Monoclonal antibodies against the acetylcholine receptor γ-subunit as site specific probes for receptor tyrosine phosphorylation

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Received 27 February 1995

Abstract Tyrosine phosphorylation of the nicotinic acetylcholine receptor (AChR) may be involved in AChR desensitization and clustering. *Torpedo* AChR γ-subunit is phosphorylated at Tyr^{365}. Using overlapping synthetic peptides, we have precisely mapped the epitopes of five anti-γ-subunit monoclonal antibodies (mAbs) and found that the epitope(s) for the mAbs 154, 165 and 168 (γ365–370) all contain Tyr^{365}. mAb 168 is a known blocker of AChR channel function. Using peptide analogues, Tyr^{365} was found to be indispensable for mAb165 binding; furthermore its binding was selectively inhibited by in vitro AChR tyrosine phosphorylation. The possible connection between γ-subunit phosphorylation and regulation of AChR function and the proven usefulness of these mAbs as tools should facilitate functional studies of AChR γ-subunit phosphorylation.

Key words: Acetylcholine receptor; Monoclonal antibody; Tyrosine phosphorylation; Epitope mapping; Synthetic peptide

1. Introduction

The nicotinic acetylcholine receptor (AChR) from fish electric organs is a transmembrane glycoprotein composed of five homologous subunits in the stoichiometry $\beta_2\beta\gamma\delta$. Acetylcholine binds to the two α-subunits and regulates the opening of the AChR ion channel. The channel is formed from all five subunits, which are arranged in a pentameric rosette (reviewed in [1,2]).

The AChR is phosphorylated by cAMP-dependent protein kinase, protein kinase C and at least two endogenous protein tyrosine kinases [3,4]. The protein tyrosine kinases phosphorylate the β, γ and δ subunits at βTyr^{355}, γTyr^{365} and δTyr^{372} [5]. AChR tyrosine phosphorylation seems to be involved in AChR clustering and in increasing its desensitization rate [6–8]. The availability of specific tools should be invaluable in studying the individual role of each single phosphorylation site.

Monoclonal antibodies (mAbs) against specific sites on the AChR offer such a set of powerful probes for studying AChR phosphorylation. Eleven anti-β-subunit mAbs bind to a *very immunogenic cytoplasmic epitope* (β352–359) containing the β-subunit phosphorylation site (βTyr^{355}) [9]. One of these mAbs (No. 148) is known to block the AChR channel [10,11] and its binding is partially inhibited by AChR tyrosine phosphorylation [9]. The epitope for one anti-δ-subunit mAb contains δTyr^{372} and that for another contains the serine phosphorylation site δSer^{377} [12]. The epitopes for our anti-γ-subunitmAbs were previously mapped at a relatively low resolution [13] which did not permit the accurate localization of the epitopes involved and their relationship to the phosphorylation sites.

In this paper, the fine epitope mapping of five mAbs on the *Torpedo* AChR γ-subunit revealed an interesting association between γ-subunit phosphorylation and regulation of AChR function; it also demonstrated that these mAbs should prove to be highly specific tools in subsequent studies on the role of AChR γ-subunit tyrosine phosphorylation.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized on the tips of small polyethylene rods coated with polymers of acrylic acid formed by radiation grafting [9,12,14–16]. For each sequence, at least 2–3 copies (i.e. 2–3 identical rods) were synthesized.

2.2. Monoclonal antibodies

mAbs were derived from rats immunized with sodium dodecyl sulfate (SDS)-denatured *Torpedo* AChR or purified γ-subunit [17,18]. The preparations used were 50% ammonium sulfate precipitates from hybridoma supernatants; the solubilized precipitates were dialyzed against 145 mM NaCl, 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, pH 7.4 (PBS), containing 0.05% NaN_3.

2.3. ELISA assays

Enzyme-linked immunosorbent assays (ELISA) were performed as previously described [9]. In brief, each peptide-bearing rod was preincubated for 1 h at room temperature with 200 μl of PBS, containing 0.1% Tween-20, 0.8% bovine serum albumin and 0.2% ovalbumin, followed by overnight incubation at 4°C with 200 μl of a dilution of test mAb (1/200 to 1/5,000) in the above solution. They were washed with PBS containing 0.05% Tween-20, then incubated with peroxidase-labelled rabbit anti-rat γ-globulin (DACO) for 1 h at room temperature and washed again. Bound mAbs were detected by reaction for 10 min with 0.05% azino-di-3-ethylbenzthiazodinsulphonate and 0.03% H_2O_2 in 0.1 M Na_2HPO_4/0.08 M citric acid buffer, pH 4, followed by measurement of the optical density of the solution at 405 nm.

2.4. AChR preparation, tyrosine phosphorylation and mAb binding

AChR-rich membranes were prepared from frozen *Torpedo californica* electric organs (Marinus, Long Beach, Ca) and treated as described [9]. ^{32}P-tyrosine phosphorylated AChR was prepared using the method of Wagner et al. [5] with slight modifications [9]. Extracts containing 1–4 pmol ^{32}P-labelled AChR were incubated with 0.5–2 pmol test mAb immobilized on CNBr activated Sepharose beads (Pharmacia) for 2 h at 4°C in 50 μl PBS containing 0.5% Triton X-100, 0.05% NaN_3. [9]. The radioactive Sepharose beads were washed and the labelled AChR subunits eluted with SDS-mercaptoethanol sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% w/v polyacrylamide) [19] without boiling. After electrophoresis, the gels were fixed,dried and subjected to autoradiography. SDS-PAGE gels were occasionally subjected to alkaline treatment [20] which confirmed that phosphorylation involved mainly tyrosines.

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Abbreviations: AChR, acetylcholine receptor; mAb, monoclonal antibody; PBS, 145 mM NaCl/7.5 mM Na_2HPO_4/2.5 mM NaH_2PO_4, pH 7.4 buffer; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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SSDI 0014-5793(95)00316-9

3. Results

3.1. Monoclonal antibody binding to overlapping γ-subunit undecapeptides

Fifteen undecapeptides, overlapping by eight residues and covering the region γ356–408, were initially synthesized. The anti-α mAb 6, used as a negative control, did not bind to any peptide (not shown). mAbs 154, 165 and 168 bound mainly to γ362–372 and γ365–375, suggesting that the critical binding segment lay within the common segment γ365–372. These results also suggested that mAb 7 should bind to γ380–387 and mAb 145 to γ392–399 (Fig. 1A).

Subsequently, to further localize the critical binding segments, we synthesized overlapping peptides differing by only one residue and tested them for their ability to bind the corresponding mAbs. Fig. 1B suggests, but does not prove, that the critical binding segment for mAb 168 is ILKKP (γ366–370) and that for mAbs 154 and 165 is YILKKP (γ365–370).

On the basis of the above results, we then synthesized selected peptides of various lengths to delineate the corresponding epitopes. Fig. 2 shows that the binding of mAbs 154, 165 and 168 required peptides (E)YILKKP (γ364–370), YILKK(P) (γ365–370) and (Y)ILKK(PRS) (γ365–372), respectively, i.e. sequences which are similar, but not identical, to those previously implicated. Residues in parenthesis denote a partial contribution to mAb binding. mAb 7 needed the hexapeptide RHGLKR (γ382–387) for efficient binding, but showed significant binding even to the tetrapeptide RHGL. mAb 145 required the hexapeptide TSDIDI (γ392–397). Fig. 3 summarises the epitope mapping results.

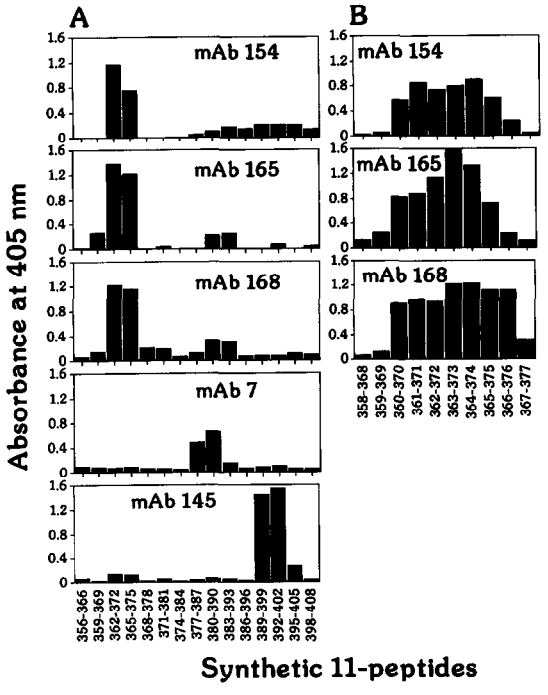

Fig. 1. Epitope analysis of 5 anti-γ-subunit mAbs. Antibodies were tested forbinding to undecapeptides, overlapping by eight (A) or ten (B) residues, derived from the *Torpedo* AChR γ-subunit sequence 356–408. Peptides were attached to polyethylene rods and tested for mAb binding by ELISA using peroxidase-labelled anti-rat γ-globulin antibody.

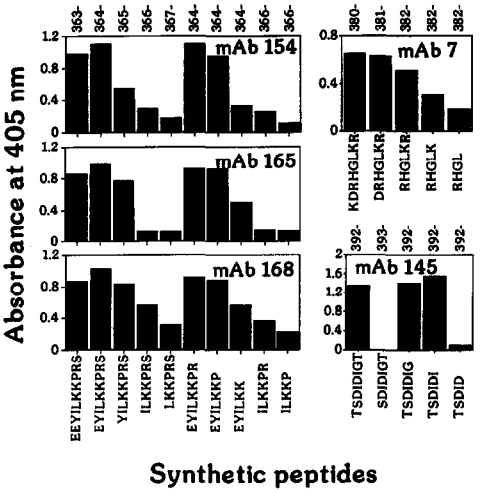

Fig. 2. Binding pattern of mAbs to selected peptides of various sizes. Both the peptide sequences and the corresponding positions of their N-terminal ends on the γ-subunit are shown. Experimental conditions as in Fig. 1.

3.2. Binding role of single residues within the phosphorylation site-containing epitopes

Since mAbs 154, 165 and 168 are important candidates for site-specific phosphorylation studies, we then examined the binding role of individual residues within their epitopes, paying particular attention to the role of γTyr^{365}. Fig. 4 shows the binding results for a series of analogues of the original nonapeptide in which one residue at a time was substituted by alanine or other test amino acids. Almost any single residue could be replaced by Ala without any dramatic reduction in the binding ability of the peptide with two exceptions: γLys^{368} was critical for binding of mAbs 154 and 168, while the phosphorylatable γTyr^{365} was indispensable for mAb 165 binding.

3.3. Tyrosine phosphorylation of AChR inhibits mAb 165 binding

We then tested the effect of tyrosine phosphorylation of the AChR on the binding of mAbs which bind to γTyr^{365}. Membrane-bound AChR was partially tyrosine phosphorylated by endogenous protein tyrosine kinases and [γ-^{32}P]ATP. Control samples, in which $MnCl_2$ was omitted, resulted in very weak labelling. The ^{32}P-labelled membranes were then solubilized using Triton X-100 and their ^{32}P-labelled AChR molecules immunoprecipitated by anti-AChR mAbs. Since the fraction of the AChR phosphorylated in vitro was approximately 0.1–0.2, most labelled molecules would be expected to have only one ^{32}P-labelled subunit (β, γ or δ). Any mAb unable to bind to the phosphorylated γ-subunit would thus still be able to precipitate AChR molecules labelled on their β and/or δ subunits.

Although differences in mAb affinity for the AChR would be expected to result insignificant differences in the total amount of precipitated radioactivity, the distribution of the radioactivity precipitated between the three subunits δ/γ/β should be constant for all mAbs whose binding is not affected by AChR tyrosine phosphorylation and should differ for those mAbs whose binding is affected by AChR tyrosine phosphorylation.

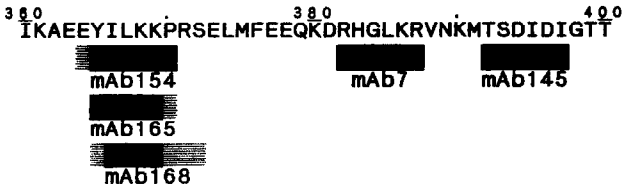

Fig. 3. Summary of the epitope locations. The epitopes determined in the study are represented by horizontal bars corresponding to the shown *Torpedo* γ-subunit segment. Shadowed parts of the bars denote that the corresponding residues enhanced, but were not essential for, mAb binding.

The resulting autoradiograms, shown in Fig. 5, demonstrate that mAb 165 precipitated proportionally a much lower amount of labelled γ-subunit than did the other mAbs and that mAbs 114 and 134, which bind to the tyrosine phosphorylation sites of the β and δ subunits [9,12], precipitated AChRs with proportionally a much lower amount of the corresponding labelled subunits (Fig. 5).

4. Discussion

In the present study, we have precisely localised the sequential epitopes of five mAbs directed against the AChR γ-subunit and provided a molecular basis for previously noted characteristics of these mAbs (see below). The epitopes containing the tyrosine phosphorylation site of the γ-subunit were characterized in depth in order to determine the basis of binding and to provide highly specific tools for subsequent phosphorylation studies. The observations that one of the mAbs directed against this site blocks AChR channel function and that another differentiates between phosphorylated and non-phosphorylated γ-subunit enhances the potential of these mAbs.

Nelson et al. [13] found that mAbs 154, 165 and 168 bind to the 20-peptide γ358–377 (IMIKAEEYILKKPRSELMFE) which contains the presently identified epitopes. They also found that mAbs 154 and 168, but not mAb 165, bind to the adult, but not the embryonic, bovine AChR. The homologous 20-peptides of adult and embryonic AChR are LLLR-**AEELILKKPRSEY-FE** and PITAGEEVALCL**PRSELLFR**, respectively. Based on sequence homologies and mAb cross-reactivity, the authors suggested that mAbs 154 and 168 bind between γ364–373 (which contains the presently identified epitopes γ364–372) but that mAb 165 binds between γ357–362 (distinct from theactual epitope). Our results (Fig. 4) now explain the different cross-reactivity pattern seen between mAb 165 and mAbs 154 and 168 with adult bovine AChR. Although these mAbs have almost identical epitopes which differ from the bovine adult sequence only at position 365 (Y versus L), Tyr^{365} is indispensable for mAb 165 binding to the peptide, but is not required in the case of mAbs 154 and 168; in fact, it can be substituted by any of the tested amino acids (including Leu, which occurs inthe adult bovine AChR) without any effect on their binding (see Fig. 4). In contrast, the epitopes of the three mAbs differ dramatically in the corresponding bovine embryonic γ-subunit, thus explaining the lack of binding to embryonic bovine AChR.

In earlier competition experiments, using mAbs which bind to the intact *Torpedo* AChR [21], we found, in agreement with the present mapping studies, that mAbs 154, 165 and 168 completely competed with one another for binding to the AChR. However, although they did not compete with many other mAbs directed against other subunits, two (mAbs 154 and 168) competed well with mAbs 128 and 129 which bind to the apparently extracellular C-terminal end of the δ-subunit [12]. mAbs 128 and 129 also compete with a mAb directed against the cytoplasmic region δ363–374 [21]. It is probable that the C-terminal end of the δ-subunit is able to move freely and that, in the Triton-solubilized AChR, a conformational rearrangement occurs, allowing this segment to approach the cytoplasmic regions. Nevertheless, we cannot exclude an allosteric interaction between extracellular and cytoplasmic sites.

mAb 7 binds to both the γ and δ subunits of the *Torpedo* AChR [17]. We recently identified its epitope on the δ-subunit as segment δ389–396 (**RHGL**VPRV) [12]. Its presently mapped epitope γ382–387 (**RHGL**KR) contains the conserved segment **RHGL** and this might account for part of the cross-reactive character of this mAb. Infact, mAb 7 binds detectably to the RHGL peptide (35% of its binding to theRHGLKR peptide, Fig. 2). This epitope falls within the 20-peptide γ373–392 of Nelsonet al. [13], although it differs significantly from their deduced putative epitope (mainly γ373–380, possibly extending to 384). mAb 7 also binds to both adult and embryonic bovine AChR. Although the RHGL residues are not preserved in the bovine γ and ε subunits, they are preserved in the bovine δ-subunit which probably accounts for the observed cross-reactivity.

Binding of mAb 145 is *Torpedo*-specific; this is explained by the fact that its identified epitope (γ392–397, TSDIDI) is completely different in the corresponding segments of the γ and ε-subunits of mammalian species.

The epitopes of mAbs 154, 165 and 168 contain the γ-subunit tyrosine phosphorylation site. The binding of mAbs 165 is much weaker when the γ-subunit of the AChR is phosphorylated. In addition, even conservative substitutions of γTyr^{365} dramatically inhibited mAb 165 binding to the peptide. mAb 168, together with the anti-β-subunit mAb 148 (which also

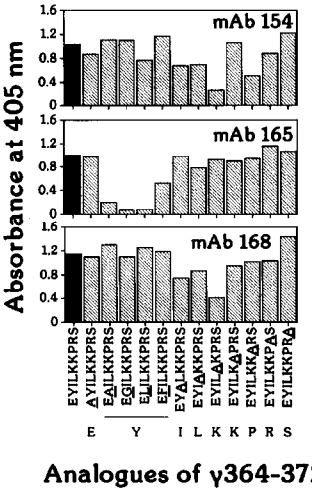

Fig. 4. Binding of mAbs to single residue analogues of the peptide γ364–373. Experimental conditions as in Fig. 1. Black bars show binding to the original peptide. Original substituted residues are shown below the peptides. Each residue was substituted by alanine, except in the case of γTyr^{365} which was substituted by three additional amino acids. Substitutions are underlined.

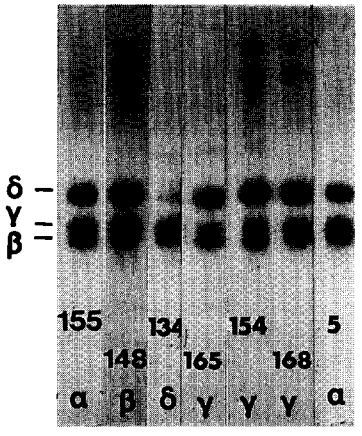

Fig. 5. mAb binding to ^{32}P tyrosine phosphorylated *Torpedo* AChR. AChR-rich *Torpedo* membranes were partially phosphorylated by endogenous tyrosine kinase, using [γ-^{32}P]ATP. The subsequent Triton X-100-solubilized extracts were incubated with the Sepharose-immobilized test mAbs. The pellets were analysed by SDS-PAGE and autoradiography. mAbs whose binding was inhibited by phosphorylation of the corresponding epitopes would selectively precipitate AChRs in which the corresponding subunit was unlabelled; thus the relative intensities of the precipitated subunits corresponding to such mAbs would be different from the control mAbs. Note that mAb 165 precipitated a proportionally much lower amount of labelled γ-subunit compared to the other mAbs. mAbs 148 and 134, which bind to non-phosphorylated βTyr^{372} [9] and δTyr^{372} [12], bound weakly to the phosphorylated β- and δ-subunits, respectively.

binds at the β-subunit tyrosine phosphorylation site [9]), are the only two mAbs, out of 60 mAbs previously tested, that effectively inhibit AChR function [10,11] (two more mAbs are weak inhibitors). Therefore, the previously observed association between overall tyrosine phosphorylation of the AChR and its ion channel function [6] has now been dissected at the level of individual tyrosine phosphorylation sites: i.e. each of at least two tyrosine phosphorylation sites on the β- and γ-subunits is itself capable of strongly influencing the channel.

Acknowledgements: We thank A. Kokla and R. Kouvatsou for valuable help and T. Barkas for useful suggestions. This work was supported by grants to S.J.T. from the Association Francaise contre les Myopathies, the BIOMED-1 program of EC (BMH1CT93–1100) and the Human Capital and Mobility program of EC (CHRXCT94–0547).

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