Biochemistry **1983**, *22*, 3312–3320

Mapping the Binding of Monoclonal Antibodies to the Acetylcholine Receptor from *Torpedo californica*[†]

W. J. Gullick[‡] and J. M. Lindstrom*

ABSTRACT: One hundred and fifty-four monoclonal antibodies (mAbs) raised against acetylcholine receptors from several species were tested for their ability to bind iodinated dissociated receptor subunits from *Torpedo californica* electric organ. Seventy-four mAbs bound detectably, and their primary subunit binding preference was established by immunoprecipitation followed by gel electrophoresis. Several mAbs cross-reacted between subunits. In order to quantitate their relative affinity we determined the apparent titer of each mAb against each subunit. Ninety-four percent of the mAbs tested were at least 95% specific for a single subunit. Most cross-reacting mAbs bound predominantly to one subunit and weakly to others. However, two mAbs bound to some degree with all four subunits. To assess their specificity of binding, we tested them all for binding to bovine serum albumin, ovalbumin, and phosvitin. Only one mAb which bound to phosvitin showed any extraneous cross-reaction. We also tested five mAbs raised against unrelated antigens for reaction with iodinated receptor subunits. No reaction was detected, emphasizing the specific binding properties of the anti-receptor mAbs. Those mAbs which reacted with denatured subunits were tested for their ability to bind *Staphylococcus aureus* V8 protease generated fragments of each iodinated receptor subunit. The fragment binding patterns were analyzed by electrophoresis on polyacrylamide gels. Those mAbs that bound the same subsets of subunit fragments were arranged into groups. mAbs that cross-reacted with other subunits cross-reacted as groups, indicating that there are several homologous determinants shared between subunits. Treatment of native receptor with trypsin in general effected the binding of all of the mAbs within a group in some way, either not altering binding or destroying binding completely, thereby confirming the group definitions. We observed at least seven groups against α subunits, nine against β, seven against γ, and five against δ. This suggested a minimum estimate of 28 determinants detectable with denatured receptor subunits.

A great potential virtue of monoclonal antibodies (mAbs) as probes of the structure and function of acetylcholine receptors is that mAbs can be prepared against many parts of the receptor macromolecule. In principal, the site at which a mAb binds can be determined rather precisely. However, the first steps in mapping a library of mAbs to the receptor, and thereby characterizing the antigenic structure of the receptor, are simply to determine the receptor subunit specificity of each mAb and then determine how many different antigenic determinants can be detected within each receptor subunit. In this paper we undertake these first steps for a library of 154 mAbs raised against receptors from the electric organs of *Torpedo californica* and *Electrophorus electricus* and the muscles of cattle and humans (Tzartos & Lindstrom, 1980, 1981; Tzartos et al., 1981; S. J. Tzartos, L. W. Swanson, L. K. Langeberg, S. M. Hochschwender, and J. M. Lindstrom, unpublished results) using subunits of receptor from *Torpedo californica*. In subsequent papers we will order these antigenic determinants and other structural features of the subunits along the amino acid sequence of the α (W. J. Gullick et al., unpublished results) and δ subunits (Anderson et al., 1983).

Acetylcholine receptors from the electric organs of *Torpedo californica* and *Electrophorus electricus* have the subunit stoichiometry $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979a; Raftery et al., 1980; Conti-Tronconi et al., 1982b). This indicates that the subunit stoichiometry of nicotinic acetylcholine receptors was established more than 400 million years ago before the evolutionary divergence of cartilaginous and bony fish (Conti-Tronconi et al., 1982b). Therefore, it is not surprising that acetylcholine receptors from mammalian skeletal muscle apparently also have an $\alpha_2\beta\gamma\delta$ subunit stoichiometry (Einarson et al., 1982; Conti-Tronconi et al., 1982a; S. J. Tzartos et al., unpublished results) and a similar size and shape (Einarson et al., 1982). That there is antigenic homology between receptors from fish electric organs and mammalian muscle was first shown by the observation that immunization with receptor from electric organs produced an autoimmune response to receptors in muscle (Patrick & Lindstrom, 1973). Later, antigenic determinants corresponding to all four subunits of receptor from *Torpedo* electric organ were found in receptors from mammalian muscles (Lindstrom et al., 1978b, 1979b), and still later subunits were found in receptors from the muscles of both fish (Lindstrom et al., 1983) and mammals (Einarson et al., 1982; Gullick & Lindstrom, 1982a,b) which corresponded immunochemically to each of the subunits of receptor from *Torpedo* electric organ. Despite these fundamental structural homologies between receptors from fish electric organs and mammalian muscle, the exquisite specificities of antibodies cause anti-receptor sera to cross-react only a few percent between species (Lindstrom et al., 1978a). Studies of mAbs to receptors revealed that a large fraction of the antibodies made to intact receptors are directed at a small part of the external surface of α subunits termed the main immunogenic region (MIR) (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1982; Gullick et al., 1981). Many of the antibodies which cross-react between species are directed at this conserved, conformationally dependent region. These studies with mAbs also demonstrated that some mAbs recognized more than one receptor subunit (Tzartos & Lindstrom, 1980). Subsequently it was shown that there is extensive amino acid sequence homology between

[†] From the Salk Institute for Biological Studies, San Diego, California 92138. *Received January 24, 1983*. Supported by grants from the National Institutes of Health (NS11323), The Muscular Dystrophy Association, the Los Angeles Chapter of the Myasthenia Gravis Foundation, The McKnight Foundation, and the Office of Naval Research. W.J.G. is a recipient of a Muscular Dystrophy Association Postdoctoral Fellowship and the J. Aron Postdoctoral Fellowship.

[‡] Present address: The Protein Chemistry Laboratory, Imperial Cancer Research Fund, Lincolns Inn Fields, London WC2A 3PX, England.

0006-2960/83/0422-3312$01.50/0

subunits (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b; Noda et al., 1982, 1983; Claudio et al., 1983). These studies make it evident that the subunit structure of receptor evolved through duplication and reduplication of a primordial subunit and that immunological cross-reaction between subunits is a consequence of the resulting sequence homologies.

Affinity-labeling reagents for the acetylcholine binding sites of the receptor show that these binding sites are located on α subunits (Karlin, 1980). The binding sites for acetylcholine (M_r 134) comprise a small part of the receptor (M_r 250 000) [reviewed in Karlin (1980); Changeux, 1981; Conti-Tronconi & Raftery, 1982; Anholt et al., 1983]. The cation channel whose transient opening is triggered by the binding of acetylcholine is an integral part of the $\alpha_2\beta\gamma\delta$ structure of the receptor monomer (Anholt et al., 1980), but which subunits compose the channel is unknown. Subunit-specific mAbs are known which effect receptor function (Lindstrom et al., 1981b). Which subunits interact with specific components of the cytoskeleton or basal lamina to localize receptors at the tips of folds in the postsynaptic membrane or regulate the large difference in turnover between synaptic and extrasynaptic receptors is also unknown. mAbs may prove valuable as probes for identifying and characterizing such potential receptor substructures. The MIR (Tzartos & Lindstrom, 1980, 1981) is a specific example of a conserved structural feature only recognized through the use of mAbs. Defining the antigenic structure of the receptor is also important for defining pathologically significant specificities of anti-receptor autoantibodies. It is clear that in both myasthenia gravis and its animal model a spectrum of anti-receptor antibodies is produced (Tzartos et al., 1982) including antibodies which differ in their pathological effects on neuromuscular transmission (S. J. Tzartos et al., unpublished results).

mAbs to acetylcholine receptors have been raised by several laboratories (Dwyer et al., 1981; Fuchs et al., 1982; Gomez et al., 1982; James et al., 1980; Lennon & Lambert, 1982; Tzartos & Lindstrom, 1980). Those mAbs which we have raised have been mapped to receptor subunits (Tzartos & Lindstrom, 1980, 1981; Tzartos et al., 1981; S. J. Tzartos et al., unpublished results). Some mAbs specific for α subunits have been mapped into subgroups both by competitive binding experiments (Tzartos & Lindstrom, 1980; Tzartos et al., 1981), by determination of the number of mAbs which can bind to a receptor (Conti-Tronconi et al., 1981), and by their ability to bind patterns of proteolytic peptide fragments of the subunit (Gullick et al., 1981). In all cases, the subgroups determined by all three methods are consistent. The groups of mAbs recognized by binding to peptide fragments of denatured subunits correspond to the groups of mAbs recognized by binding studies using intact receptors. Thus, each group of mAbs must define an antigenic determinant on the receptor, although it is possible that more than one closely spaced determinant might fall within such a group.

In this paper we extend this grouping of mAbs by the peptide fragments of receptor subunits they bind to all the mAbs in our library which cross-react with denatured subunits of *Torpedo californica*. This provides a minimum estimate of the number of antigenic determinants on each subunit, analyzes the extent of cross-reaction between subunits, and is a prelude to ordering of these antigenic determinants along the amino acid sequences of the subunits (Anderson et al., 1983; W. J. Gullick et al., unpublished results) and precise mapping of particular mAbs which are of special structural, functional, or immunological interest.

Materials and Methods

mAbs. mAbs were prepared by fusion of spleen cells from immunized rats with rat or mouse myeloma cell lines as previously described. mAbs 1–19 (Tzartos & Lindstrom, 1980) and 91–188 were raised against receptor from *Torpedo californica* or its subunits (S. J. Tzartos et al., unpublished results). mAbs 21–61 were raised against receptor from *Electrophorus electricus* (Tzartos et al., 1981). mAbs 62–74 were raised against receptor from fetal bovine muscle (S. J. Tzartos et al., unpublished results). mAbs 189–207 were raised against receptor from human muscle (S. J. Tzartos et al., unpublished results).

Acetylcholine Receptor. The acetylcholine receptor from *Torpedo californica* was prepared by affinity chromatography (Lindstrom et al., 1981a), and the individual subunits were purified by preparative gel electrophoresis (Lindstrom et al., 1979a). Subunits or whole receptors were iodinated by using lactoperoxidase coupled to Sepharose CL-4B to specific activities of (1–3) $\times 10^{18}$ cpm/mol (Lindstrom et al., 1979a).

Determination of Primary Subunit Specificities of mAbs. Iodinated whole receptor was denatured in 2% (w/v) sodium dodecyl sulfate and diluted into 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, 0.5% Triton X-100, and 0.1% sodium dodecyl sulfate (0.5%–0.1% buffer) to a final concentration of 1×10^{-9} M. One picomole of mAb was added to 1 mL of iodinated receptor subunits and allowed to bind overnight at 4 °C. Twenty microliters of goat anti-rat antibody coupled to cyanogen bromide activated Sepharose CL-4B (10 mg of affinity purified antibody/mL of Sepharose) was added and the mixture shaken for 2 h at 4 °C. The Sepharose–antibody complex was washed 5 times with 1 mL of 0.5%–0.1% buffer lacking sodium dodecyl sulfate (0.5% buffer) and then twice with 1 mL of water. The bound subunit was dissociated by adding 70 μL of 2% (w/v) sodium dodecyl sulfate. After 30 min at room temperature a 10-μL aliquot was counted for radioactivity. In general, equal amounts of radioactivity were then applied to lanes of a 10% polyacrylamide gel and electrophoresed, fixed, dried onto Whatman 3MM filter paper, and autoradiographed by using preflashed Kodak XAR-5 film and an intensifying screen as described previously (Lindstrom et al., 1979b).

Measurement of Approximate Titers of mAbs against Individual ^{125}I-Labeled Receptor Subunits. Individual ^{125}I-labeled AChR subunits were diluted in triplicate to 1×10^{-8} M in 0.5%–0.1% buffer and 5 μL of normal rat serum/100 μL. Then 1×10^{-11} mol of mAb titer were added to give a final concentration of 1×10^{-8} M in titer. mAb titer was measured by indirect immunoprecipitation using intact receptor from *Torpedo* labeled with ^{125}I toxin (Lindstrom et al., 1981a) in units of moles of toxin binding sites of receptor bound per liter of mAb. Thus, because purified receptor from *Torpedo* consists of about two-thirds dimers in which each monomer can bind two toxin molecules (Karlin, 1980), 1×10^{-8} M in titer actually corresponds to about 3×10^{-9} M in mAb molecules, depending on whether the mAb cross-links monomers (Conti-Tronconi et al., 1981) and the affinity of the mAb. Therefore, binding of subunits of mAbs was measured in antigen excess. The mixture was incubated overnight at 4 °C. Then 100 μL of goat anti-rat antibody was added and allowed to bind for 4 h at 4 °C. The immunoprecipitates were spun down and washed with 1 mL of 0.5% buffer before their radioactivity was determined. Antibody titer in moles of subunits bound per liter of mAb was calculated when 5–30% of the subunit was precipitated. For mAb 142, the titer against the α subunit was determined with 1×10^{-12} mol of mAb titer so that the amount of precipitated subunit did not exceed 30% of the total available. Several mAbs precipitated less than 5% of the total labeled subunit. These were tested again by adding 1×10^{-10} mol of mAb titer to each assay to give a final antibody concentration of 1×10^{-7} M (Table II).

mAb Mapping to Subunit Fragments. Individual ^{125}I-labeled subunits (2×10^{-7} M) were digested with 0.5 volume of *S. aureus* V8 protease (Miles) in 125 mM Tris-HCl buffer, pH 6.8, containing 0.1% sodium dodecyl sulfate for 30 min at room temperature. The β, γ, and δ subunits were treated with 0.1 mg/mL protease and the more protease-resistant α subunit with 1.0 mg/mL V8 protease. The enzyme was inactivated by heating at 100 °C for 5 min. The fragments were diluted to 5×10^{-8} M in 0.5%–0.1% buffer, and 10 μL of mAb was added to 100 μL of fragments to give ~0.1–4 μM mAb titer (against *Torpedo* receptor). The bound fragments were determined as described above for intact subunits except that 15% polyacrylamide gels were used (Gullick et al., 1981).

Control Experiments for mAb Binding. All the mAbs employed here were tested for the possibility of cross-reaction with apparently unrelated antigens. Bovine serum albumin, ovalbumin, and phosvitin (Sigma) were iodinated to similar specific activities as the receptor subunits, denatured in 2% (w/v) sodium dodecyl sulfate, and diluted to 5×10^{-8} M in 0.5%–0.1% buffer. Ten microliters of mAb stocks (~1–40 μM) was added to each of the three test proteins and treated as in the subunit fragment mapping experiment.

Five mAbs raised against unrelated antigens (mouse immunoglobulin κ, λ, α, and μ chains, a gift from Dr. Kathrin Lehman) were also tested for their ability to bind to each receptor subunit. The conditions were as in the subunit fragment mapping experiment.

mAbs Binding to Trypsinized Receptor. Receptor from *Torpedo* (1 mg/mL) in 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 0.2% (w/v) sodium cholate was digested with 1:1000 (w/w) TPCK-treated trypsin (Worthington) for 90 min at room temperature. The enzyme was inactivated by adding a 1000-fold mole excess of phenylmethanesulfonyl fluoride. The digested receptor was diluted to 1×10^{-9} M in 0.5% buffer containing 5 μL of normal rat serum/mL and labeled with a 2-fold excess of [^{125}I]-α-bungarotoxin (specific activity 4×10^{17} cpm/mol). To 1 mL aliquots in triplicate were added 1×10^{-11} mol (a 10-fold excess) of each mAb, and the mixtures were incubated overnight at 4 °C. The receptor–antibody complexes were precipitated with 100 μL of goat anti-rat antibody for 4 h at 4 °C. Precipitates were spun down and washed, and the extent of mAb binding was calculated by using a normal serum control as 0% binding and 5 μL of a 10 μM anti-*Torpedo* receptor serum as 100%. Undigested receptor was also tested for mAb binding in parallel.

Results

Monoclonal antibodies were tested for their ability to bind denatured iodinated receptor subunits. Figure 1 shows two representative mAbs with primary binding specificities against each of the four receptor subunit types. The complete list of the subunit binding specificities of the mAbs used in this work is shown in Table I. Several of these have been reported previously (Tzartos & Lindstrom, 1980; Tzartos et al., 1981; S. J. Tzartos et al., unpublished results), but all were confirmed here. Since the mAbs and the iodinated receptor subunits were incubated in equimolar amounts, the subunit binding reflects the primary binding specificities of each mAb. Few mAbs precipitated more than one subunit under these conditions, although cross-reaction with other subunit was occasionally observed. In order to quantitate this cross-reaction, we measured the apparent titer of mAbs against each iodinated subunit (Table II). Of the 63 mAbs tested in antigen excess, only 4 (mAbs, 7, 142, 147, and 168) showed cross-reaction of ≥5% with more than one subunit. However, 13 of these 63 mAbs (21%) showed some cross-reaction between subunits in antigen excess. When mAb was added in large excess over antigen, slight apparent cross-reaction was detectable with a further 11 mAbs, or about 40% of the total. Only two mAbs showed extensive reaction with two or more subunits (mAbs 142 and 147). These two mAbs cross-reacted detectably with α, β, γ, and δ subunits despite reacting prefrentially with α. The extensive amino acid sequence homology between subunits would be expected to account for cross-reaction of mAbs

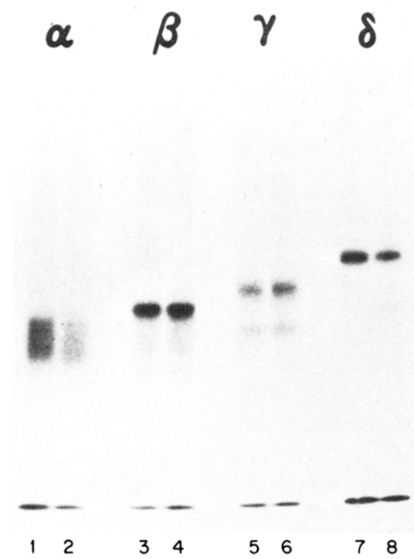

FIGURE 1: Immunoprecipitation of iodinated subunits from *Torpedo* receptor by mAbs. Subunits dissociated from the Sepharose–antibody complex were electrophoresed on a 10% polyacrylamide gel containing sodium dodecyl sulfate. (Lane 1) mAb 142, (lane 2) mAb 147, (lane 3) mAb 111, (lane 4) mAb 118, (lane 5) mAb 154, (lane 6) mAb 168, (lane 7) mAb 128, and (lane 8) mAb 150.

Table I: Primary Acetylcholine Receptor Subunit Specificities of the mAbs Used in This Work Determined by Immunoprecipitation and Polyacrylamide Gel Electrophoresis

mAb	subunit	mAb	subunit	mAb	subunit
3	α	102	β	141	δ
5	α	110	β	142	α
6	α	111	β	145	γ
7	δ	112	β	147	α
8	α	113	β	148	β
9	δ	114	β	149	α
10	β	116	β	150	δ
11	β	117	β	151	β
13	α	118	β	152	α
19	α	120	β	153	α
26	δ	121	β	154	γ
42	α	123	β	155	α
59	β	124	β	157	α
61	α	125	β	162	γ
65	α	127	δ	163	β
71	α	128	δ	164	α
91	β	129	δ	165	γ
92	β	130	δ	166	δ
94	β	131	δ	168	γ
95	β	132	γ	169	β
96	β	134	δ	170	β
97	β	136	δ	172	β
98	β	137	δ	187	α
99	β	139	δ	203	α
108	β	140	δ		

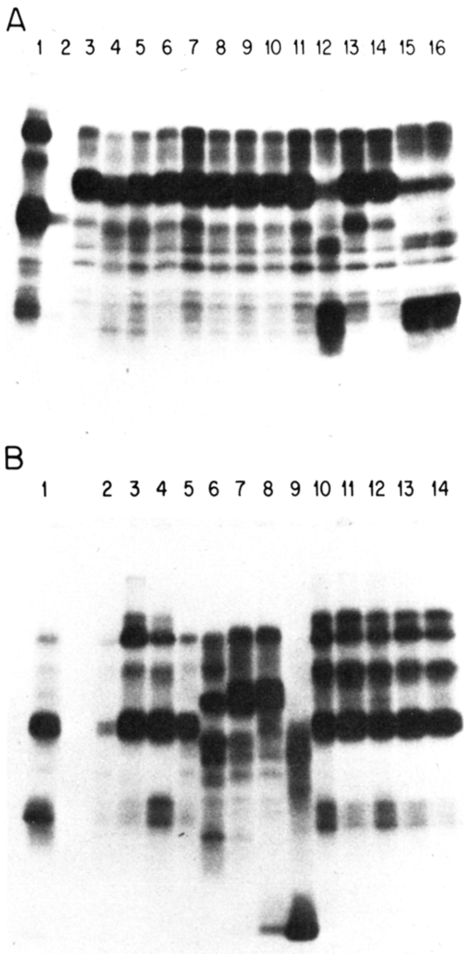

FIGURE 2: Immunoprecipitation of V8 protease generated iodinated subunit fragments run on a 15% polyacrylamide gel containing sodium dodecyl sulfate. (A) (Lane 1) Complete γ-subunit fragments, (lane 2) normal serum, and (lanes 3–16) mAbs 11, 97, 110, 111, 112, 113, 120, 123, 125, 148, 151, 163, and 170, respectively. (B) (Lane 1) Complete γ-subunit fragments, (lane 2) normal serum, (lane 3) anti-γ serum, and (lanes 4–14) mAbs, 7, 60, 94, 118, 124, 132, 145, 154, 162, 165, and 168, respectively.

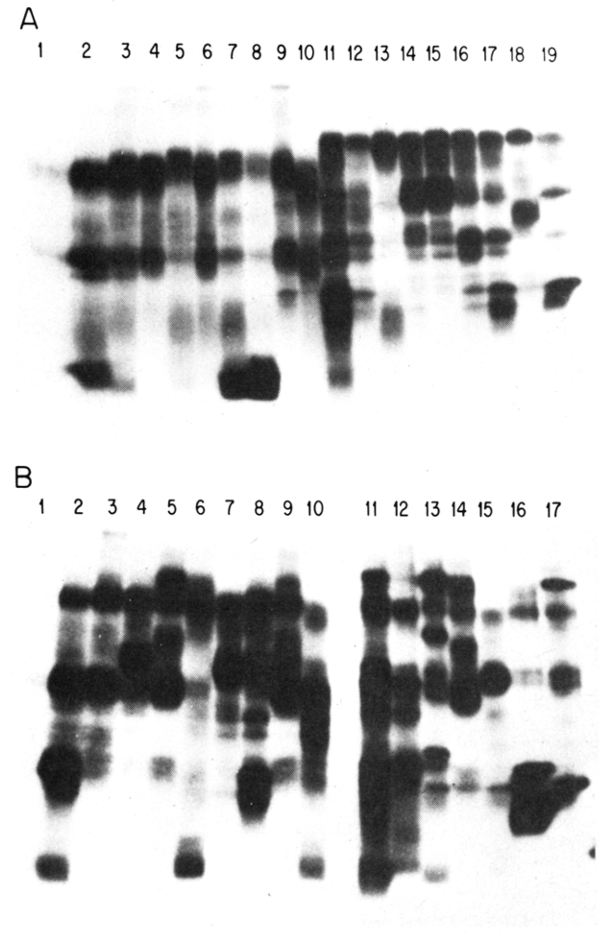

FIGURE 3: Immunoprecipitation of V8 protease generated fragments of iodinated *Torpedo* receptor subunits by representative mAbs of each group. (A) Lanes 1–10 use α subunit fragments. Lane 1 shows a normal serum precipitate. Lane 2 shows unfractionated α-subunit fragments. Lanes 3–10 are from precipitates using the following antibodies: (3) anti-α subunit serum, (4) mAb 148, (5) mAb 142, (6) mAb 111, (7) mAb 152, (8) mAb 147, (9) mAb 10, and (10) mAb 94. Lanes 11–19 use β-subunit fragments. Lane 11 shows unfractionated β-subunit fragments. Lanes 12–19 are from precipitates using the following antibodies: (12) anti-β subunit serum, (13) mAb 187, (14) mAb 109, (15) mAb 11, (16) mAb 117, (17) mAb 125, (18) mAb 154, and (19) mAb 96. (B) Lanes 1–10 use γ-subunit fragments. Lane 1 shows a normal serum precipitate. Lane 2 shows fractionated γ-subunit fragments. Lanes 3–10 are from precipitates using the following antibodies: (3) anti-γ subunit serum, (4) mAb 148, (5) mAb 168, (6) mAb 147, (7) mAb 92, (8) mAb 163, (9) mAb 124, and (10) mAb 132. Lanes 11–17 use δ-subunit fragments. Lane 11 shows unfractionated δ-subunit fragments. Lanes 12–17 are from precipitates using the following antibodies: (12) anti-δ subunit serum, (13) mAb 150, (14) mAb 154, (15) mAb 121, (16) mAb 140, and (17) mAb 170.

between subunits (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b; Noda et al., 1982, 1983; Claudio et al., 1983).

We next sought to determine whether this cross-reaction of some of the mAbs with more than one subunit was artifactual. Several reports have appeared of cross-reaction of mAbs or antisera with apparently unrelated antigens (Walter & Werchau, 1982; Nigg et al., 1982). We tested each mAb used here against three other iodinated proteins with bovine serum albumin as a representative nonglycosylated protein, ovalbumin as a glycosylated protein, and phosvitin as a phosphorylated protein. Each of these was denatured in sodium dodecyl sulfate and labeled with ^{125}I to a specific activity similar to that of the receptor subunits. Under the same conditions used to assay primary subunit specificity in Table I, only 1 of the 74 mAbs tested bound any of these 3 proteins (data not shown). Thus, these mAbs do not bind promiscuously to extraneous proteins under these conditions. Monoclonal antibody 163 bound quite strongly to iodinated phosvitin, but not to bovine serum albumin or ovalbumin. This binding could not be prevented by adding 1 mM phosphoserine or phosphothreonine, a 10 000-fold molar excess over iodinated protein. We next tested five mAbs raised against unrelated antigens (immunoglobulin chains) for binding to receptor subunits. No reaction was observed above background. These results indicate that the binding of ^{125}I-labeled receptor subunits by anti-receptor mAbs are the results of highly specific interactions.

Having established the specific primary subunit binding specificities of these mAbs and some of their cross-reactions, we then tested them for binding to protease generated fragments of each subunit. This in itself is a useful test of specificity, since in the preceding experiments we could not exclude the possibility that our purified iodinated subunits were contaminated by small amounts of other subunits, thereby artifactually producing the appearance of slight cross-reaction between subunits in some cases. Since we generate characteristic fragment patterns from each subunit, we could compare the subset of fragments immunoprecipitated by a mAb to this complete pattern to judge whether the reaction was indeed specific. In these experiments, mAbs were used in large excess

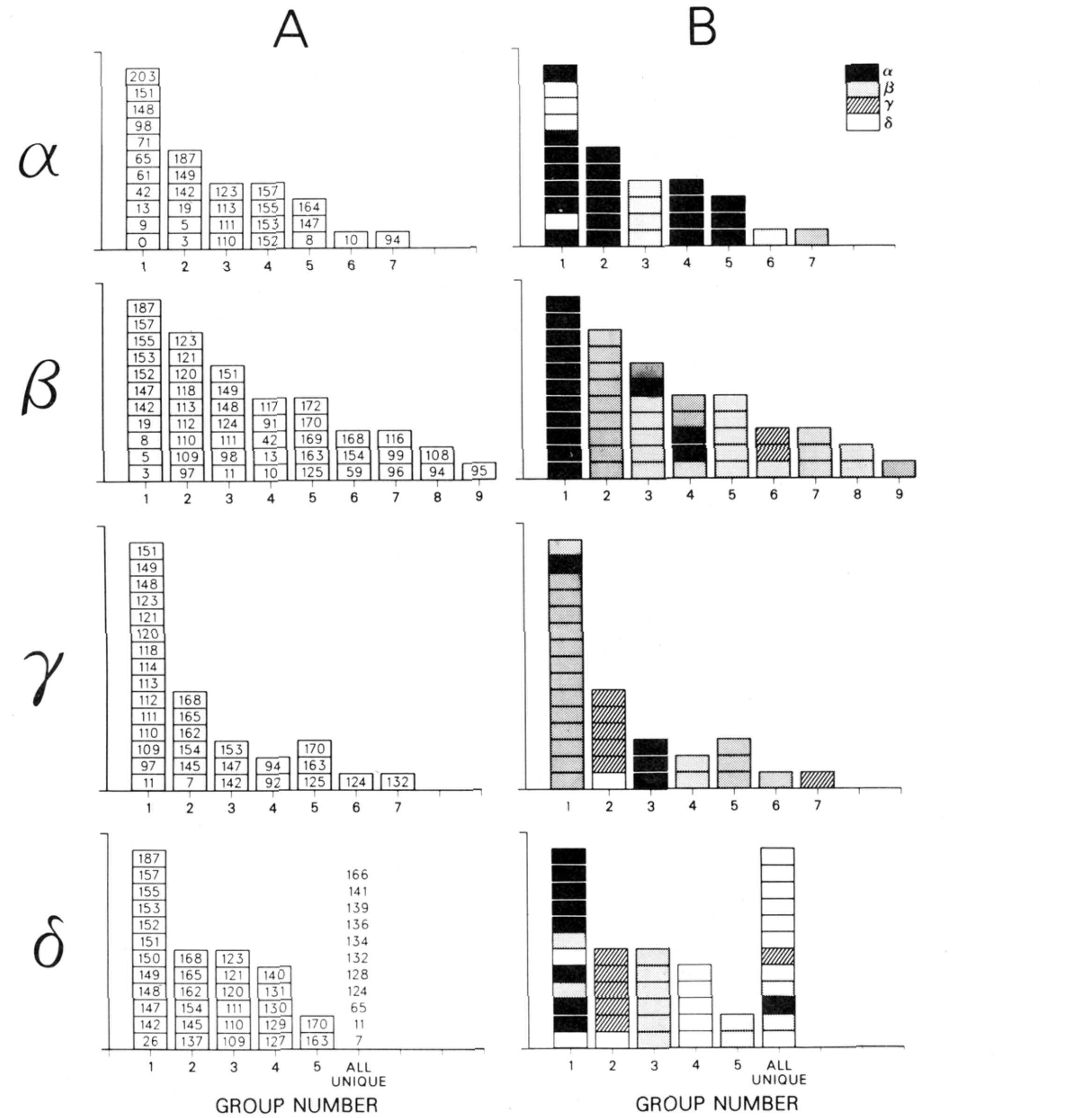

FIGURE 4: Diagrammatic representation of the groups of mAbs defined against each of the four subunits from *Torpedo* receptor. (A) mAb number is indicated in each box. (B) The primary subunit binding specificity of the mAbs shown is represented.

over antigen to exaggerate cross-reaction. In all cases, cross-reaction detected by measurement of apparent titers was confirmed by gel electrophoresis of bound subunit fragments. Occasionally we observed cross-reaction of mAbs with subunit fragments which had not been detected by the apparent titer measurements, probably as a result of the excess of mAbs added.

mAbs were classified into groups against each subunit on the basis of the subsets of fragments they bound. For example, Figure 2 is an autoradiograph of two gels that were obtained by using several mAbs whose binding patterns were unknown. Lane 1 of Figure 2A shows the pattern obtained from a complete mixture of V8 protease generated fragments of the γ subunit. Lane 2 of Figure 2A is a normal serum control, and lanes 3–16 are immunoprecipitates obtained by individual mAbs. The antibodies used here bound primarily to the β subunit but cross-reacted with the γ subunit. In this case it was easy to define lanes 3–11, 13, and 14 as being members of one group and lanes 12, 15, and 16 as belonging to another group. In Figure 2B, lane 1 shows another experiment using V8 protease fragments of the γ subunit, lane 2 is the normal serum control, and lane 3 shows the fragments precipitated by anti-γ subunit serum. In this case, lanes 5–9 represent mAbs from different groups, whereas lanes 4 and 10–14 are all members of another group. The most difficult subunit to deal with was the α subunit, first because it gave less well-defined bands than did other subunits (see Figure 3A in which lanes 1–10 are α-subunit fragments and lanes 11–19 are β fragments, all run on the same gel) and second because most mAbs tested bound small amounts of radioactivity. From these experiments we defined several groups of mAbs against each subunit. Figure 3 shows immunoprecipitates by representatives from each group against each of the four subunits. The groups we have defined are shown in Figure 4A. The same groups are presented in Figure 4B, but in this case their primary subunit binding properties have been indicated.

As another test of our group definitions we investigated the ability of each mAb to bind to trypsin-treated receptor (Figure 5). Receptor treated with relatively low levels of trypsin showed degrees of precipitation by different mAbs varying from complete to not detectable. If our group mapping defines individual determinants, one would expect that members of a group would be similarly affected by proteolysis. This was generally observed. For example, mAbs that form group 3

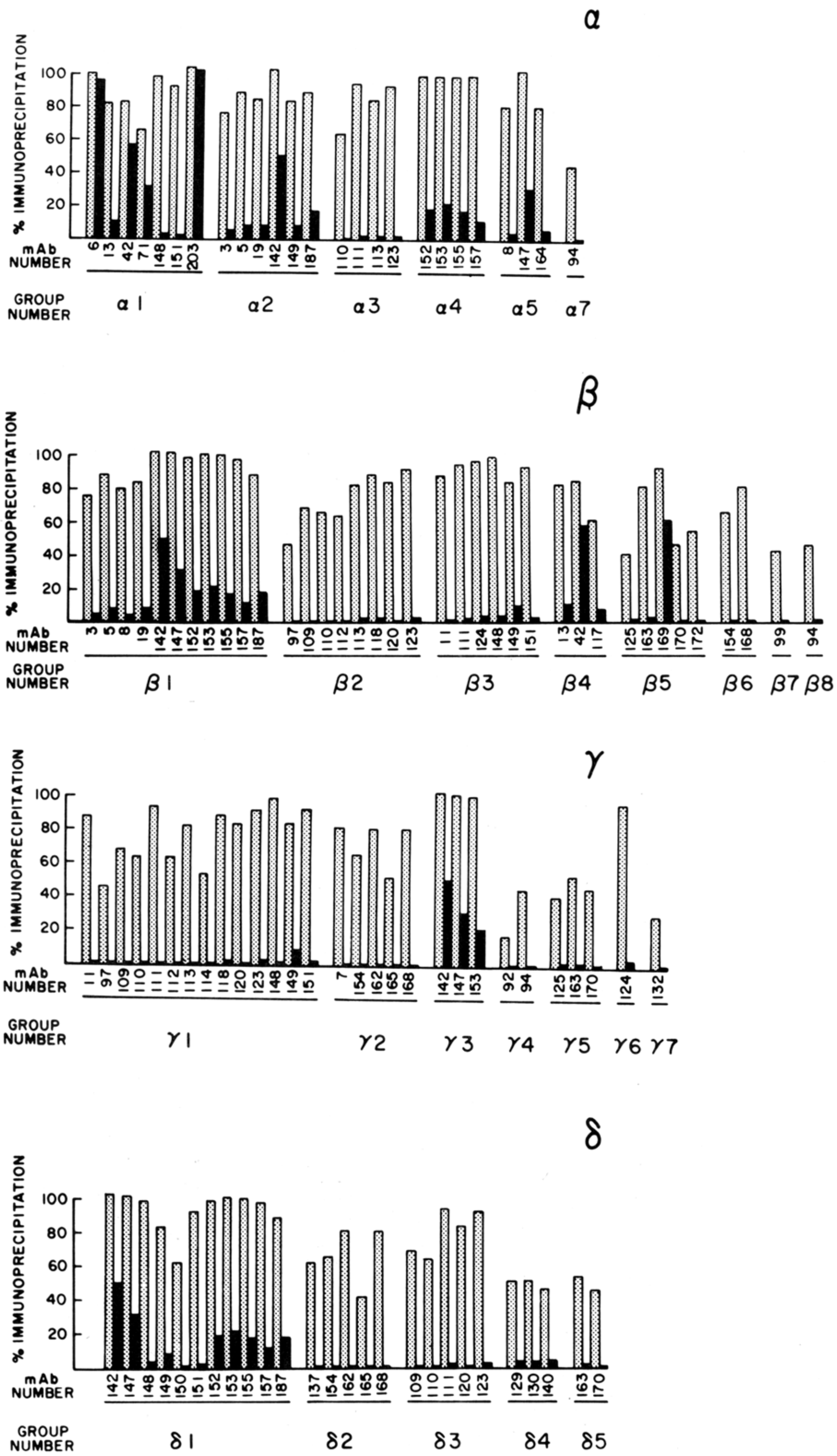

FIGURE 5: Effect of trypsin treatment of native *Torpedo* receptor on mAb binding. Shaded bars show the percent of untreated receptor immunoprecipitated by each mAb. Solid bars show the amount of proteolyzed receptor immunoprecipitated under the same conditions. The 100% figure in each case is the amount of receptor precipitated by an anti-receptor serum. All figures are corrected for nonspecific precipitation by a normal serum control.

against the γ subunit retain considerable binding, whereas all other anti γ mAbs are no longer capable of binding (Figure 5). However, in some cases members of a group were clearly distinguished by the trypsin sensitivity of their antigenic determinants. For example, mAbs 125 and 169 are anti-β antibodies in group 5, but the antigenic determinant of mAb 125 is proteolytically sensitive, whereas the determinant for mAb 169 is not. Thus, although the groups of mAbs defined by the pattern of V8 protease generated peptide fragments they bind in general may identify a single antigenic determinant on a subunit, in some cases more than one determinant may be recognized by the group of mAbs.

Discussion

We found that 74 of 154 mAbs raised against acetylcholine receptors from *Torpedo californica*, *Electrophorus electricus*, cattle, and humans reacted with denatured subunits of receptor from *Torpedo* (Figure 1, Tables I and II). The specificity of these reactions was shown by the failure of five mAbs to extraneous proteins to react with any receptor subunit and by

Table II: Monoclonal Antibody Titer (nM) against Individual ^{125}I-Labeled Subunits from *Torpedo* Acetylcholine Receptor

mAb[a]	α	β	γ	δ	mAb	α	β	γ	δ
3	628				129				127
5	3300				130				96
6					132		5^b	69	
7			513	1910	134				83
8	270				136				9^b
10	5	14			137				1250
11		3470	119		138				
13	539	2^b			139				52^b
19	2590	3^b			140				272
42					141				317
61	9^b				142	27600^c	3710	1780	939
65	0.5				147	3580	617	199	30^b
71					148	49^b	11700	563	
92		108			149	2220	276^b	50^b	
94		75			150				1950
96		15			151		9510	55^b	
97		98			152	1250	18^b		
98		10			153	1400	20^b	17^b	
127				26	154		115^b	2780	34
128				420					
99		136			155	4350			
109		5180			157	996			
110		8370	147		162			51	5^b
111	58^b	11800	52		163		55		
112		1820^b	12		164	659			
113		12000	81^b		165			159	
114		144			166				496
116		17			168		950	2920	37^b
117		96			169		830	37	
118		11700	448		170		43		
120		5310	34^b		172		2800	17^b	
123		9000	349		173				
124	111^b	25100	1270		187	2400		17^b	
125		190			203				

[a] Unless otherwise indicated, mAb titers against subunits (expressed in nM of subunit bound per L of mAb) were measured by using subunits at 1×10^{-8} M and mAb at 1×10^{-8} M (expressed in moles of ^{125}I toxin binding sites of native *Torpedo* receptor bound per liter of antibody). Because two toxin molecules are bound per receptor monomer and because purified receptor is about two-thirds dimers (Karlin, 1980), 1×10^{-8} M in titer against native receptor corresponds to about 3×10^{-9} M in IgG concentration, depending on the affinity of the mAb and whether it cross-links monomers (Conti-Tronconi et al., 1981). Thus, mAb titers against subunits were measured in antigen excess. [b] In these cases, mAbs were added in about 3-fold molar excess over antigen by adding 1×10^{-7} M of mAb titer against native receptor. [c] In these cases mAbs were very effective at binding subunits, and only 1×10^{-9} M of mAb titer against native receptor was added to ensure antigen excess.

the failure of all but one mAb to receptor to react with any of three extraneous proteins. Of the mAbs tested, 94% were at least 95% specific for a single receptor subunit (Table II). However, when large excesses of mAbs over subunits were added, at least some low-affinity cross-reaction with more than one subunit was detectable with 40% of the antibodies. Two mAbs cross-reacted with all four receptor subunits. These results show that mAbs are highly specific probes for receptor subunits and that some can detect structural homologies between subunits which are the consequence of the extensive amino acid sequence homologies between subunits (Raftery et al., 1980; Conti-Tronconi et al., 1982b; Noda et al., 1982, 1983).

mAbs were further tested for their ability to bind characteristic patterns of peptide fragments generated by proteolysis of each subunit by V8 protease (Figures 2–4). These results permitted the grouping of mAbs according to the pattern of peptide fragments which they bound. The grouping of mAbs was further tested by determining the trypsin sensitivity of the antigens for each mAb (Figure 5). These results suggest that several antigenic determinants are identifiable on each subunit by this approach. (Figure 4 suggests about seven on α, nine on β, six on γ, and at least five on δ.)

These results can be compared with the results of other studies. In the case of α subunits, we have previously defined three antigenic determinants by peptide mapping [corresponding to α groups 1 (the MIR), 2, and 5 in Figure 4] (Gullick et al., 1981) and shown that these mAbs also react as groups when tested with native receptor by competitive binding (Tzartos & Lindstrom, 1980) and by formation of characteristic antibody complexes on sucrose gradients (Conti-Tronconi et al., 1981). We know that the MIR is located on the extracellular surface of α subunits (Gullick et al., 1981) and that another of the determinants (α group 5) is inaccessible in receptors incorporated in membranes (Gullick et al., 1981; Lindstrom et al., 1981b). We have also succeeded in ordering these three determinants, the carbohydrate binding site, and the MBTA binding site along the α subunit (W. J. Gullick et al., unpublished results). Anderson et al. (1983) have ordered the binding sites for several of these mAbs along the sequence of δ and determined their transmembrane orientation. All of the mAbs to δ react with domains on the cytoplasmic surface of the receptor. The antigenic determinants for mAbs, 7, 141, and 166 are on the cytoplasmic surface of the 65-kilodalton δ subunit approximately 44–52 kilodaltons from the N terminus, whereas mAbs 127, 129, 131, and 140 react with a determinant or determinants on the cytoplasmic surface of the δ subunit between 52 and 65 kilodaltons from the N terminus in a domain that is solubilized as a 12-kilodalton fragment by trypsinization (Anderson et al., 1983). These results are consistent with the results presented here. Anderson et al. (1983) also showed that mAb 169 uniquely reacts with the extracellular domain of the β subunit, while mAb 125 reacts with the cytoplasmic domain. Figures 3 and 4 indicate that the complex pattern of V8 peptides produced by mAbs 169 and 125 are indistinguishable, but Figure 5 shows that the antigens for mAbs 169 and 125 are clearly distinguished by their sensitivity to proteolysis. The proteolytic sensitivity of the determinant for mAb 125 may result from solubilization of the cytoplasmic domain to which it is directed. Studies with antisera have indicated that the cytoplasmic (C-terminal; Anderson et al., 1982) domains of denatured receptor subunits are the most immunogenic (Froehner, 1981). This is consistent with our results (Anderson et al., 1983; J. M. Lindstrom et al., unpublished results) and an interesting contrast to the dominant immunogenicity of the extracellular domain of the α subunit in the intact receptor (Tzartos & Lindstrom, 1980; Tzartos et al., 1981). Fuchs et al. (1982) reported that trypsinized receptor was not immunoprecipitated by antisera to denatured receptor. Similarly, most of our mAbs made to denatured receptor are unable to bind to trypsinized receptor, but mAbs to native receptor, which are primarily to the MIR on a α subunits, are quite trypsin resistant. The observations that the extracellular surface of the receptor molecule is remarkably resistant to trypsin while the cytoplasmic surface is very sensitive (Klymkowski et al., 1980), that trypsin solubilizes a cytoplasmic domain of δ subunits but does not effect an extracellular domain of β subunits (Anderson et al., 1983), and that the receptor subunits are extensively homologous (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b; Noda et al., 1982, 1983; Claudio et al., 1983) suggest that the proteolytic sensitivity of most subunit-specific mAbs made against denatured receptor may be due to the cleavage and dissociation of small cytoplasmic domains of each subunit of the receptor of trypsinization. In this context it is interesting to note that even extensive proteolysis of receptor does not dissociate a large fraction of its mass, prevent its integration in the membrane, or prevent opening of its cation channel in response to agonists (Lindstrom et al., 1980; Huganir & Racker, 1980).

This report provides base-line informatioin which will aid in the much more precise mapping experiments with mAbs which will be required in the future to adequately define the antigenic structure of the receptor and map the binding sites of mAbs which effect receptor functions.

Acknowledgments

We thank John Cooper for isolating the receptor from *Torpedo* and performing the iodinations. We are grateful to Dr. Susan Hochschwender for preparing the goat anti-rat immunoglobulin coupled to agarose and to Dr. Kathrin Lehman for the gift of the anti-mouse immunoglobulin mAbs. We are especially grateful to Dr. David Anderson for discussions of his unpublished experiments.

Registry No. Acetylcholine, 51-84-3.

References

Anderson, D., Walter, P., & Blobel, G. (1982) *J. Cell Biol. 93*, 501–506.

Anderson, D., Blobel, G., Tzartos, S., Gullick, W., & Lindstrom, J. (1983) *J. Neurosci.* (in press).

Anholt, R., Lindstrom, J., & Montal, M. (1980) *Eur. J. Biochem. 109*, 481–487.

Anholt, R., Lindstrom, J., & Montal, M. (1983) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) Plenum Press, New York (in press).

Changeux, J. P. (1981) *Harvey Lect. 75*, 85–254.

Claudio, T., Ballivet, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1111–1115.

Conti-Tronconi, B., & Raftery, M. (1982) *Annu. Rev. Biochem. 51*, 491–530.

Conti-Tronconi, B. M., Tzartos, S. J., & Lindstrom, J. M. (1981) *Biochemistry 20*, 2181–2191.

Conti-Tronconi, B., Gotti, G., Hunkapiller, M., & Raftery, M. (1982a) *Science (Washington, D.C.) 218*, 1227–1229.

Conti-Tronconi, B., Hunkapiller, M., Lindstrom, J., & Raftery, M. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6489–6483.

Dwyer, D., Kearney, J., Bradley, R., Kemp, G., & Oh, S. (1981) *Ann. N.Y. Acad. Sci. 377*, 143–157.

Einarson, B., Gullick, W., Conti-Tronconi, B., Ellisman, M., & Lindstrom, J. (1982) *Biochemistry 21*, 5295–5302.

Froehner, S. (1981) *Biochemistry 20*, 4905–4915.

Fuchs, S., Bartfeld, D., Mochly-Rosen, D., Souroujon, M., & Feingold, C. (1982) *Ann. N.Y. Acad. Sci. 377*, 110–124.

Gomez, C. M., Richman, D. P., Burres, S. A., & Arnason, B. G. W. (1982) *Ann. N.Y. Acad. Sci. 377*, 97–109.

Gullick, W., & Lindstrom, J. (1982a) *J. Cell. Biochem. 19*, 223–230.

Gullick, W., & Lindstrom, J. (1982b) *Biochemistry 21*, 4563–4569.

Gullick, W. J., Tzartos, S. J., & Lindstrom, J. M. (1981) *Biochemistry 20*, 2173–2180.

Huganir, R., & Racker, E. (1980) *J. Supramol. Struct. 14*, 215–221.

James, R., Kato, A., Rey, M., & Fulpius, B. (1980) *FEBS Lett. 120*, 145–148.

Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Poste, G., & Nicolson, G. L., Eds.) pp 191–260, Elsevier, Amsterdam.

Klymkowski, M., Heuser, J., & Stroud, R. (1980) *J. Cell Biol. 85*, 823–838.

Lennon, V. A., & Lambert, E. H. (1982) *Ann. N.Y. Acad. Sci. 377*, 77–96.

Lindstrom, J., Campbell, M., & Nave, B. (1978a) *Muscle Nerve 1*, 140–145.

Lindstrom, J. M., Einarson, B., & Merlie, J. P. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 769–773.

Lindstrom, J. M., Merlie, J. P., & Yogeeswaran, G. (1979a) *Biochemistry 18*, 4465–4470.

Lindstrom, J., Walter, B., & Einarson, B. (1979b) *Biochemistry 18*, 4470–4480.

Lindstrom, J., Gullick, W., Conti-Tronconi, B., & Ellisman, M. (1980) *Biochemistry 19*, 4791–4795.

Lindstrom, J., Einarson, B., & Tzartos, S. (1981a) *Methods Enzymol. 74*, 432–459.

Lindstrom, J. M., Tzartos, S. J., & Gullick, W. J. (1981b) *Ann. N.Y. Acad. Sci. 377*, 1–19.

Lindstrom, J. M., Cooper, J. F., & Swanson, L. W. (1983) *Biochemistry* (in press).

Nigg, E. A., Walter, G., & Singer, S. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5939–5943.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature (London) 299*, 793–797.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983) *Nature (London) 301*, 251–255.

Patrick, J., & Lindstrom, J. (1973) *Science (Washington, D.C.) 180*, 871–872.

Raftery, M. A., Hunkapiller, M. W., Strader, C. D., & Hood,

L. E. (1980) *Science (Washington, D.C.) 208*, 1454–1457.
Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035–2038.
Tzartos, S. J., & Lindstrom, J. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 755–759.
Tzartos, S. J., & Lindstrom, J. M. (1981) in *Monoclonal Antibodies in Endocrine Research* (Fellows, R., & Eisenbarth, G., Eds.) pp 69–86, Raven Press, New York.
Tzartos, S. J., Rand, D. E., Einarson, B. L., & Linstrom, J. M. (1981) *J. Biol. Chem. 256*, 8635–8645.
Tzartos, S. J., Seybold, M. E., & Lindstrom, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 188–192.
Walter, G., & Werchau, H. (1982) *J. Cell. Biochem. 19*, 119–125.

Kinetics of Divalent Cation Induced Fusion of Phosphatidylserine Vesicles: Correlation between Fusogenic Capacities and Binding Affinities†

Joe Bentz,* Nejat Düzgüneş, and Shlomo Nir

ABSTRACT: Fusion and aggregation of sonicated phosphatidylserine small unilamellar vesicles (PS SUV) induced by the divalent cations Ba^{2+}, Ca^{2+}, Sr^{2+}, and Mg^{2+} are studied and correlated with cation binding. Fusion is monitored with the terbium/dipicolinic acid (Tb/DPA) assay, which is supplemented by measuring the dissociation of preencapsulated Tb/DPA complex due to leakage of contents and entry of medium into the vesicles. The separate contributions of aggregation and fusion rates to the overall kinetics of membrane fusion are analyzed. In the presence of Na^+ or Li^+ concentrations of 300 mM or less, the rate of the overall fusion reaction induced by the divalent cations decreases in the sequence $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$ with respect to bulk concentrations. Under these conditions, both aggregation kinetics and bilayer destabilization are shown to affect the overall rate of fusion. In the presence of 500 mM Na^+ or Li^+ and subfusogenic concentrations of each of the divalent cations, the PS SUV reversibly aggregate; thus, the effect of the divalent cations (at larger, fusing concentrations) on the rate of bilayer destabilization can be examined directly. Here, Ba^{2+} and Ca^{2+} appear to be equally effective at inducing fusion. However, when the rate constant of fusion (which can be easily obtained in these cases) is compared with the amount of divalent cation bound per PS (calculated from binding constants which apply to the vesicles before aggregation and fusion), the fusogenic capacities of these divalent cations follow the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$.

The fusion of acidic phospholipid vesicles provides a basic model for biological membrane fusion in that there are three distinct, kinetically coupled stages common to both systems. First, the close apposition of bilayers is obtained by the aggregation of two or more vesicles. Second, the closely apposed bilayers must undergo a destabilization leading to an initial merging of the bilayers. Third, the merged bilayers must re-form into a continuous bilayer which encapsulates the mixed contents of each vesicle. Hence, we can use the vesicle systems to develop the biophysical theory required to examine the molecular mechanisms of membrane fusion. [See Nir et al. (1983) and Düzgüneş & Papahadjopoulos (1983) for reviews on phospholipid vesicle fusion.]

While there are many methods of monitoring fusion (Papahadjopoulos et al., 1974, 1976; Hoekstra et al., 1979; Liao & Prestegard, 1980a,b; Schullery et al., 1980; Schmidt et al., 1981; Vistnes & Puskin, 1981), those methods employing resonance or exchange energy transfer between fluorescent molecules are well suited for continuously monitoring the mixing of vesicle contents and bilayers (Struck et al., 1981; Wilschut & Papahadjopoulos, 1979). The terbium/dipicolinic acid (Tb/DPA)[1] assay (Wilschut et al., 1980, 1981), which measures the mixing of vesicle contents via the enhancement of Tb^{3+} fluorescence following chelation by DPA, is particularly suitable as the measured Tb fluorescence can be directly related to the number of fused vesicles (Nir et al., 1980b). The assay is sufficiently sensitive to allow the use of low vesicle concentrations, which is essential to examine the initial interaction of two vesicles. We have used this assay to determine both aggregation and fusion rate constants for PS SUV and LUV in Ca^{2+} (Nir et al., 1982; Bentz et al., 1983).

Here we will use the assay to quantitate the fusion kinetics of PS SUV induced by Ba^{2+}, Ca^{2+}, Sr^{2+}, Mg^{2+} in media containing either Na^+ or Li^+. We have introduced a new element to this assay: the dissociation of preencapsulated Tb/DPA complex which is due to both the leakage of complex into the medium and the influx of divalent cations and EDTA into the vesicles. This natural complement to the fusion assay is necessary because the fusion of PS SUV is somewhat leaky (Wilschut et al., 1980), and it is known that with Ca^{2+} the vesicles eventually transform into collapsed anhydrous structures (cochelates; Papahadjopoulos et al., 1975) which is the final equilibrium state. As models for biological membrane

† From the Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy (J.B.), and the Cancer Research Institute and Department of Anesthesia, School of Medicine (N.D.), University of California, San Francisco, California 94143, the Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center, Oakland, California 94609 (N.D.), and The Seagram Centre for Soil and Water Sciences, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel (S.N.). *Received December 20, 1982.* This investigation was supported by Research Grants GM-31506 (J.B. and S.N.) and GM-28117 (N.D.) from the National Institutes of Health and Grant 1-768 from the March of Dimes Foundation (N.D.).

[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetate; LUV, large unilamellar vesicle(s) (diameter ~100 nm); MLV, multilamellar vesicle(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicle(s) (diameter ~30 nm); Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; TMA^+, tetramethylammonium.

0006-2960/83/0422-3320$01.50/0