Structural Similarities between Acetylcholine Receptors from Fish Electric Organs and Mammalian Muscle†

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ABSTRACT: Acetylcholine receptors from fish electric organ tissue and mammalian muscle were compared by peptide mapping. The α subunits from receptors of *Torpedo californica* and *Electrophorus electricus* electric organ tissue were digested with V8 protease and the resulting fragments separated on polyacrylamide gels and stained for protein or for carbohydrate. ^{125}I-Labeled α subunits of acetylcholine receptors from *Electrophorus* electric organ tissue and bovine muscle were digested with V8 protease, and the resulting fragments were also separated on polyacrylamide gels. Intact receptors from both the fish electric organs and mammalian muscle were labeled with [4-(*N*-maleimido)benzyl]tri[^{3}H]-methylammonium iodide which binds specifically to the acetylcholine binding site on α subunits, and the isolated α subunits were subjected to the same peptide mapping procedure. The fragment patterns produced were stained for protein and fluorographed to identify active site containing polypeptides. None of these peptide mapping approaches revealed extensive homologies between α subunits. Intact and V8 proteolyzed sodium dodecyl sulfate denatured receptors from *Torpedo* and *Electrophorus* electric organs and bovine muscle were electrophoretically transferred to diazophenyl thioether paper and probed with antisera to *Torpedo* receptor subunits and two monoclonal antibodies. Unique fragment patterns were obtained with each antisubunit serum. A fragment of the same size was derived from the β subunit of each acetylcholine receptor and was shown to specifically bind the same monoclonal antibody in each case. These results indicate that only in the β subunits from all of the species examined is a large sequence nearly identical. However, it is likely that corresponding receptor subunits from receptors of all of these species have extensive structural homologies.

The acetylcholine receptors (AcChRs)[1] from *Torpedo* and *Electrophorus* have been purified [review by Karlin (1980) and Changeux (1981)] and have been shown to consist of four glycoprotein subunits with molecular weights of approximately 40 000, 50 000, 57 000, and 64 000 for *Torpedo* AcChR (Weill et al., 1974; Raftery et al., 1975; Lindstrom et al., 1978) and 41 000, 50 000, 55 000, and 62 000 for *Electrophorus* AcChR (Lindstrom et al., 1980a). The *Torpedo* subunits are present in the mole ratio $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980). Mammalian muscle contains much smaller quantities of AcChR than do fish electric organs; consequently, muscle AcChR has proven difficult to isolate. A particular problem in the purification of muscle AcChR is proteolysis of the molecule either during tissue preparation or during the isolation procedure (Lindstrom et al., 1980b; Einarson et al., 1982). Reports have shown one to five subunits to be present when analyzed by $NaDodSO_4$ gel electrophoresis, depending on the tissue and method of preparation (Boulter & Patrick, 1977; Dolly & Barnard, 1977; Shorr et al., 1978, 1981; Nathanson & Hall, 1979; Stephenson et al., 1981; Merlie & Sebbane, 1981). The reported molecular weights of the polypeptide chains roughly approximate those of fish AcChR. Particular structures on α subunits have been recognized in AcChRs from all species examined, such as a critical disulfide bond near the acetylcholine binding site (Karlin, 1980) and a main immunogenic region (Tzartos & Lindstrom, 1980; Tzartos et al., 1981). Immunochemical techniques have indicated the presence of antigenic determinants in AcChR from muscle which correspond to the four subunits of *Torpedo* and *Electrophorus* (Lindstrom et al., 1978, 1979b). Recently we have shown that muscle AcChR is, in fact, composed of four kinds of subunits corresponding to α, β, γ and δ of *Torpedo* AcChR (Einarson et al., 1982). Monoclonal antibodies (mAbs) also revealed similarities and have indicated the presence of two α subunits present in AcChRs from *Electrophorus* (Conti-Tronconi et al., 1981) and human muscle (Lindstrom et al., 1981b). It seems likely that the basic arrangement of four types of subunits forming a pentameric $\alpha_2\beta\gamma\delta$ complex has been conserved during evolution and is therefore probably necessary for AcChR function and interaction with suprasynaptic and subsynaptic structures. Peptide mapping on polyacrylamide gels has shown that there are few, if any, extensive regions of identical amino acid sequences between the four subunits of *Torpedo* AcChR (Froehner & Rafto, 1979; Gullick et al., 1981) or rat AcChR (Nathanson & Hall, 1979). In principal, two identical proteins compared in this way should generate identical peptide maps. This has proven useful, for instance, when an identified protein is compared to a cell-free translation product presumed to be the same molecule (Merlie et al., 1981). Conversely, two different proteins will give different peptide maps, with the provision that there may be similar-sized fragments generated entirely coincidentally. The primary structures of the subunits from *Torpedo* AcChR provide an example of an intermediate situation in which N-terminal sequence analysis has shown that there is 20–50% sequence homology between subunits in the first 54 amino acids (Raftery et al., 1980). Also, the N-terminal 24 amino acid residues of the corresponding

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[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; *Electrophorus*, *Electrophorus electricus*; mAbs, monoclonal antibodies; [^{3}H]MBTA, [4-(*N*-maleimido)benzyl]tri[^{3}H]methylammonium iodide; $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff reagent; *Torpedo*, *Torpedo californica*; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DPT, diazophenyl thioether.

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subunits of *Torpedo* and *Electrophorus* AcChR have been compared and exhibit 46–71% sequence homology (Conti-Tronconi et al., 1982b). There are, however, only very short (<10 amino acids) regions of complete homology. Peptide mapping will generally not detect this level of identity since critical amino acid changes at points of enzymic cleavage will generate different maps and the fragments analyzed by gel electrophoresis are at least 40 amino acids long (i.e., $M_r \simeq 5000$). Monoclonal antibodies have been obtained which cross-react between the subunits of AcChR from *Torpedo* (Tzartos & Lindstrom, 1980) or from *Electrophorus* AcChR (Tzartos et al., 1981). However, determinants which antibodies recognize are only about six to seven amino acids long, and alteration of a single amino acid can reduce or eliminate antibody binding (Atassi, 1978). Thus, the extreme specificities of both peptide mapping and immunological cross-reaction with mAbs may obscure fundamental structural homologies between related proteins, but the detection of homologies by these techniques is quite significant.

In this work, we compare the equivalent subunits of *Torpedo*, *Electrophorus*, and bovine AcChR to each other by peptide mapping (Cleveland et al., 1977) using iodinated, [^{3}H]MBTA-labeled, or unlabeled AcChR. Since mammalian AcChR is available in small amounts, we used the rather sensitive method of electrophoretic transfer of polypeptides separated on NaDodSO$_4$–polyacrylamide gels onto diazotized paper (Symington et al., 1981). Antisera and monoclonal antibodies were used to visualize the subunit fragments. In this way, we combine peptide mapping, with its recognized limitations, with the use of an acetylcholine binding site specific affinity label and antibodies to study the extent of differences and the degrees of sequence conservation between AcChRs from diverse species.

Materials and Methods

The AcChRs from *Torpedo californica* (Lindstrom et al., 1978), *Electrophorus electricus* (Lindstrom et al., 1980a), and fetal calf muscle (Einarson et al., 1982) were prepared by affinity chromatography on toxin–agarose (Lindstrom et al., 1981a). The unlabeled α subunits of *Torpedo* and *Electrophorus* AcChRs were purified by preparative gel electrophoresis. The intact AcChRs from *Electrophorus* electric organ and bovine muscle were iodinated by the lactoperoxidase–glucose oxidase method (Bio-Rad, Richmond, CA) and the ^{125}I-labeled α subunits isolated by preparative gel electrophoresis.

Native AcChRs were affinity labeled with [4-(*N*-maleimido)benzyl]tri[^{3}H]methylammonium iodide ([^{3}H]MBTA) (Karlin & Cowburn, 1973), and the labeled α subunits were prepared by gel electrophoresis.

Peptide mapping was performed as described previously (Gullick et al., 1981). V8 protease (Miles, Elkhart, IN), papain (Sigma, St. Louis, MO), α-chymotrypsin (type VII, TLCK treated; Sigma, St. Louis, MO), and elastase (type 1; Sigma, St. Louis, MO) were used to digest the subunits. Gels were stained for protein by using Coomassie brilliant blue R250 (Cleveland et al., 1977). Peptide fragments that contained carbohydrate were visualized on NaDodSO$_4$ gels by the periodic acid–Schiff reagent (PAS) glycoprotein staining method (Glossman & Neville, 1971) with modifications (Gullick et al., 1981).

Autoradiography of ^{125}I-labeled proteins was performed by using Kodak X-OMAT AR film preflashed to an optical density of 0.125 unit (Laskey & Mills, 1975) and an intensifying screen (Du Pont, Cronex). For rapid visualization of the [^{3}H]MBTA-containing proteins, the gels were fluorographed according to Bonner & Lasky (1974) and exposed at −70 °C.

Electrophoretic transfer of proteins from NaDodSO$_4$ gels onto diazotized paper (Western blotting) was performed at 0.8 A and 7.5 V for 3 h on an E-C electroblot apparatus (E-C Apparatus Corp., St. Petersburg, FL) essentially as described by Symington et al. (1981). The buffer was 25 mM sodium phosphate, pH 6.5 at 23 °C. DPT paper was prepared and activated according to Brian Seed (1982). After transfer, excess reactive groups were inactivated by incubating the paper in 100 mL of 0.1 M Tris-HCl, pH 9.0, 10% (v/v) ethanolamine, and 0.25% (w/v) gelatin for 2 h at 23 °C.

The paper was incubated for 18 h at 23 °C with 20 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 5 mm EDTA, 0.25% (w/v) gelatin, and 0.05% (v/v) Triton X-100 (buffer A) containing 10 μL of antiserum to each *Torpedo* AcChR subunit (titer approximately 4×10^{-6} M; Lindstrom et al., 1979b), a mixture of all four antisubunit sera, or individual monoclonal antibodies (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; S. J. Tzartos and J. M. Lindstrom, unpublished experiments). The paper was then washed with 2 × 100 mL of buffer A for 1 h at 23 °C to remove unbound antibodies and then incubated for 2 h in 20 mL of this buffer containing 5×10^5 cpm/mL ^{125}I-labeled goat anti-rat IgG (specific activity approximately 10^{18} cpm/mol). The unbound antibodies were removed by washing the paper in 3 × 100 mL of buffer A containing 0.1% (w/v) NaDodSO$_4$ and 0.5% (w/v) Triton X-100. The paper was then wrapped in cellophane and autoradiographed for 16–48 h at −70 °C by using preflashed X-OMAT AR film (Kodak) and an intensifying screen.

The paper was erased by incubating for 1 h at 60 °C on a shaker in 100 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M β-mercaptoethanol and 2% (w/v) NaDodSO$_4$ (Pierce). Prior to subsequent probings, the paper was reequilibrated into buffer A by washing several times to remove residual NaDodSO$_4$.

Results

The α subunits from *Torpedo* and *Electrophorus* AcChRs were prepared by gel electrophoresis in quantities that allowed us to perform conventional one-dimensional peptide maps on NaDodSO$_4$ gels which were stained for protein (Cleveland et al., 1977). Figure 1 shows the α subunit of Electrophorus AcChR digested with increasing amounts of V8 protease (tracks 1–5) and, for comparison (track 7), the α subunit from *Torpedo* AcChR digested with the same enzyme/protein ratio as in track 5. Several discreet fragments of the *Electrophorus* α subunit can be seen with molecular weights ranging from approximately 35 000 to 10 000. As the amount of enzyme was increased, the larger fragments were progressively broken down to the pattern shown in track 4. Increasing the level of enzyme a further 2-fold had little effect on this pattern (track 5). Comparison of the fragments of the *Electrophorus* α subunit (track 5) and *Torpedo* α subunit (track 7) generated by the same enzyme to subunit ratio showed essentially different patterns although the smaller (<14 000 molecular weight) pieces appeared similar in size. It is not possible to say, however, whether this is coincidental or represents cleavage at the same residues within homologous sequences.

The same subunit fragments were therefore stained with PAS to see if equivalently sized polypeptide(s) contained carbohydrate (Figure 2). The heavy stained pair of bands in tracks 2 and 3 of Figure 2 with approximate molecular weights of 40 000 are the V8 protease polypeptides. The molecular weight standards were also stained for protein with Coomassie blue (Figure 2, track 1) or for carbohydrate with

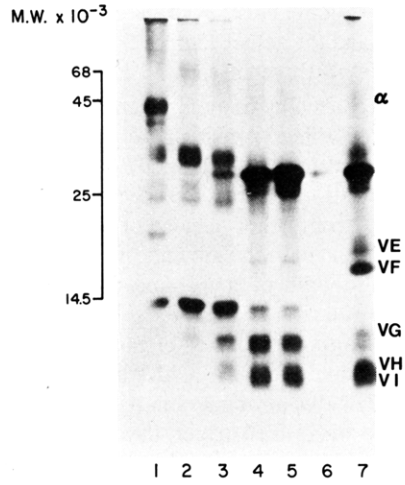

FIGURE 1: Peptide mapping on 15% polyacrylamide gels in $NaDodSO_4$ of the α subunit from *Electrophorus* AcChR and *Torpedo* AcChR. (Lanes 1–5) 10 μg of *Electrophorus* α subunit digested with 0.02, 0.1, 1, 10, and 20 μg of V8 protease, respectively, for 30 min at 22 °C; (lane 6) 0.5 μg of V8 protease only; (lane 7) 10 μg of *Torpedo* α subunit digested with 20 μg of V8 protease. The nomenclature of the fragments is as reported in Gullick et al. (1981).

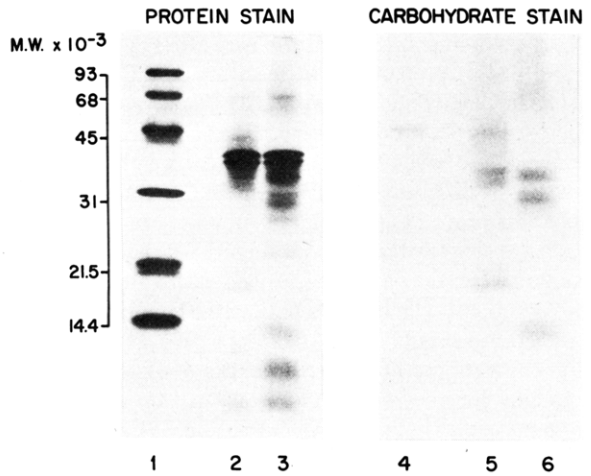

FIGURE 2: Peptide mapping on 15% polyacrylamide gels in $NaDodSO_4$ of the α subunit from *Electrophorus* AcChR and *Torpedo* AcChR stained for protein or carbohydrate. (Lane 1) Molecular weight marker proteins; (lane 2) 5 μg of *Torpedo* α digested with 10 μg of V8 protease; (lane 3) 5 μg of *Electrophorus* α subunit digested with 10 μg of V8 protease; (lane 4) molecular weight marker proteins as in lane 1 but stained with PAS glycoprotein; (lanes 5 and 6) same as lanes 2 and 3 but twice as much material was electrophoresed and stained with PAS.

PAS (Figure 2, track 4). Ovalbumin, the only glycoprotein in the mixture, gave a positive reaction with the PAS stain (Figure 2, track 4), showing that the staining was specific. As previously shown (Gullick et al., 1981), fragment VF (M_r 17 000) of the α subunit from *Torpedo* and some higher molecular weight fragments were stained (Figure 2, track 5). Three major staining bands are present in the pattern from the α subunit of *Electrophorus* AcChR (Figure 2, track 6), none of which is coincident in size with the positively stained fragments of the *Torpedo* α subunit. Thus, the similar-sized fragments seen in Figure 1 appear to be coincidental.

We then compared the α subunit of *Electrophorus* AcChR with that of bovine AcChR. Since bovine AcChR was available in limited amounts (<0.1 mg), we iodinated both of these AcChRs and isolated the α subunits by preparative gel electrophoresis (Figure 3, tracks 1 and 6). Peptide maps with increasing enzyme/subunit ratios are shown in Figure 3.

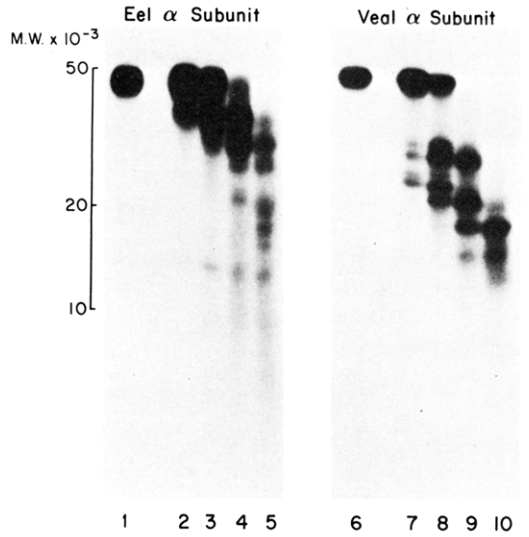

FIGURE 3: Autoradiography of peptide maps on 15% polyacrylamide gels in $NaDodSO_4$ of ^{125}I-labeled *Electrophorus* α and bovine α subunits digested with V8 protease. (Lane 1) Intact *Electrophorus* α subunit (10^5 cpm, 10^{-13} mol); (lanes 2–5) *Electrophorus* α subunit digested with 0.01, 0.1, 1, and 10 μg of V8 protease, respectively, for 30 min at 22 °C; (lanes 6–10) same as lanes 1–5 but with bovine α subunit.

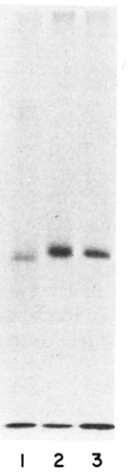

FIGURE 4: Fluorograph of *Torpedo*, *Electrophorus*, and bovine AcChR subunits labeled with [^{3}H]MBTA on 10% polyacrylamide gels in $NaDodSO_4$. (Lane 1) *Torpedo* AcChR; (lane 2) *Electrophorus* AcChR; (lane 3) bovine AcChR.

Several fragments were obtained in each case, but the patterns were clearly different. Thus, it seems that there are significant differences in primary sequence between the α subunits from *Torpedo*, *Electrophorus*, and bovine AcChRs.

We next generated peptide maps of the [^{3}H]MBTA-labeled α subunits from *Torpedo*, *Electrophorus*, and bovine AcChRs. All were labeled specifically on the α subunit only (Figure 4). We isolated these labeled subunits and generated peptide maps of each by using V8 protease, papain, α-chymotrypsin, and elastase to see whether similar-sized fragments were generated which contained the active-site label (Figure 5). Coomassie staining of the α-subunit fragments (Figure 5B), although weak, indicated few, if any, coincident proteolytic fragments (the rather strongly Coomassie blue stained high molecular weight bands in lanes 10–12 are the V8 protease polypeptides). Autoradiography of the stained gels (Figure 5A) gave clear patterns of tritiated fragments which again were unique to each subunit. Therefore, there do not appear to be any *extensive* sequence homologies around the acetylcholine binding site;

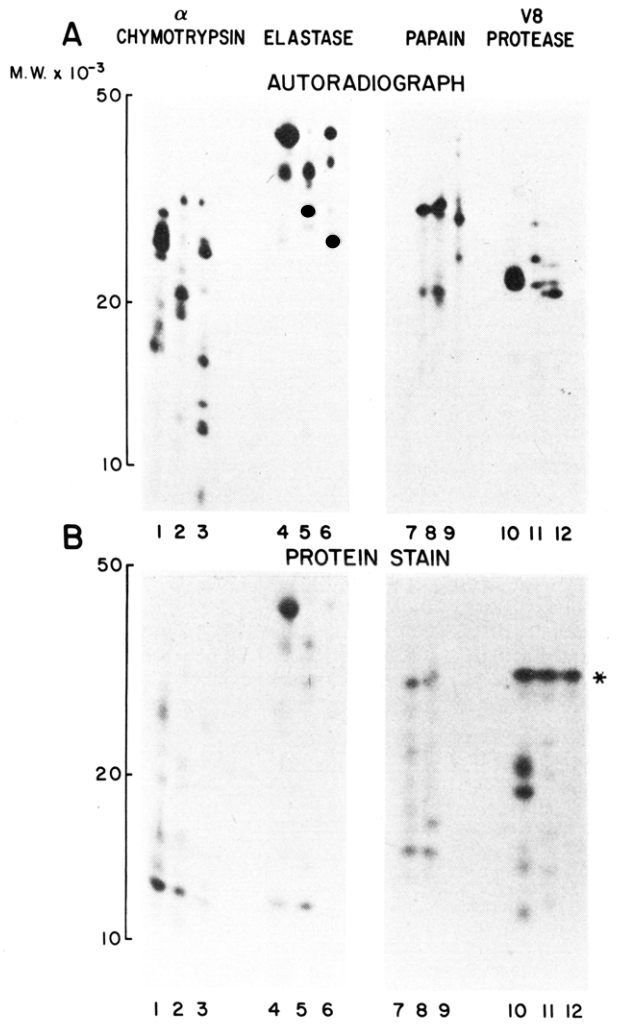

FIGURE 5: Peptide maps on 15% polyacrylamide gels in $NaDodSO_4$ of [^{3}H]MBTA-labeled α subunits isolated from *Torpedo*, *Electrophorus*, and bovine AcChRs. (A) Autoradiograph of 5 μg of *Torpedo* (lanes 1, 4, 7, and 10), *Electrophorus* (lanes 2, 5, 8, and 11), and bovine (lanes 3, 6, 9, and 12) α subunits digested with 1 μg of α-chymotrypsin (lanes 1–3), 1 μg of elastase (lanes 4–6), 0.2 μg of papain (lanes 7–9), or 2 μg of V8 protease (lanes 10–12). (B) The same gels as in (A) but stained with Coomassie brilliant blue R250.

however, the fragments resolved here are much larger than the site itself, and the actual composition of the site could even be identical in all three species.

Antisera produced by immunization of rats with individual subunits from *Torpedo* AcChR react specifically with those subunits (Lindstrom et al., 1978, 1979b). Antisera prepared in this manner also cross-reacted specifically with the analogous subunits from other species of *Torpedo* (Claudio & Raftery, 1977) and with the subunits of *Electrophorus* AcChR as well as the subunits of bovine and rat AcChR (Lindstrom et al., 1979b; Einarson et al., 1982). We therefore sought to compare the fragment patterns of each subunit of *Torpedo*, *Electrophorus*, and bovine AcChRs by the sensitive method of immunologically probing small amounts of material transferred to diazotized paper (Symington et al., 1981). Unlabeled AcChR was denatured in $NaDodSO_4$ and digested with V8 protease. Samples of both undigested and digested material were then electrophoresed on 15% $NaDodSO_4$–polyacrylamide slab gels and transferred to DPT paper. The paper was probed with a mixture of all four anti-*Torpedo* subunit sera to identify all fragments present (Figure 6A) and then erased and sequentially probed with each antisubunit serum individually (Figure 6B–E). The mixture of sera (Figure 6A) visualized all four subunits of the *Torpedo* (Figure 6A, track 1) and *Electrophorus* AcChRs (Figure 6A, track 2) and in this preparation at least the α and β subunits of bovine AcChR (Figure 6A, track 3). The fragment patterns generated by V8 protease were rather complex, as expected, but again, few bands of similar molecular weight were evident between the different AcChRs (Figure 6A, tracks 4–6). The paper was probed with each antisubunit serum individually in order to simplify the patterns. Figure 6B shows the patterns obtained when the paper was probed with antiserum to *Torpedo* α subunit which reacts only with the intact α subunits from each species (Figure 6B, tracks 1–3). The proteolytic fragment patterns (Figure 6B, tracks 4–6) showed several fragments for the α subunit from *Torpedo* and one main fragment each from *Electrophorus* and bovine α subunits. None of these were of the same molecular weight. However, when the same paper was probed with antiserum to *Torpedo* β (which again was specific for the β subunit in each case)

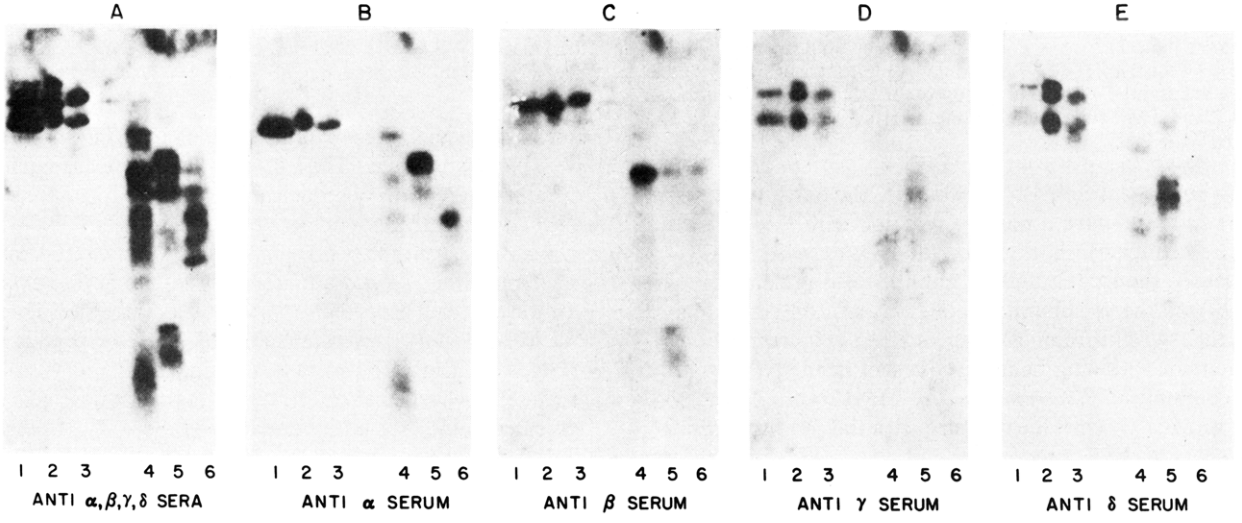

FIGURE 6: Intact and V8-proteolyzed AcChRs from *Torpedo*, *Electrophorus*, and bovine muscle separated on a 15% polyacrylamide gel in $NaDodSO_4$ and then transferred electrophoretically to DPT paper. (Lane 1) *Torpedo* AcChR (10 ng); (lane 2) *Electrophorus* AcChR (20 ng); (lane 3) bovine AcChR (50 ng); (lane 4) *Torpedo* AcChR (40 ng) digested with V8 protease (1:10 w/w); (lane 5) *Electrophorus* AcChR (80 ng) treated as in lane 4; (lane 6) bovine AcChR (100 ng) treated as in lane 4 probed with (A) a mixture of equal amounts of antisera to *Torpedo* α, β, γ, and δ subunits, (B) anti-α serum, (C) anti-β serum, (D) anti-γ serum, and (E) anti-δ serum. Autoradiography varied from 16 to 48 h.

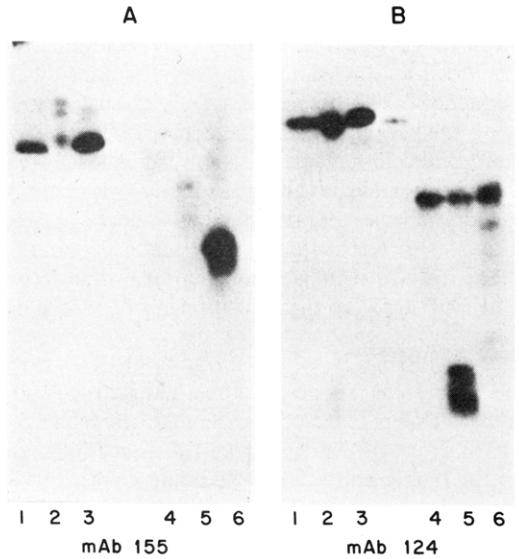

FIGURE 7: Same transfer as in Figure 6 but probed with (A) mAb 155 or (B) mAb 124.

(Figure 6C, tracks 1–3), one major band of high molecular weight was found to be common to each AcChR (Figure 6C, tracks 4–6). This finding was analyzed further in additional experiments below. Antiserum to *Torpedo* γ reacted with the γ subunit from each species but also cross-reacted with material with the same apparent molecular weight as the α subunits in each case and with the δ subunit of *Electrophorus* AcChR (Figure 6D, tracks 1–3). The reaction with the subunit fragments was weak, making this pattern difficult to analyze, but there were few coincident polypeptide fragments visible. Antiserum to *Torpedo* δ reacted with the δ subunit from *Torpedo* and *Electrophorus* (and bovine muscle) but also reacted with material with the same relative mobility as the γ and α subunits of *Electrophorus* (strongly), the α subunit of bovine AcChR (weakly), and all the other subunits of *Torpedo* AcChR (weakly) (Figure 6D, tracks 1–3). The fragment patterns of the proteolyzed molecules were again substantially different from each other (Figure 6D, tracks 4–6). Thus, it seems that the β, γ, and δ subunits of *Torpedo*, *Electrophorus*, and bovine AcChRs are different in sequence to each other within and between species, but equivalent subunits are sufficiently similar that antisera may cross-react between them.

One of the objectives of this laboratory has been to prepare a library of monoclonal antibodies as probes for AcChRs. Since the peptide maps referred to above showed a few polypeptides of similar size in receptors from different species, we investigated whether these polypeptides were truly homologous by testing for their reaction with mAbs. Figure 7 shows two mAbs, 124 which binds to the β subunit and 155 which binds to the α subunit of *Torpedo* AcChR (S. J. Tzartos et al., unpublished experiments). Monoclonal antibody 155 reacts strongly with the α subunit from *Torpedo* and bovine AcChRs (Figure 7A, tracks 1 and 3, respectively) but cross-reacts rather weakly with the α, β, and δ subunits of *Electrophorus* (Figure 7A, track 2). The mAb bound rather weakly to the *Torpedo* and *Electrophorus* AcChR subunit fragment pattern, but several bands were visible (Figure 7A, tracks 4 and 5). The bovine sample showed one strongly reacting component and some weakly reactive higher molecular weight material (Figure 7A, track 6). There were no common-sized polypeptides that reacted with this mAb. Monoclonal antibody 124, however, reacted strongly and specifically with the intact β subunit from each AcChR (Figure 7B, tracks 1–3). Moreover, it reacted specifically with a large fragment from each proteolyzed subunit (previously visualized with antisera to *Torpedo* β; Figure 6C, tracks 4–6) together with some other dissimilar-sized polypeptides (Figure 7B, tracks 4–6). Thus, a fragment of the same molecular weight from each species is generated by the same enzyme and contains the same antigenic determinant. This suggests the existence of a rather extensive region of conserved sequence in the β subunits from these molecules.

Discussion

In this work, we have employed the one-dimensional Cleveland-type peptide mapping technique to compare the equivalent subunits of AcChRs from three different species of animals. "Equivalent" in this context means accepting the subunit pattern from *Torpedo* as a paradigm for the nomenclature of the subunits from other AcChRs. In most instances, this is obvious. For example, the similarity in size, immunological reactivity, and [^{3}H]MBTA labeling of each of the smallest subunits from the three AcChRs studied here makes their classification as α subunits easy. It would be, however, rather more difficult to assign equivalence with AcChRs in which subunits were of similar size and for which specific sera or labeling reagents were unavailable. Another significant problem is that subunits break down to give material that coelectrophoreses with intact subunits of lower molecular weight, leading to false assignments of cross-reaction with antisera, mAbs, or affinity labels that may be, in fact, quite subunit specific. Wennogle et al. (1981), for example, have reported that the δ subunit from *Torpedo* breaks down to give a fragment of the same size as the intact β subunit, but which can be distinguished from β by the fact that it is labeled with a local anesthetic analogue which binds only to the δ subunit. Here we observed that γ breaks down to a fragment the size of α (to be discussed later). *Torpedo* subunits appear to be derived from a common ancestral gene, but since they are of different lengths, there must be additional sequences in the larger subunits. It is possible that the genes for these subunits are composed of a series of exons coding for protein domains, some of which may have been duplicated to generate additional translated sequences. These exon products may form structural domains linked by rather more exposed connecting sequences which would be sensitive to proteolysis. This would generate breakdown products of the same size as the smaller intact subunits. Only direct analysis of the primary protein sequence or cDNA made from the mRNA template, in comparison to the sequence of the DNA of the gene of preferably more than one subunit, would test this speculative hypothesis. Obviously, the high molecular weight subunits cannot be contaminated by lower molecular weight subunits by this mechanism; for example, breakdown of the δ subunit might occur to pieces of the same size as α, β, or γ subunits but not visa versa. One way of dealing with this identification problem which is available at the moment is to generate fragments of each subunit by using a specific protease. Genetic drift leading to small changes in primary sequence by point mutations, deletions, and insertions (which have already been observed in the N-terminal regions of the AcChR subunits) of molecules derived from a common ancestor provides sufficient differences for the protease to generate characteristic unique fragment patterns. Comparison of these would allow unambiguous identification of subunits or subunit fragments if reagents were available with adequate subunit specificity.

In this work, we show that the total fragment patterns of isolated α subunits from each species are basically different. There are, however, some bands in common with similar

molecular weights. We simplify the pattern by looking at special features on the sequence such as points of attachment of carbohydrate or the acetylcholine binding site (strictly the cysteine residue labeled by [^{3}H]MBTA), both of which are likely to be within rather conserved sequences. These fragment patterns were no more similar than complete patterns, indicating that extensive regions around these features are not absolutely identical. However, from amino acid sequence studies, we known that there is extensive *homology* between the corresponding subunits of receptor from *Torpedo* and *Electrophorus* (Conti-Tronconi et al., 1982a,b). The N-terminal 35 amino acids of *Electrophorus* α subunits show 69% amino acid identity with α subunits from *Torpedo* receptor (Conti-Tronconi et al., 1982a), which is a higher degree of homology than the 37–43% identity which *Torpedo* α showed with β, γ, and δ subunits from *Torpedo* (Raftery et al., 1980).

In order to study all of the subunits from each AcChR, we proteolyzed a mixture of each set of subunits from native AcChR which had been denatured in $NaDodSO_4$. The fragments produced were separated by gel electrophoresis and transferred to DPT paper. As little as 1 ng of each intact subunit from *Torpedo* AcChR may be visualized with the appropriate antisera in 24 h, although this is by no means the limit of sensitivity (W. J. Gullick and J. M. Lindstrom, unpublished experiments). The complete pattern of fragments was visualized by a mixture of the four antisera to *Torpedo* AcChR subunits. Antiserum to α subunits reacts with all the V8 protease generated fragments of the α subunit from *Torpedo* (Gullick et al., 1981), and the same applies for the other subunits and antisubunit sera (W. J. Gullick and J. M. Lindstrom, unpublished experiments). Therefore, it is unlikely that any major fragments are not visualized. Since this complete pattern is rather complicated, we used antisera to each subunit individually and probed the same transfer sequentially. Each unproteolyzed AcChR was included to provide a control to show how subunit specific the reaction of each antiserum was. Interestingly, both the antisera to α and β subunits are very specific. However, the anti-γ and -δ subunit sera visualize material with lower molecular weights with the same mobilities as α and γ (but not β) subunits. It is not possible to say from this alone whether the material at these positions is the result of breakdown of higher molecular weight subunits or represents real cross-reaction between intact subunits. However, when one inspects the V8 protease generated fragment pattern visualized with antiserum to γ, it does not resemble that revealed by antiserum to α, indicating that the apparent cross-reactivity of γ with α is in fact due to a breakdown product of γ. The situation is not, however, quite as clear with the antiserum to δ which reveals material in the position of the γ and α subunits in the unproteolyzed AcChR patterns. The fragments visualized do not resemble α fragments but clearly do contain some seen with antiserum to γ as well as some unique ones. It seems, therefore, that the antisera to γ and δ cross-react with each other and with fragments of γ which comigrate with α. This type of analysis then, with suitable reagents and controls, can establish if a band on a gel contains one or more AcChR-derived polypeptide(s).

We found some similar-sized pieces of equivalent subunits. In order to determine whether these are truly homologous, we tested their cross-reaction with mAbs. We found, in one case, high molecular weight fragments of equivalent β subunits of the same size which react specifically with mAb 124. This strongly suggests that long stretches of the primary sequence of β subunits are highly conserved.

In summary, we find that one-dimensional peptide mapping on its own does not generally indicate that equivalent subunits from the AcChRs studied have long regions of identical amino acid sequence. The detailed analysis of such patterns by observing special features or the presence of cross-reactive immunological determinants does, however, allow us to predict that there are homologies between subunits within an AcChR from a single species and tissue and also between equivalent subunits from different species. Ultimately, amino acid sequence and DNA sequence information, when it becomes available, will describe these relationships in more detail.

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Minimal Size Phosphatidylcholine Vesicles: Effects of Radius of Curvature on Head Group Packing and Conformation†

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ABSTRACT: Egg phosphatidylcholine small unilamellar vesicles ranging from 150 to 270 Å in diameter have been studied by proton nuclear magnetic resonance (400 MHz) to investigate the relationship between phosphatidylcholine head group conformation and small changes in the vesicle radius of curvature. We find that as the vesicle size decreases, the split between the choline *N*-methyl resonances, corresponding to lipids residing in the outer and inner monolayer, becomes more pronounced. The increasing split is due to the dramatic upfield shift of the inner monolayer choline resonance with decreasing vesicle size. We also investigated the formation of deoxycholate–phosphatidylcholine mixed micelles by following the changes in the choline *N*-methyl resonances of small unilamellar vesicles with the progressive addition of deoxycholate. Our data provide additional support for the proposal by Mazer et al. [Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601] of the existence of distorted bilayer structures at low deoxycholate:lipid molar ratios (<0.5 mM), which decay to mixed micelles at higher deoxycholate concentrations. Taken together, our results demonstrate that the choline *N*-methyl chemical shift is a sensitive indicator of head group surface area. Furthermore, we infer from our results that (1) the inner monolayer head group packing significantly influences the size limitations of a small unilamellar vesicle, (2) the inner phosphatidylcholine *N*-methyl chemical shift is indicative of the vesicle radius, and (3) the chemical shift of a phosphatidylcholine *N*-methyl residing in a planar bilayer will be very similar to that in an outer monolayer of a small unilamellar vesicle.

Small unilamellar vesicles (SUV)[1] are of interest from a biological as well as a physical chemical point of view due to the special properties a small radius of curvature imparts to them. In contrast to larger, multilamellar vesicles (MLV), SUV are unstable below the phase transition temperature (T_m), which results in spontaneous enlargement of the vesicles, probably via fusion (Suurkuusk et al., 1976; Kantor et al., 1977; Lichtenberg et al., 1981). The enthalpy associated with the SUV phase transition is 30–50% of that observed for MLV, and the T_m is depressed (Mabrey & Sturtevant, 1978; Gruenewald et al., 1979). The individual leaflets of SUV have been shown by NMR (Schmidt et al., 1978; Eigenberg & Chan, 1980) to respond independently to thermal perturbation.

The flexibility in the structure of a phosphatidylcholine (PC) molecule allows it to adapt somewhat to the geometric constraints imposed by a highly curved surface. On the basis of hydrodynamic and NMR data, the calculated lipid packing densities of the inner and outer monolayers of PC SUV are quite different, and both differ from values for phospholipids in a planar bilayer obtained by X-ray diffraction studies (Reiss-Husson, 1967; Small, 1967a). For an egg PC vesicle of Stokes radius 105 Å, the hydrated outer and inner surface area per lipid molecule has been calculated to be 84 and 56 Å^2, respectively (Cornell et al., 1980). The corresponding surface area of a maximally hydrated planar lipid molecule is about 72 Å^2 (Small, 1967a). The difference in packing requirements between the outer and inner monolayers is manifested in transbilayer compositional asymmetries of mixed lipid vesicles (Berden et al., 1975; Lentz & Litman, 1978) and a greater average freedom of motion for the hydrocarbon chains compared to MLV, deduced from Raman (Gaber & Peticolas, 1977) and NMR (Petersen & Chan, 1977) spectroscopies.

NMR spectroscopy has been successfully employed to gain information about phospholipid head group conformation in aqueous dispersions (Seelig, 1977; Hauser & Phillip, 1979).

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[1] Abbreviations: SUV, small unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; ^{1}H NMR, proton nuclear magnetic resonance; DOC, deoxycholate; O:I, outer monolayer:inner monolayer small unilamellar vesicle ratio; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

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