# An anti-acetylcholine receptor monoclonal antibody cross-reacts with phosvitin

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Received 15 June 1983

Rabbit and mouse anti-*Torpedo* acetylcholine receptor antibodies cross-reacted partially with the highly phosphorylated protein, phosvitin. We have selected an anti-*Torpedo* acetylcholine receptor monoclonal antibody which binds specifically to phosvitin; this binding is inhibited by acetylcholine receptor. These findings suggest that a phosphorylated amino acid residue may be a part of the determinant on the acetylcholine receptor recognized by this monoclonal antibody.

*Acetylcholine receptor* *Monoclonal antibody* *Phosvitin* *Antigenic determinant*

## 1. INTRODUCTION

Two molecular forms of acetylcholine receptor (AChR) are found in skeletal muscle. In the embryonic myotube the AChR molecules are uniformly distributed over the cell surface in a low density. In the adult muscle, AChR is almost exclusively present in the subsynaptic membrane in a densely packed form [1–4]. Those two receptor forms, referred to as extrajunctional receptor (EJR) and junctional receptor (JR), respectively, differ in several properties such as metabolic turnover time [5,6], channel open time [7,8] and some pharmacological properties [9,10].

In *Torpedo*, there is a difference in the degree of phosphorylation of junctional (adult) and extrajunctional (newborn) AChR. JR appears to be more heavily phosphorylated than EJR. This has been related to some physico-chemical characteristics of the two molecules [11]. *Torpedo* JR is more heat-resistant than EJR and has a lower isoelectric point. The latter is also true for the two forms of muscle AChR [12]. After treatment of adult *Torpedo* AChR with alkaline phosphatase, there is a decrease in heat resistance, and the isoelectric point becomes 0.1 pH units higher; these are properties similar to those of the immature *Torpedo* AChR [11].

AChR from electric organ of adult *Torpedo californica* contains phosphorylated amino acids [13], and the receptor itself is a specific substrate for phosphorylation by an endogenous receptor kinase [14–16]. To assess whether phosphorylated amino acid residues participate in antigenic determinants in AChR and contribute to its antigenic specificity, we analyzed the immunological reactivity of our monoclonal anti-AChR antibodies (mcAbs) with two highly phosphorylated proteins, phosvitin and casein. Here, we describe an anti-AChR mcAb prepared against purified adult *Torpedo* receptor, which reacts specifically with phosvitin. This suggests that a phosphorylated amino acid residue may be a part of the determinant on AChR recognized by this mcAb.

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## 2. MATERIALS AND METHODS

Casein and phosvitin were purchased from Sigma (St Louis MO). Radioiodination of goat-anti-mouse or anti-rabbit immunoglobulins and of AChR was performed by the chloramine T method [17].

### 2.1. *Antisera preparation*

Rabbit anti-casein and rabbit anti-phosvitin antisera were obtained following 3 intradermal injections at 20 day intervals of 100 $\mu$g immunogen emulsified in complete Freund's adjuvant. Rabbit anti-AChR [18], mouse anti-AChR [19] and anti-AChR mcAbs [20] were prepared as described. We used ascitic fluids as the source of mcAbs.

### 2.2. *Solid phase radioimmunoassay (SphRIA)*

This test was performed as in [21]. For binding experiments, 100 $\mu$l of the test antigen (50 $\mu$g/ml) in phosphate-buffered saline (PBS) was added to the wells of 96-well-polystyrene plates and incubated overnight at 4°C. The plates were then washed with PBS containing 1% (w/v) bovine serum albumin (BSA) and this wash buffer was left in the wells for 2 h at room temperature. Of each mcAb or antiserum, 75 $\mu$l properly diluted in 1% PBS–BSA, was added to the wells and incubated for 2 h at room temperature. After washing, 50 $\mu$l $^{125}$I-goat anti-mouse or anti-rabbit immunoglobulin (corresponding to the antibody tested), diluted in 1% PBS–BSA to give a total of $10^5$ cpm, was added. After washing with PBS, wells were dried, cut and counted in an autogammacounter. Non-immune sera, antisera against unrelated antigens and a mcAb with an unrelated specificity were used as controls. For inhibition experiments the antibody was preincubated with the tested inhibitor for 12 h at 4°C and then added to the antigen-coated plates. The test was continued as described above for binding experiments.

### 2.3. *Radioimmunoassay (RIA) with $^{125}$I-AChR*

This test was performed as in [21]. 25 $\mu$l of $^{125}$I-AChR [2.5 ng in 0.01 M Tris–HCl buffer (pH 7.4) and 0.1% Triton X-100, containing 1 mg/ml BSA], was incubated with 25 $\mu$l mcAb, properly diluted in PBS, containing 5% (v/v) normal mouse serum (NMS), for 1 h at 37°C, then precipitated by goat-anti-mouse immunoglobulins and counted. For inhibition experiments, the test inhibitor was preincubated with the antibodies, as described above for SphRIA, prior to the addition of the radiolabelled AChR.

## 3. RESULTS

Both rabbit and mouse anti-AChR antisera bind in SphRIA to phosvitin, although to a much lesser extent than their binding to the homologous immunogen, AChR (table 1). Rabbit anti-AChR antiserum did not bind at all to casein, whereas weak binding is observed with mouse anti-AChR antiserum. Rabbit antisera against casein and phosvitin failed to cross-react with *Torpedo californica* AChR. Also, rabbit anti-phosvitin antiserum does not cross-react with casein; limited cross-reactivity between rabbit anti-casein antiserum and phosvitin is observed.

### 3.1. *Binding of mcAbs*

Our anti-*Torpedo* AChR mcAbs were tested here for their reactivity with casein and phosvitin.

Table 1

Binding of polyclonal antisera to phosvitin, casein and AChR

| Antiserum | Dilution | Binding to[a] | | |
|---|---|---|---|---|
| | | Phosvitin | Casein | AChR |
| Rabbit anti-AChR | 1/10 | 9320 | – | 22340 |
| | 1/100 | 1490 | – | 20780 |
| | 1/1000 | 590 | – | 23650 |
| Mouse anti-AChR | 1/10 | 10300 | 5020 | 13650 |
| | 1/100 | 6400 | 1480 | 16890 |
| | 1/1000 | 980 | – | 14630 |
| Rabbit anti-casein | 1/10 | 3750 | 10450 | – |
| | 1/100 | 600 | 13600 | – |
| | 1/1000 | – | 6640 | – |
| Rabbit anti-phosvitin | 1/10 | 10740 | – | – |
| | 1/100 | 7720 | – | – |
| | 1/1000 | 6380 | – | – |

[a] Experiments were performed in triplicates

Results are expressed in average cpm after subtraction of the value obtained with control normal serum

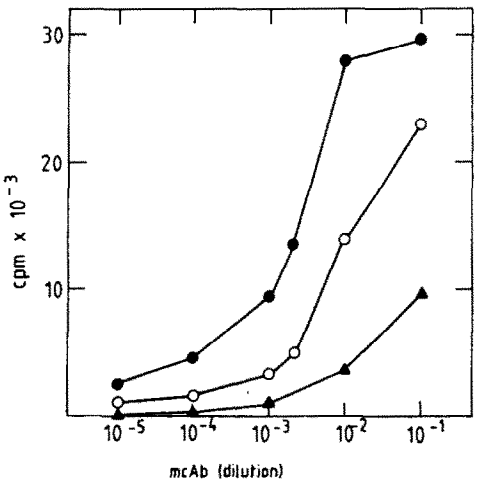


Fig.1. Binding (solid phase radioimmunoassay) of anti-AChR mcAbs to phosvitin: (●) mcAb 5.46; (○) mcAb 5.14; (▲) control, an mcAb of a non-related specificity.

About 30 different mcAbs from hybridizations no.1 and 5 were tested. The characterization of some of these mcAbs is described in [20–23]. None of the mcAbs exhibited significant binding to casein. Two mcAbs, 5.46 and 5.14, bound to phosvitin (fig.1). However, of the two, 5.46 showed a much higher binding, which was constant and highly reproducible throughout all experiments, while 5.14 gave somewhat variable results. No significant binding was detected below 1/500 dilution of 5.14 ascitic fluid. In order to determine whether the binding to phosvitin was due to electrostatic interactions with the negative charges on phosvitin, we tested the binding of these two mcAbs to negatively charged polyglutamic acid (PGA). McAb 5.46 showed no significant binding to PGA (table 2). However, mcAb 5.14 exhibited binding to PGA which amounted to about 35% of the binding obtained with phosvitin.

Table 2

Binding of mcAb 5.46 and mcAb 5.14 to phosvitin and polyglutamic acid

| mcAb | Dilution | Binding to (cpm) | |
|---|---|---|---|
| | | Phosvitin | PGA[a] |
| 5.46 | 1/100 | 27300 | 3100 |
| | 1/500 | 12800 | 1380 |
| 5.14 | 1/100 | 13800 | 6000 |
| | 1/500 | 4700 | 1800 |
| URS[b] | 1/100 | 3920 | 2480 |
| | 1/500 | 1140 | 960 |

[a] Polyglutamic acid
[b] McAb with a non-related specificity

Table 3

Specificity of the binding of mcAb 5.46 to phosvitin[a]

| Inhibitor (mg/ml) | Inhibition by | | | |
|---|---|---|---|---|
| | AChR | | Phosvitin | |
| | cpm | % Inhibition | cpm | % Inhibition |
| – | 11280 | – | 11280 | – |
| 0.001 | 10600 | 6 | 7780 | 39 |
| 0.01 | 8700 | 23 | 6150 | 45 |
| 0.025 | 7220 | 36 | n.d. | n.d.[b] |
| 0.1 | 4400 | 61 | 5190 | 54 |
| 1 | n.d. | n.d. | 4060 | 64 |

[a] Inhibition of the binding in solid phase radioimmunoassay of mcAb 5.46 (1/500) to phosvitin by AChR and phosvitin
[b] n.d., not done

### 3.2. *Inhibition experiments*

Preincubation of mcAb 5.46 with AChR or with phosvitin resulted in inhibition of the mcAb binding to phosvitin in a dose-dependent way (table 3). However, preincubation with phosvitin does not prevent the binding of 5.46 to AChR in either RIA or SphRIA. Control proteins did not affect the binding of 5.46 to phosvitin.

## 4. DISCUSSION

This study was designed to find an anti-AChR monoclonal antibody directed against an antigenic determinant containing a phosphorylated amino acid. Our strategy was to search for an anti-AChR mcAb which could react with highly phosphorylated non-related proteins such as casein and phosvitin. Early screening with polyclonal anti-AChR antisera showed cross-reactivity with either casein or phosvitin. Both rabbit anti-AChR and mouse

anti-AChR bound to phosvitin, while only mouse anti-AChR gave a weak positive reaction with casein. Normal rabbit and mouse sera did not react with either casein or phosvitin. Screening of anti-AChR mcAbs failed to show reactivity with casein. However, in screening mcAbs from another hybridization (no.6; unpublished) obtained from a fusion of spleen cells of mice immunized with AChR-enriched *Torpedo californica* membranes, we found one mcAb (6.27) which exhibited a very strong binding (up to $1/10^5$ dilution) to casein. This mcAb exhibited some cross-reactivity with phosvitin (up to $1/10^4$) but did not bind AChR at all, and was therefore not appropriate for our present study. McAb 5.46, shown to be directed against a conformation-dependent antigenic determinant on AChR [23], was found to cross-react with phosvitin. The binding of mcAb 5.46 to phosvitin, although lower than with AChR, was nonetheless very significant. The different binding of 5.46 towards phosvitin and AChR may suggest either that 5.46 binds to antigenic determinants on AChR and phosvitin which are similar, but not identical, or alternatively, that the antigenic determinants are identical but differ in the structures that surround them. Such a determinant on phosvitin may not be immunogenic or may represent only a small percentage of the total antibodies. This may explain why we did not observe cross-reactivity of anti-phosvitin antisera with AChR.

The fact that phosvitin is a very acidic protein suggested to us that charge interactions could play a significant role in the binding of mcAb 5.46 to phosvitin. This was ruled out by the fact that mcAb 5.46 is not significantly different in its isoelectric point from the other anti-AChR mcAbs tested, and by its inability to bind to polyglutamic acid coated wells. The charge was more likely an important component in the binding of another anti-AChR mcAb (5.14) to phosvitin. However, we could not rule out the possibility that 5.14 also recognizes some structure on phosvitin.

We screened our anti-AChR mcAbs with casein and phosvitin to determine whether these two highly phosphorylated proteins could be used to select antibodies directed against antigenic determinants involving phosphorylated groups. Although mcAb 5.46 binds phosvitin specifically, we could not unequivocably demonstrate that this cross-reactivity between phosvitin and AChR involves a phosphorylated group. Phosphoserine and phosphothreonine, products of the kinase reaction, did not inhibit mcAb 5.46 binding to the AChR (not shown). However, this does not rule out the possibility that these phosphorylated amino acids are part of the cross-reacting determinant.

The degree of phosphorylation may be one of the differences between EJR and JR; this was suggested to account for differences between neonatal and adult AChR of *Torpedo marmorata* [11]. It should be noted that in binding studies of various anti-AChR mcAbs to rat muscle homogenates at different ages, we observed that mcAb 5.46 bound to membrane-bound AChR only from rats $>17$ days of age. However, all the other mcAbs bound to membranous AChR at all ages (in preparation). These results suggest that mcAb 5.46 recognizes an antigenic determinant involving a phosphorylated amino acid which appears late during muscle development.

## ACKNOWLEDGEMENTS

This study was supported by grants from the Muscular Dystrophy Association and the Los Angeles Chapter of the Myasthenia Gravis Foundation to S.F. and from the NIH and NSF to A.S.G.

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