropylene Pegs. Polypropylene pegs held on racks in a 96-well format compatible with ELISA plates, Fmoc-amino acid active esters, and the software for directing the synthesis and subsequent data analysis were purchased from Cambridge Research Biochemicals. The polypropylene pegs serving as supports for synthesis of peptides are provided with an Fmoc-protected amino group, which subsequently serves as an anchor on which peptides are synthesized. Following the deprotection of the amino group, the synthesis of the individual peptides is carried out by using (fluorenylmethyloxycarbonyl)amino acid pentafluorophenyl esters (Fmoc-aa-OPfp), according to the manufacturer's instructions. Each coupling was allowed to proceed for 18 h, and, thus, 430 octameric peptides were synthesized in 10 days. An IBM-PC was used to plan the peptide synthesis and direct the addition of the correct amino acid to each peg on each day. At the completion of the final coupling reaction, the peg-coupled peptides are finally deprotected on the N-terminal α-amino group and acetylated by acetic anhydride. The final side chain deprotection was carried out by treatment of the polypropylene pegs with a mixture of trifluoroacetic acid–phenol–ethanedithiol (95:2.5:2.5 v/v/v) for 4 h. In later experiments, two changes were made in the synthesis procedure: one to decrease cost and the other to increase coupling efficiency. The volumes of expensive solvents used were greatly reduced by carrying out the couplings and washings for each peg in individual 1 mL 8.8 × 45 mm polypropylene microtubes (catalog no. RN0946-01RS, National Scientific) held in 96-well test tube racks into which the sets of pegs could be easily dipped. The absorption of piperidine by the polypropylene pegs was reported by Cambridge Research Biochemicals to reduce coupling efficiency; so in later experiments an additional 30-min washing step with 30 mM 1-hydroxybenzotriazole in dimethylformamide, followed by washing with dimethylformamide, was introduced prior to the coupling step with the Fmoc-aa-OPfp to neutralize any remaining piperidine.

Enzyme-Linked Immunosorbent Assay (ELISA). The octapeptides coupled to polypropylene supports on which they were synthesized were subjected to an ELISA for antibody binding essentially according to Geysen et al. (1987). The tips of the polypropylene pegs bearing synthetic peptides were agitated for 1 h in a solution of 10 mM sodium phosphate buffer, pH 7.2, 145 mM NaCl, 1% ovalbumin, 1% bovine serum albumin, and 0.1% Tween 20 for 1 h at 22 °C to block nonspecific binding. The pegs were then treated with antisera diluted in the same buffer overnight at 4 °C. The pegs were given four washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20 at 23 °C, after which they are agitated at 22 °C for 1 h with the reagent antibody diluted in the same buffer as the antisera. The pegs were again

washed as before. Finally, the presence of antibody was detected by reaction for 15–30 min with 150 μL of a freshly made substrate solution consisting of ABTS (5×10^{-4} M), H_2O_2 (22 μL) in 72 mL of Na_2HPO_4 (0.1 M), and citric acid (0.08 M), pH 4.0. Following the development of blue-green color, the wells were read at 405 nm in a Titertek ELISA reader. ELISA absorbance values were transmitted to a microcomputer and stored on the diskette for later analysis.

To remove bound antibody from the polypropylene rods prior to reassaying, the pegs were subjected to sonication for 30 min in an aqueous solution, prewarmed to 60 °C, containing 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.1 M sodium phosphate, pH 7.2. They were then immersed in boiling water followed by boiling methanol and air dried.

Results and Discussion

Antisera to denatured α subunits were tested on overlapping synthetic octapeptides corresponding to the complete sequence of α subunits, to determine the pattern of epitopes on α subunits (Figure 1). Both rat and rabbit antisera gave similar patterns, showing prominent epitopes at α150–164, α338–349, α356–366, and α364–372, though there were some interspecies differences with the rabbit antisera, detecting two additional prominent epitopes at α40–50 and α75–85. Antisera to native AChR gave similar patterns. The observation of several epitopes in the region α338–372 was consistent with the pattern expected from previous mapping of rat anti-AChR subunit mAbs by using subunit peptide fragments on Western blots and larger soluble synthetic peptides in immunoprecipitation assays (Ratnam et al., 1986a,b). These results suggest that a majority of serum antibodies to denatured α subunits are directed at only a few parts of the α sequence. The sequences of α subunits are highly conserved, with 80% sequence identity between α subunits of AChRs from *Torpedo* and humans (Noda et al., 1983a,b). When the sequence of α subunits from *Torpedo*, *Xenopus*, chicken, calf, mouse, and human are compared (Luther et al., 1988), prominent regions of nonidentity occur in the sequences α154–159, α337–346, and α355–364, which are regions in which prominent epitopes occur.

It turns out that mAbs are available in our library that are directed at the prominent epitopes on α subunits. Several of these were mapped in detail to precisely define their epitopes for use in future studies. To evaluate the specificities of these mAbs, it is as important to note the hundreds of peptide octamers that are not bound by the mAb as it is to note precisely which peptides are bound.

mAb 142 is an IgG2a that was raised against denatured *Torpedo* AChR (Tzartos et al., 1986). It was found to immunoprecipitate the peptide α349–365 and was found, by electron microscopy, to bind to the cytoplasmic surface of AChR-rich electric organ vesicles (Ratnam et al., 1986b). By use of a solid-phase radioimmunoassay employing peptides 18–21 amino acids long, mapping of the epitope for mAb 142 was confirmed to be within α346–364 (Tzartos et al., 1990). It is species specific for *Torpedo* AChR and binds to both native AChR and denatured subunits. Figure 1 shows that by using the peg-coupled peptide octamer system, mAb 142 binds quite specifically in the region α356–366, in close agreement with the previous results. The sequence α355–364 is interesting because it is the longest sequence of the 437 amino acids of α subunits that does not contain a single amino acid shared by all six species of α subunits that have been sequenced [see Figure 5 in Luther et al. (1988)]. This unique sequence accounts for the species specificity of mAb 142 and may contribute to the immunogenicity of this sequence. The

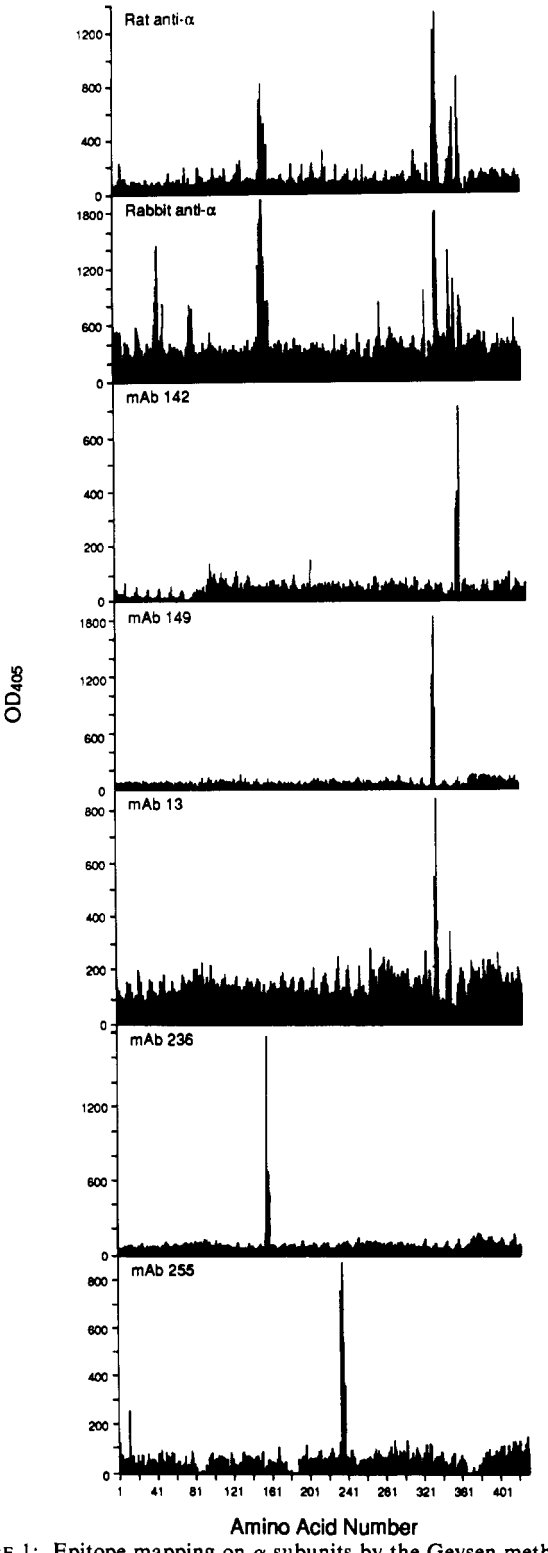

FIGURE 1: Epitope mapping on α subunits by the Geysen method. On each panel the antiserum or mAb assayed is indicated. Numbers on the *x*-axis denote the location within the *Torpedo* AChR α subunit sequence of the N-terminal amino acid of each overlapping octapeptide. Rat and rabbit antisera to *Torpedo* α subunits purified by electrophoresis in sodium dodecyl sulfate were diluted 1:280 and 1:420, respectively. The binding of antibodies to synthetic peptides was detected by peroxidase-conjugated goat anti-rat or anti-rabbit IgG in the presence of a substrate solution consisting of ABTS and H_2O_2. The CRB program multiplies the measured A_{405} by a factor of 10^3; thus the data are plotted in these units. Bound mAbs were identified by using the mouse anti-rat IgG mAb MAR 18.5 coupled to horseradish peroxidase. mAb 142 was used at 3.5×10^{-4} M. mAb 149 was used at 4.8×10^{-8} M. mAb 13 was used at 1.8×10^{-9} M. mAb 236 was used at a dilution of 1/400. mAb 255 was used at a concentration of 2.5×10^{-9} M.

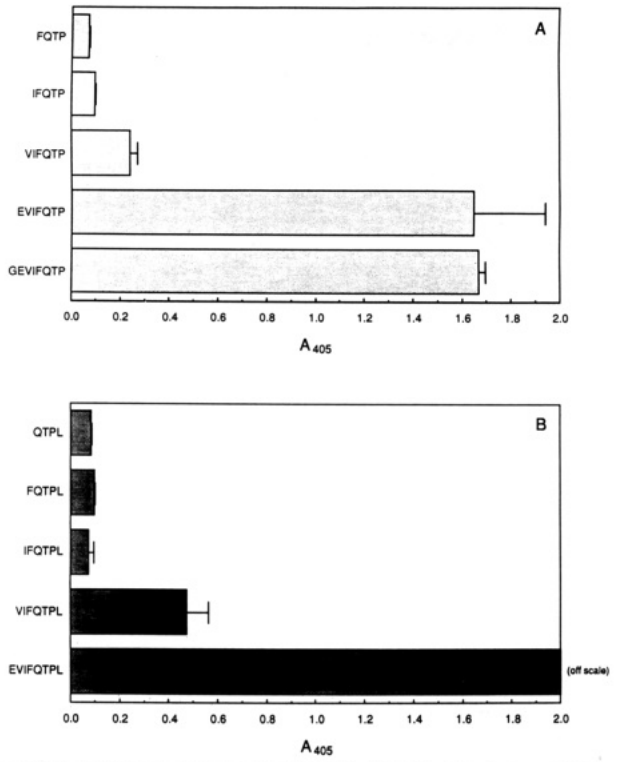

FIGURE 2: Minimal epitope of mAb 142. ELISA showing reactivities of coupled peptides with mAb 142. Shorter peptides derived from two neighboring octamers, *Torpedo* AChR α358–365 (GEVIFQTP) and α359–366 (EVIFQTPL), were synthesized on the polypropylene pegs and subjected to ELISA (see under Materials and Methods). The concentration of mAb 142 was 3.6×10^{-4} M. Peptide–antibody binding was detected by using MAR 18.5 conjugated to horseradish peroxidase in the presence of ABTS–H_2O_2 substrate, as in Figure 1. Comparison of the 7-mers EVIFQTP and VIFQTPL shows EVIFQTP to be more reactive, and therefore the minimal epitope. Peptide sequences are shown beside the histograms. Each peptide is coupled to the polypropylene peg by the C-terminal end.

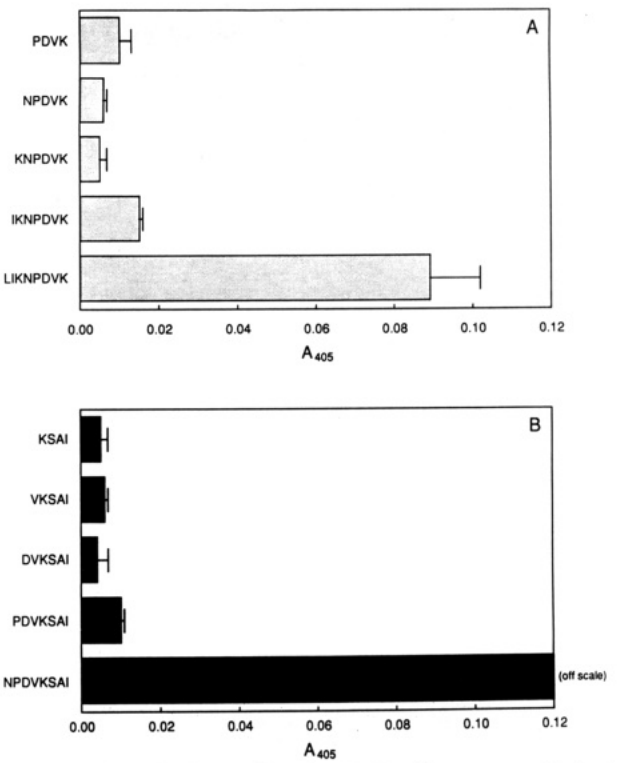

FIGURE 3: Minimal epitope of mAb 147. Peptides were synthesized on pegs, then mAb 147 at 2×10^{-7} M was allowed to bind, and bound mAb was detected by ELISA, as in Figure 2. (A) Peptides corresponding to α366–373; (B) peptides corresponding to α369–376.

observations that these 10 amino acids are uniquely variable between species and that they are immunogenic and antigenic for mAbs that can bind to both native AChRs and denatured subunits suggest that α355–364 is exposed on the cytoplasmic surface of the AChR and is not severely conformationally restrained. In two different experiments, octameric peptides were found to be potent in binding mAb 142: α358–365 (GEVIFQTP) and α359–366 (EVIFQTPL). To try to define the minimal epitope, smaller peptides were synthesized. Figure 2 shows that little or no binding was seen with peptides of four, five, or six amino acids and that the seven amino acid peptide EVIFQTP was more effective than the seven amino acid peptide VIFQTPL and nearly as effective as the octamer EVIFQTPL. Thus, mAb 142 requires a peptide of at least seven amino acids corresponding to α359–365 to bind effectively.

The epitope for mAb 147 maps just after the C-terminal end of the epitope for mAb 142. These two mAbs compete for binding to native AChR (Kordossi & Tzartos, 1987). mAb 147 is an IgG2a raised against denatured *Torpedo* AChR (Tzartos et al., 1986). It was found to immunoprecipitate the synthetic peptide α360–378 (Ratnam et al., 1986b) and in a solid-phase assay was reported to bind the synthetic *Torpedo* peptide α360–378 (Tzartos et al., 1990) and the human peptide α360–370 (Tzartos et al., 1988). Using overlapping *Torpedo* octameric peptides we mapped it to α364–376 (Lindstrom et al., 1989). These assays revealed that mAb 147 bound very specifically to the α364–376 region. To map its epitope more precisely, two sets of peptides of increasing lengths were synthesized on pegs (Figure 3). One set of peptides started with one amino acid increments N-terminally from the tetrapeptide α369–372 (PDVK), and binding of mAb 147 was detected only with the octamer α365–372 (LIKNPDVK) (Figure 3A). Another set of peptides started with one amino acid increments N-terminally from the tetrapeptide α372–375 (KSAI), and binding of mAb 147 was detected only with the octamer α368–375 (NPDVKSAI) (Figure 3B). Curiously, both peptides share the sequence NPDVK, but this sequence alone does not bind the mAb. However, if two hydrophobic residues are present at either the N-terminus (LIKNPDVK) or the C-terminus (NPDVKSAI), the mAb does bind.

mAb 149 is an IgM that was raised against denatured *Torpedo* AChR (Tzartos et al., 1986). It was found to immunoprecipitate the peptide α339–346 and, like mAb 142, was found, by electron microscopy, to bind to the cytoplasmic surface of AChR-rich electric organ vesicles (Ratnam et al., 1986b). By use of a solid-phase radioimmunoassay with 18–21 amino acid peptides, mapping of the epitope for mAb 149 was confirmed to be within α332–350 (Tzartos et al., 1990). It is species specific for *Torpedo* AChR and binds to both native AChR and denatured subunits. Figure 1 shows that it binds very specifically to the octapeptide α340–347 (KIFADDID), in good agreement with the previous results. Shorter peptides of four, five, or six amino acids were ineffective at binding, but the seven amino acid peptide α341–347 (IFADDID) was quite effective (data not shown). The sequence α340–347 contains only a single amino acid (Fα342) shared by all of the sequenced α subunits. As in the case of α355–364, α340–347 is probably exposed on the cytoplasic surface and not conformationally restrained. mAb 149 is surprising for the high specificity of its binding, since most other IgM mAbs that we tested were much less specific.

mAb 13 is an IgM that was raised against a mixture of native *Torpedo* and *Electrophorus* AChR (Tzartos & Lind-

strom, 1980). By use of a solid-phase radioimmunoassay with 18–21 amino acid peptides, the epitope for mAb 13 was mapped to α332–350 (Tzartos et al., 1990). It recognizes the same epitope as does mAb 149 (α340–347) but also binds promiscuously to many other peptides (Figure 1), which is typical of our experience with IgM antibodies in this assay. Its low specificity makes this mAb basically useless as a structural probe.

mAb 236 is an IgG2a raised against the synthetic peptide α152–167 (Criado et al., 1985a). It does not bind well to native AChRs in AChR-rich membrane vesicles or to Triton X-100 solubilized AChRs, but it binds very well to denatured *Torpedo* AChRs on Western blots and to AChR in membranes after denaturation with 3 M KSCN (unpublished data). This mAb is interesting because there is some evidence that it can bind weakly to the cytoplasmic surface of intact AChRs (Criado et al., 1985a), which is surprising, since analysis of the sequence reveals no hydrophobic sequences N-terminal of α210, suggesting that all of the α subunit between the N-terminus and the first cytoplasmic domain starting at α210 may form an extracellular domain. mAb 236 does not bind well to detergent-solubilized AChRs. However, after denaturation of AChRs with 3 M KSCN, mAb 236 binds very strongly to the extracellular surface of membrane-bound AChRs (unpublished data). Thus, the transmembrane orientation of the epitope for mAb 236 in native membrane-bound AChRs is uncertain. Pedersen et al. (1990) have recently obtained similar data using mAbs mapped by using peptide fragments to epitopes between α156 and α179. Their mAbs, also, did not bind to detergent-solubilized AChRs but did bind to the cytoplasmic surface of native membrane-bound AChRs. mAb 236 binds very specifically to α subunit octameric peptides with a peak at α159–165, and it shows no binding to other α-subunit peptides (Figure 1). By testing with the series of peptides SDRP, ESDRP, ...ISPESDRP, the minimal epitope was found to be α161–165 (ESDRP) (data not shown). This is the shortest AChR mAb epitope that we have mapped.

mAb 255 is an IgM raised against the synthetic peptide α235–242 (Criado et al., 1985b). Evidence suggests that it binds to the cytoplasmic surface. This sequence is expected to be between the first two hydrophobic sequences and consequently on the cytoplasmic surface in all the models for the transmembrane orientation of the AChR subunit polypeptide chains (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983b). Surprisingly for an IgM, the binding of mAb 255 is quite specific (Figure 1). By testing with a series of peptides GEKM, SGEKM, ...PTDSGEKM, its minimal epitope was mapped to α238–243 (DSGEKM) (data not shown). Unlike the other mAbs described here, the mAb does not bind to an epitope that is normally highly immunogenic. The sequence α235–242 is identical in all six α-subunit sequences that have been reported. Like mAb 236 and many other mAbs we have made to synthetic peptides, mAb 255 does not bind well to native detergent-solubilized AChR, presumably because in the native AChR this sequence is either highly constrained and therefore unrecognizable to the mAb or buried and inaccessible to the mAb.

Table I summarizes the mapping of sequential epitopes by the Geysen method reported here. Epitopes of mAbs were mapped corresponding to each of the epitopes prominent for rat antisera to α subunits. These epitopes were all associated with poorly conserved sequences. Immunization with a synthetic peptide corresponding to a conserved sequence that was not normally immunogenic produced mAb 255, whose epitope was also mapped. The minimal epitopes of all of these mAbs, mapped as illustrated in Figures 2 and 3, ranged from five to eight amino acids.

Table I: Summary of Antibodies and Epitopes Mapped by the Geysen Method

antibody	antigen	epitope	sequence
antiserum	α subunits	α150–164	TYDGTKVSISPESDR
		α338–349	ENKIFADDIDIS
		α356–366	VTGEVIFQTPL
		α364–372	TPLIKNPDVK
mAb 13	native AChR	α340–347	KIFADDID
mAb 142	denatured AChR	α359–365	GEVIFQTP
mAb 147	denatured AChR	α368–375	NPDVKSAI
mAb 149	denatured AChR	α341–347	IFADDID
mAb 236	α152–167	α161–165	ESDRP
mAb 255	α235–242	α238–243	DSGEKM

Figure 4 summarizes the epitopes mapped on α subunits here and in our previous studies (Ratnam et al., 1986a,b; Criado et al., 1985a,b, 1986). These epitopes are compared with various analyses of the sequence of α subunits, such as for hydrophylic sequences or poorly conserved sequences, which are frequently used to predict epitopes and to predict the transmembrane orientation of the subunit polypeptide chain. Note that poorly conserved sequences are associated with the most prominent epitopes on denatured α subunits. However, not all poorly conserved sequences were highly immunogenic. Prominent sequential epitopes that are recognized in both denatured and native AChR were found only in the large, cytoplasmic domain between the third and fourth hydrophobic domains on α subunits. mAbs that recognize both native and denatured AChRs have also been mapped to this region of β, γ, and δ subunits (Ratnam et al., 1986a,b), neuronal nicotinic receptor α3 and α4 subunits (Whiting et al., 1991; Schoepfer et al., 1989), and brain α-bungarotoxin-binding protein α1 and α2 subunits (Schoepfer et al., 1990). The only other prominent sequential epitopes on α subunits were in the region α150–164, but mAbs to these epitopes do not bind well to native AChRs. The most important epitope on α subunits was not detected by using these octomeric peptides. The main immunogenic region (MIR) was detected by using a nonamer corresponding to α68–76 of the human α sequence (Das & Lindstrom, 1989), but the affinity of binding to the synthetic peptide was very low. mAbs to the MIR do not bind to nascent α subunits (Merlie & Lindstrom, 1983) but bind with moderate affinity to a synthetic intermediate conformation of α subunits formed 15–30 min after synthesis (Merlie & Lindstrom, 1983; Conroy et al., 1990) and bind with high affinity after the assembly of α subunits with γ or δ subunits (Saedi et al., 1991). It is not yet clear whether the increased affinity with which mAbs bind to the assembled α subunits reflects a conformation change in the α subunits or a direct contribution of amino acids from γ and δ subunits. There are, not doubt, other conformation-dependent epitopes that are not detected by this synthetic peptide binding technique. Even sequential epitope are not prominent over the vast majority of the sequence; however, it is possible to raise mAbs to some of these conserved sequences, e.g., mAb 255 to α238–243 (Criado et al., 1985b) or mAbs 257–261 to α127–143 (Criado et al., 1986).

The mAbs whose epitopes were mapped in detail here and that have previously been used in studies of the transmembrane orientation of the α subunit polypeptide chain proved to be highly specific (i.e., mAbs 142, 149, 236, and 255; Ratnam et al., 1986a,b; Criado et al., 1985a,b). In the cases of mAbs 142 and 149, which bind well to both native and denatured AChRs, this leaves no doubt as to the specificity of their binding to the native AChR and transmembrane orientation of the mapped epitope sequences. For mAbs 236 and 255,

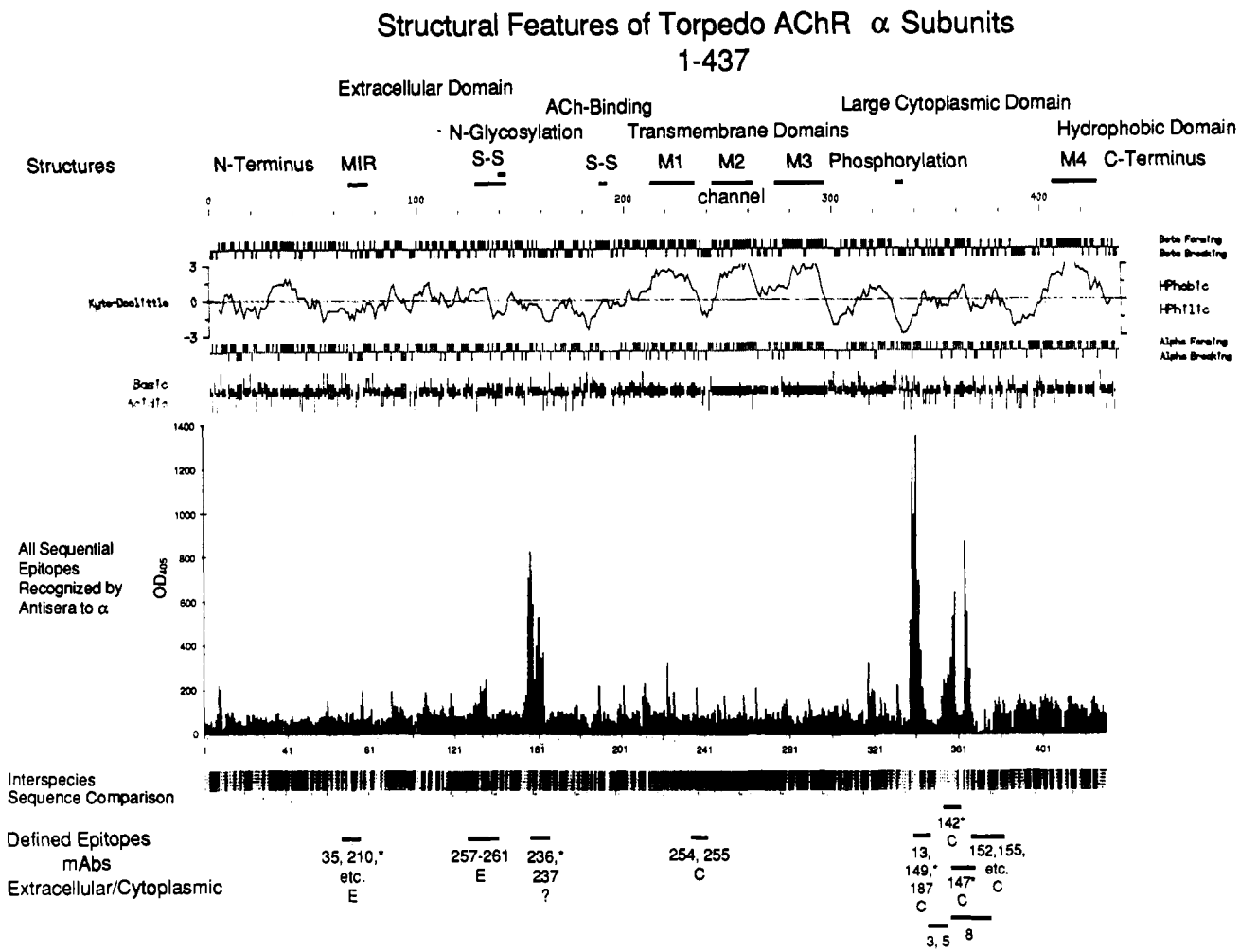

FIGURE 4: Summary of antigenic structure of the α subunit. The epitope map of antisera to α and a summary of α subunit epitopes of mAbs mapped in this paper and others from the laboratory (Ratnam et al., 1986a,b; Criado et al., 1985a,b, 1986; Das & Lindstrom, 1989; Saedi et al., 1990) are shown. These are compared above with various standard analyses of the α-subunit sequence for hydrophilicity etc., which are typically used to predict epitopes and transmembrane domains [obtained by using PEPPLOT, Genetics Computer Group (Devereux et al., 1984), and the sequence of the *Torpedo* α subunit (Noda et al., 1982)]. Below the epitope map is a comparison of the sequence of α subunits from *Torpedo*, *Xenopus*, chicken, calf, mouse, and human, modified from Luther et al. (1989), where the sequences are presented at a legible magnification. Identical amino acids in all of the subunits are in black boxes, so the white gaps in the sequence indicate regions of unconserved sequence. The most prominent epitopes on denatured α subunits occur at unconserved hydrophilic sequences, and most are in the large cytoplasmic domain between the third (M3) and fourth (M4) hydrophobic sequences.

which do not bind well to detergent-solubilized native AChRs, questions remain. It may be possible to take advantage of the knowledge of their short and precisely mapped epitopes, along with the techniques of in vitro mutagenesis and expression, to insert these epitopes in sequences where the corresponding mAbs can be used as highly specific reagents to localize them.

To briefly summarize our experience with making mAbs to AChRs and mapping their epitopes, the data reported here at high resolution with antisera and a few mAbs to α subunits are typical of our previous experiences at lower resolution with α, β, γ, and δ subunits (Ratnam et al., 1986a,b). There are several epitopes in the large cytoplasmic domain of α, β, γ, and δ subunits, and these are recognized by mAbs that can bind to both native and denatured AChRs, suggesting that the epitopes are loosely organized sequences on the surface. In all of the subunits of receptors in the gene superfamily, the putative cytoplasmic domain is the most variable in sequence. In α subunits, the sequential epitopes typically occur in the most variable sequences. When native AChRs are used as immunogens, the conformation-dependent and highly immunogenic MIR dominates the immune response (Lindstrom et al., 1987a), but when denatured subunits are used as immunogens, the less immunogenic epitopes in the cytoplasmic domain remain to dominate the immunogenicity. The region α161–165 recognized by mAb 236 is highly immunogenic in denatured α subunits but inaccessible in the native detergent-solubilized molecule. Antibodies like mAb 255 can be made to synthetic peptides that are not normally very immunogenic, but mAbs to synthetic peptides, even though they may have high affinity and specificity for denatured subunits, can rarely react with the native AChR because the sequences that they recognize are normally either buried or constrained to an unrecognizable conformation (unpublished results). We have taken advantage of the data indicating that the putative large cytoplasmic domain of AChR subunits contains unique sequences that are immunogenic and accessible in both native and denatured subunits by using as immunogens bacterially expressed, large cytoplasmic domains from human AChR α, β, γ, and δ subunits (unpublished results), chicken neuronal AChR α3 subunits (Schoepfer et al., 1989; Whiting et al., 1991), and chicken neuronal α-bungarotoxin-binding protein α1 and α2 subunits (Schoepfer et al., 1990) to raise mAbs that are subunit-specific and able to bind both native proteins and denatured subunits. Raising mAbs by using bacterially expressed, relatively long sequences from cDNA fragments has the advantage over chemical synthesis of shorter peptides that one or more of the limited sequences that are highly immunogenic and antigenic are more likely to be included in the immunogen. The epitopes of mAbs mapped by using synthetic peptides have been confirmed in reactions with the native

protein in the case of mAbs to the MIR. Several groups detected weak, but specific, binding of some mAbs to the MIR to synthetic peptides in conventional solid-phase assays (Bellone et al., 1989; Das & Lindstrom, 1989; Tzartos et al., 1990), to bacterially expressed, denatured α subunit fragments (Barkas et al., 1988), or to peptides of nine or more amino acids synthesized on polypropylene pegs (Das & Lindstrom, 1989). Amino acids α68 and α71, which appeared critical for specific mAb binding in the peptide studies, also proved to be critical for binding of mAbs to the MIR of intact AChRs expressed in *Xenopus* oocytes with these residues altered by in vitro mutagenesis (Saedi et al., 1990).

The Geysen (1987) epitope mapping technique permitted high-resolution mapping of epitopes for some anti-AChR mAbs that recognized sequential epitopes. Although it would have been possible to map some epitopes (e.g., mAb 236) by using peptides as short as five amino acids, most mAbs required at least seven amino acid peptides. In the case of mAbs to the MIR, we found that even eight amino acid peptides were insufficient but that nine amino acid peptides gave detectable binding (Das & Lindstrom, 1989). Still longer peptides might be better in some cases. For highly conformation-dependent mAbs, like those to the MIR, synthetic peptide binding at best gives a poor approximation to the native state, but peptide binding and in vitro mutagenesis results have proven consistent (Saedi et al., 1990). The Geysen technique offers the prospect of rapid throughput and high-resolution mapping over extensive sequences. It is still expensive and laborious and has the problem that it is not easy to quality control the many solid-phase peptides. Conventional synthesis of the entire sequence of α subunits as partially overlapping 18–20 amino acid soluble peptides, which can, in principle, be quality controlled and then used in a conventional assay, has been achieved and produced results complementary to those we report here (Tzartos et al., 1990). We used the Fmoc synthetic approach to the Geysen method marketed by Cambridge Biochemicals rather than the original Boc approach reported by Geysen (1984, 1987). We found rapid decreases in coupling efficiencies after the first amino acid (at 20 nmol/peg) down to 2.4 nmol by the eighth amino acid, which is lower than the levels achieved by Geysen et al. (1987) using the Boc approach. Nonetheless, we were able to obtain some useful epitope mapping at high resolution using this technique. The high specificity of binding of some of the mAbs that could be demonstrated by this technique was impressive, resulting in negligible binding to more than 400 overlapping peptides in a single assay, while binding only to those containing a single five to seven amino acid sequence.

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Role of Phosphatidylethanol in Membranes. Effects on Membrane Fluidity, Tolerance to Ethanol, and Activity of Membrane-Bound Enzymes†

Fausta Omodeo-Salè,* Clara Lindi,‡ Paola Palestini,§ and Massimo Masserini§

Institute of General Physiology and Biochemistry, Faculty of Pharmacy, and Department of Biochemistry, Medical School, University of Milano, 20133 Milano, Italy

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ABSTRACT: We investigated the effect of phosphatidylethanol (PEt) on fluidity and membrane tolerance to the fluidization induced by ethanol as well as on the activity of two membrane-bound enzymes, Na^+/K^+ ATPase and 5′-nucleotidase. PEt was synthesized from 1,2-dimyristoylphosphatidylcholine and phosphatidylcholine from bovine brain and studies were performed to determine the optimal experimental conditions for the insertion of PEt in natural bilayers. The effects of PEt, evaluated by differential scanning calorimetry or fluorescence polarization techniques, were studied in model membranes made of synthetic phospholipids or made of total lipids extracted from rat brain crude mitochondrial fraction (P_2 fraction) and from natural membranes (P_2 fraction). The presence of PEt increased the fluidity of artificial as well of natural membranes, but tolerance to the addition of ethanol, displayed by dimyristoylphosphatidylcholine vesicles and by natural membranes containing PEt, was lacking in vesicles made of dimyristoylphosphatidylethanolamine and in artificial bilayers reconstituted from total P_2 lipid extracts, suggesting an involvement of PC on PEt-induced ethanol resistance. Na^+/K^+ ATPase activity was enhanced by the addition of small amounts of ethanol (up to 50 mM) and progressively inhibited at higher concentrations, while 5′-nucleotidase was not affected up to 400 mM ethanol. The presence of PEt in the bilayer exerted the opposite effects on the two enzymes, reducing the Na^+/K^+ ATPase activation induced by ethanol and enhancing 5′-nucleotidase activity. The mechanisms of the PEt-induced modifications are discussed.

Recently, a pathway for alcohol metabolism whose product is an unusual phospholipid, phosphatidylethanol (PEt)[1] (Alling et al., 1984), has been reported. The conformation and the properties of this phospholipid could be of considerable interest since it is at the level of the polar region that the membrane interacts with its external environment. In a previous paper (Omodeo-Salè et al., 1989) we investigated by high-sensitivity differential scanning calorimetry the thermotropic behavior of PEt in vesicular dispersions of phosphatidylcholine, showing that PEt can markedly influence the physicochemical properties of the membrane where it is occasionally synthesized. However, the role of this unusual phospholipid in the structure and functions of biological membranes has not been studied. Moreover, the mechanism of membrane tolerance to the disordering effect of ethanol observed in membranes of ethanol-treated animals is still obscure (Waring et al., 1981;

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* Address correspondence to this author at the Istituto di Fisiologia Generale e Chimica Biologica, Facoltà di Farmacia, Via Saldini 50, 20133 Milano, Italy.
‡ Institute of General Physiology and Biochemistry, Faculty of Pharmacy.
§ Department of Biochemistry, Medical School.

[1] Abbreviations: PC, phosphatidylcholine from bovine brain; DMPC, 1,2-dimyristoylphosphatidylcholine; PEt, phosphatidylethanol synthesized from bovine brain PC; DMPEt, 1,2-dimyristoylphosphatidylethanol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; DMPE, 1,2-dimyristoylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; HPTLC, high-performance thin-layer chromatography; DSC, high-sensitivity differential scanning calorimetry; T_m, temperature of transition from gel to liquid-crystalline phase.

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