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The tyrosine phosphorylation site of the acetylcholine receptor β subunit is located in a highly immunogenic epitope implicated in channel function: antibody probes for β subunit phosphorylation and function

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Tyrosine phosphorylation of the nicotinic acetylcholine receptor (AChR) seems to be involved in AChR desensitization and localization on the postsynaptic membrane. This study reveals a probable function of the single known β subunit phosphorylation site (βTyr355) and provides suitable tools for its study. The epitopes for 15 monoclonal antibodies (mAbs) against the cytoplasmic side of the AChR β subunit were precisely mapped using >100 synthetic peptides attached on polyethylene rods. Eleven mAbs bound to a very immunogenic cytoplasmic epitope (VICE-β) on *Torpedo* β352-359, which contains the βTyr355, and to the corresponding sequence of human AChR. The contribution of each VICE-β residue to mAb binding was then studied by peptide analogues having single residue substitutions. Overall, each of the residues β354-359, including βTyr355, proved critical for mAb binding. Two of our four mAbs known to block the ion channel were found to bind at (mAb148) or close (mAb10) to VICE-β. Tyrosine phosphorylation of *Torpedo* AChR by endogenous kinase(s) selectively reduced binding of some VICE-β mAbs, including the channel blocking mAb148. We conclude that VICE-β probably plays a key role in AChR function. Elucidation of this role should be facilitated by the identified mAb tools.

Key words: acetylcholine receptor/epitope/monoclonal antibodies/tyrosine phosphorylation

Introduction

The muscle-type nicotinic acetylcholine receptor (AChR), from fish electric organs and vertebrate skeletal muscles, is a transmembrane glycoprotein composed of five homologous subunits of the stoichiometry $\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\epsilon\delta$. Acetylcholine binds on the two α subunits regulating the opening of the AChR ion channel. The channel is formed by all five subunits which are arranged in a pentameric rosette. Each subunit seems to span the membrane four times with four transmembrane segments (M1-M4). The five M2 helices contribute to the lining of the channel (reviewed in Maelicke, 1987; Stroud *et al.*, 1990; Changeux *et al.*, 1992; Hall, 1992; Sakmann *et al.*, 1992; Unwin, 1993).

Protein phosphorylation is intimately involved in the regulation of synaptic function (Nestler *et al.*, 1984). AChR phosphorylation seems to be responsible for triggering several effects (Huganir *et al.*, 1986; Hopfield *et al.*, 1988; Mulle *et al.*, 1988; Wallace *et al.*, 1991; Ferrer-Montiel *et al.*, 1991; Green *et al.*, 1991; Baker and Peng, 1993). AChR is phosphorylated by at least three protein kinases. A cAMP-dependent protein kinase phosphorylates the γ and δ subunits, a protein kinase C phosphorylates the α and δ subunits, and an unidentified endogenous protein tyrosine kinase phosphorylates the β, γ and δ subunits (reviewed in Huganir and Miles, 1989). All the identified AChR phosphorylation sites are located in the major cytoplasmic domain of each subunit between M3 and M4 (Safran *et al.*, 1987; Huganir and Miles, 1989; Schroeder *et al.*, 1991). The three *Torpedo* AChR tyrosine phosphorylation sites are situated on the homologous residues βTyr355, γTyr364 and δTyr372 (Wagner *et al.*, 1991).

Tyrosine phosphorylation of the AChR seems to be involved in at least two distinct activities. (i) Tyrosine phosphorylation of the *Torpedo* electric organ AChR increases its rate of desensitization (Hopfield *et al.*, 1988). The specific subunits involved in this mechanism are not yet known. (ii) Tyrosine phosphorylation of muscle AChR is regulated by innervation through the release of a nerve-derived factor (Qu *et al.*, 1990). Agrin, a nerve-derived aggregation factor induces tyrosine phosphorylation of the chick AChR β subunit and this effect correlates very well with agrin-mediated AChR aggregation (Wallace *et al.*, 1991). It has thus been suggested that tyrosine phosphorylation of the β subunit may play a role in regulating AChR distribution on the membrane (Wallace *et al.*, 1991). Phosphorylation may alter the interaction of the β subunit with the 43 kDa protein thus resulting in AChR immobilization (Wallace *et al.*, 1991). Clearly the role of tyrosine phosphorylation of the AChR β subunit deserves extensive investigation. Specific tools for such studies should be valuable.

Monoclonal antibodies (mAbs) to the AChR have proved to be excellent tools for studying the molecule as well as the autoimmune disease myasthenia gravis, which is caused by anti-AChR autoantibodies (Lindstrom *et al.*, 1988; Tzartos, 1991). The extensive epitope characterization of several of our anti-AChR mAbs (Tzartos and Lindstrom, 1980; Gullick and Lindstrom, 1983; Sargent *et al.*, 1984; Ratnam *et al.*, 1986a,b; Kubalek *et al.*, 1987; Tzartos *et al.*, 1981, 1986, 1988; Papadouli *et al.*, 1990, 1993; Saedi *et al.*, 1990; Cung *et al.*, 1991; Nelson *et al.*, 1992; Tzartos and Remoundos, 1992) has rendered them valuable tools in numerous studies (reviewed in Tzartos, 1991). Concerning the effect of these mAbs in perturbing AChR function, it has been shown that out of ~70 tested mAbs, only four (all binding at cytoplasmic epitopes on α, β and γ subunits) could inhibit the carbamoylcholine-induced opening of the ion channel in reconstituted membrane vesicles and planar lipid bilayers (Lindstrom *et al.*, 1981a; Wan and Lindstrom, 1985; Blatt *et al.*, 1986).

The best studied group of our mAbs is that of the anti-α subunit mAbs. It contains antibodies to the extracellular main

Table I. Characteristics of the tested mAbs

mAb No.	Immune rat strain	Fusion No.	Ig class	Binding to intact AChR[a]		Earlier mapping data				Presently identified epitopes
				Torpedo	Human	Transmembrane[b]	Ab competition[c]	Peptide groups[d]	Approximate epitope[e]	
10	Lewis	27	G2a	+	−			β4		β338−346
99	Lewis	170	G2a	++	−			β7		β364−376
117	Leu	171	G2b	++	−		B	β4		β343−352
125	Leu	171	G1	+	−	cyt.	C	β5	β428−442	β408−414
VICE-β mAbs:										
11	Lewis	29	M	++	−		A	β3		β354−358
110	Lewis	170	G2a	++	+	cyt.		β2		β354−360
111	Lewis	170	G1	++	+	cyt.	A	β3	β368−407	β354−359
112	Lewis	170	G1	++	−		A	β2		β354−359
114	Lewis	170	G2a	++	−			β		β352−359
118	Leu	171	G1	++	+	cyt.	A	β2		β354−360
120	Leu	171	G1	++	+	cyt.		β2		β354−360
123	Leu	171	G1	++	+	cyt.	A	β2		β354−359
124	Leu	171	G1	++	++	cyt.		β3		β354−359
148	Lewis	178	G2	++	++	cyt.	A	β3	β368−407	β354−359
151	Lewis	180	G1	++	+	cyt.		β3		β354−359

Empty spaces denote no available data. Data of the first six columns are from Tzartos and Lindstrom (1980) and Tzartos *et al.* (1986).
[a]++,+,−: apparent antibody titer: ≥1 μM, 0.01−1 μM and ≤0.001 μM respectively.
[b]Cyt. represents cytoplasmic localization of the epitopes, determined either directly (by electron microscopy) or indirectly by saponin treatment of *Torpedo* membranes (Sargent *et al.*, 1984; Ratnam *et al.*, 1986a,b).
[c]A, B and C represent groups of mAbs as determined by antibody competition experiments (Kordossi and Tzartos, 1987): A, all mAbs of group A completely competed with each other for binding to the AChR; B, mAb117 partially competed with mAbs of group A; C, mAb125 did not significantly compete with mAbs of groups A and B.
[d]β1, β2 etc. represent mAb groups according to mAb binding patterns to proteolytic peptides of AChR subunits (Gullick and Lindstrom, 1983).
[e]According to epitope mapping by the use of proteolytic peptides (Ratnam *et al.*, 1986a). The presented residue numbers were estimated by us using the authors' diagrams.

immunogenic region (MIR) on α67−76 (reviewed in Tzartos *et al.*, 1991) and several mAbs to precisely localized epitopes on the cytoplasmic side of the AChR (Sargent *et al.*, 1984; Tzartos *et al.*, 1986, 1988; Ratnam *et al.*, 1986b) including five mAbs to the very immunogenic cytoplasmic epitope on α subunit (VICE-α, α373−380; Marx *et al.*, 1990; Tzartos and Remoundos, 1992). mAbs to the other subunits are less well characterized and consequently their use to date has been rather limited.

We present here the precise localization of the epitopes for 15 anti-β subunit mAbs by the use of synthetic peptides. Most mAbs bound to an eight residue segment (β352−359) which contains the β subunit phosphorylation site (Tyr355). Two mAbs that block AChR channel function were mapped at or near this site. Interestingly, tyrosine phosphorylation of the AChR by endogenous kinase(s) reduced the AChR binding efficiency of some mAbs to β352−359 (including a channel blocking mAb). Therefore these studies provide: (i) a clue for the possible connection between β subunit phosphorylation and regulation of AChR function and (ii) highly specific probes for further investigating the role of AChR tyrosine phosphorylation.

Results

All mAbs tested in the present study have been generated in rats immunized with SDS-denatured *Torpedo* electric organ AChR or purified β subunit. They bind well both to the denatured β subunit of *Torpedo* AChR and to the intact *Torpedo* AChR, while some also bind to human muscle AChR (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1986; see Table I). Because most, if not all, of these mAbs seem to bind to the cytoplasmic side of the AChR (Sargent *et al.*, 1984), we focused our efforts to the C-terminal domain β322−469. Over 100 7−10 residue-long peptides were synthesized on polyethylene rods and were tested for mAb binding.

Monoclonal antibody binding to Torpedo β subunit peptides

Thirty-three anti-β subunit mAbs were tested for their binding activity to 47 overlapping decapeptides of the *Torpedo* β322−469 sequence as well as to the control MIR peptide α67−76. The peptides were overlapping by seven residues so that any epitope of up to eight residue-long within β322−469 would be included in at least one peptide. Nine and ten residue-long epitopes would have only 66 and 33% chances, respectively, to be identified. Fifteen mAbs reacted quite satisfactorily. Several others did not bind to any peptide whereas a few produced very high background. The negative control anti-MIR mAb6, bound as expected only to α67−76 (not shown).

Figure 1 shows the binding patterns of the 15 anti-β mAbs which bound significantly to at least one peptide. Interestingly, 11 of these mAbs bound characteristically to the peptide β352−361. This peptide should therefore contain a VICE on the β subunit (VICE-β).

In order to localize more accurately the epitopes of the above mapped mAbs, we then synthesized all possible overlapping octa- or heptapeptides within selected small regions (Figure 2). From the binding patterns of the mAbs, five subgroups could be identified and their minimum epitope requirements could be determined. Figure 2 shows the binding patterns of representative mAbs from each subgroup and the data are summarized in Figure 4C. Thus VICE-β was restricted to β352−360 with the segment β354−359 being most critical.

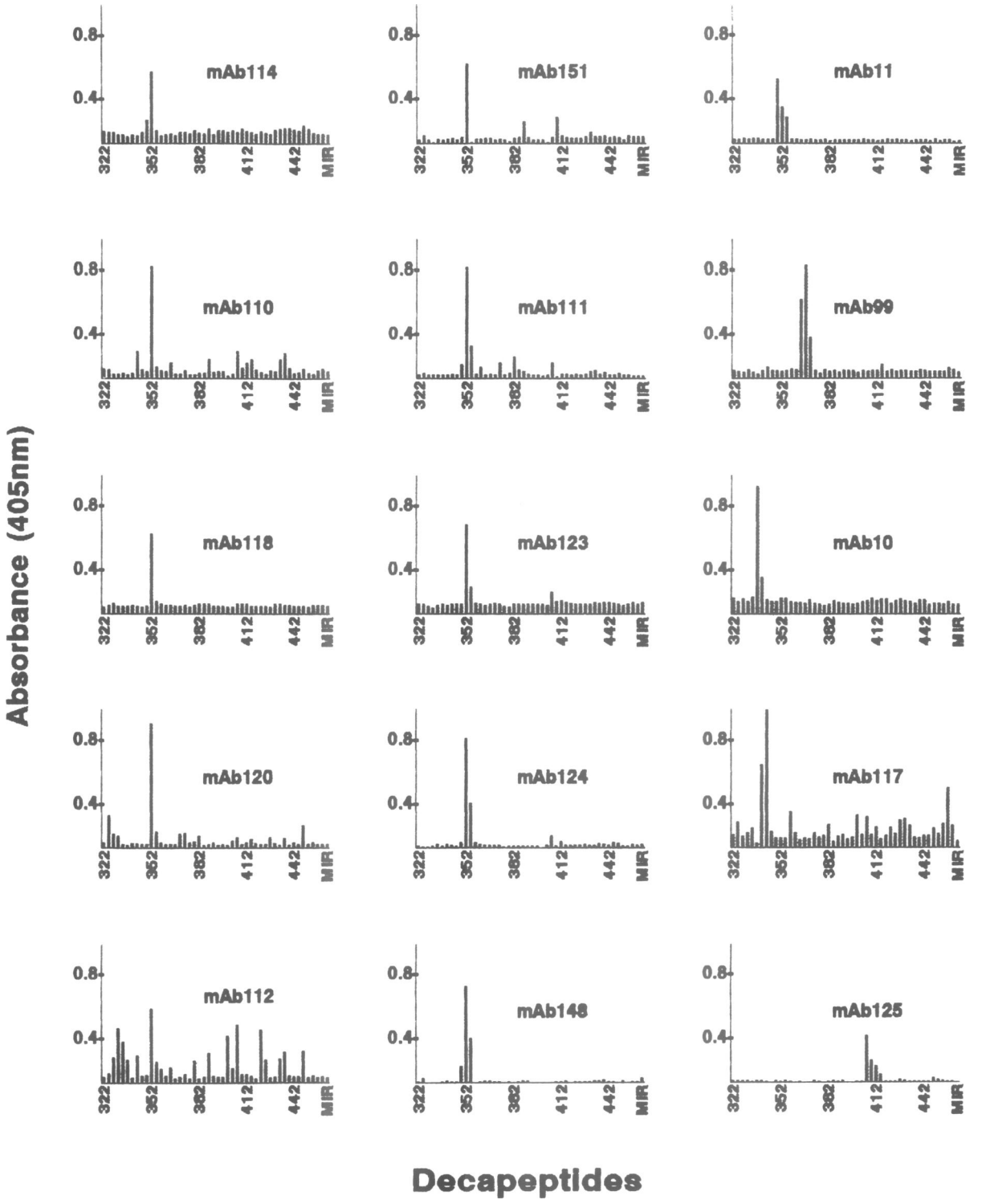

Fig. 1. Epitope analysis of 15 anti-β subunit mAbs. Antibodies were tested for binding to overlapping by seven residues decapeptides corresponding to the *Torpedo* AChR β322−469 sequence (47 peptides), plus the control MIR-peptide α67−76. Peptides were attached to polyethylene rods and tested for mAb binding by ELISA. Numbers on the abscissa denote the amino-terminal residue numbers of some decapeptides.

Monoclonal antibody binding to human VICE-β

We then synthesized overlapping decapeptides of the human β subunit segment 352−377 (corresponding to *Torpedo* β340−365 which includes VICE-β), in order to compare the cross-reactivities between peptides of the two species with the cross-reactivities between the corresponding intact AChRs. Figure 3 shows binding of three representative VICE-β mAbs on the corresponding human peptides. Most results were very similar with, and served as confirmatory of, those of Figures 1 and 2. The only mAb which did not bind to any human peptide was mAb114 (not shown), i.e. the only one which requires the *Torpedo* β352; this residue differs between the two species (Asn in *Torpedo*, Thr in human AChR).

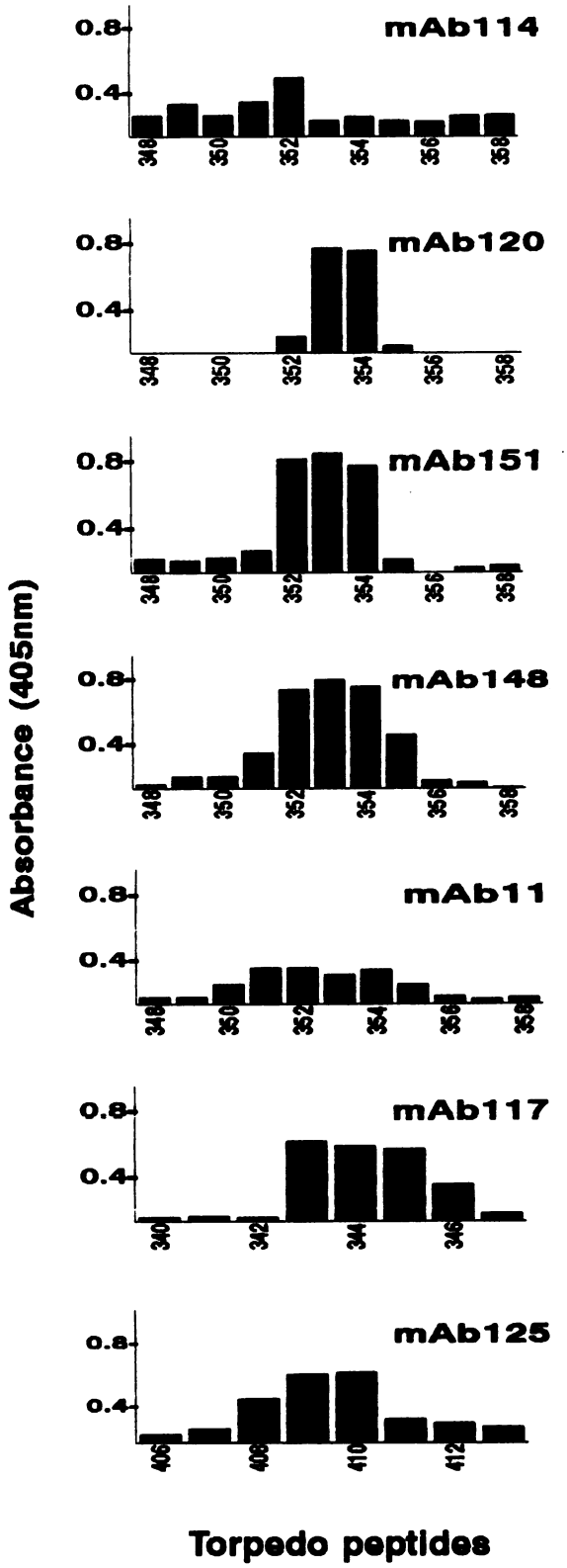

Fig. 2. Binding pattern of representative mAbs to all possible overlapping octa- and heptapeptides corresponding to selected *Torpedo* AChR β subunit sequences. Experimental conditions and symbols as in Figure 1. Three sets of peptides were used: one set of octapeptides for the VICE-β mAbs (top five mAbs) and two sets of heptapeptides for the last two mAbs (Nos 117 and 125).

Structural features of the identified epitopes

The structural profiles of the identified epitopes are shown in Figure 4A. Hydrophilicity, flexibility and high β-turn propensity have been proposed as characteristics of high antigenicity (Westhof *et al.*, 1984; Tainer *et al.*, 1985; Geysen *et al.*, 1987b; Krchnak *et al.*, 1990). Interestingly, despite its proved high antigenicity, no characteristic theoretical high antigenicity profiles could be detected for VICE-β except for its hydrophilic ends. In contrast, remarkable was the pattern of the epitope for mAb10 which presents high antigenic characteristics with all three studied structural profiles (Figure 4A). Yet this is a low affinity mAb (Tzartos and Lindstrom, 1980; Lindstrom *et al.*, 1981a).

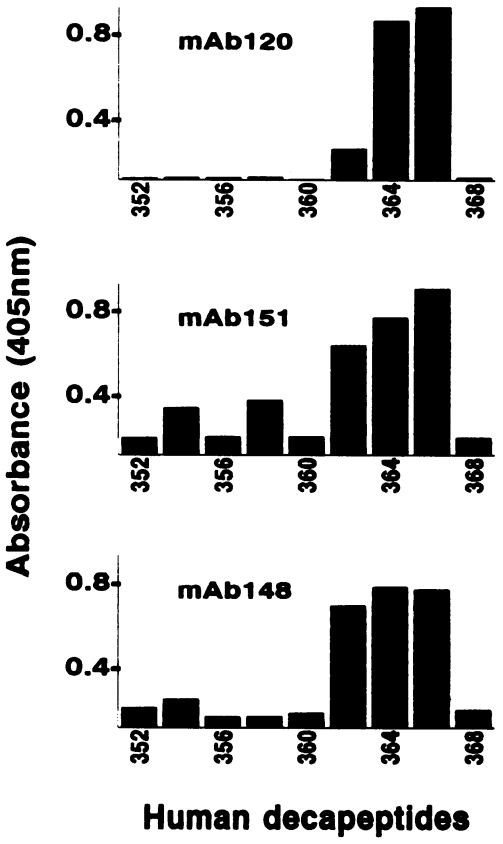

Fig. 3. Binding of representative VICE-β mAbs to overlapping human AChR β subunit synthetic decapeptides. Experimental conditions and symbols as in Figure 1. Human β352–368 corresponds to *Torpedo* β340–356.

Antigenic role of each residue within VICE-β

We subsequently determined the mAb binding role of each residue within the VICE-β octapeptide. A series of 26 different analogues of the β352–359 sequence representing both conservative and non-conservative single substitutions for each residue was produced and tested for binding of all VICE-β mAbs (representative patterns in Figure 5). As expected from Figures 1 and 2, substitutions on Asn352 and Asp353 did not affect much the binding of most mAbs. Each of the other six residues was generally found critical for mAb binding since, at least any non-conservative substitution dramatically reduced mAb binding. Glu354, Ile357 and Arg358 seemed to be the most critical residues since, in several cases, even conservative substitutions (Gln for Glu, Leu for Ile and Lys for Arg) dramatically reduced mAb binding. Despite the overall rather similar binding pattern among most VICE-β mAbs, significant differences were also observed, especially on mAb binding to the analogues of

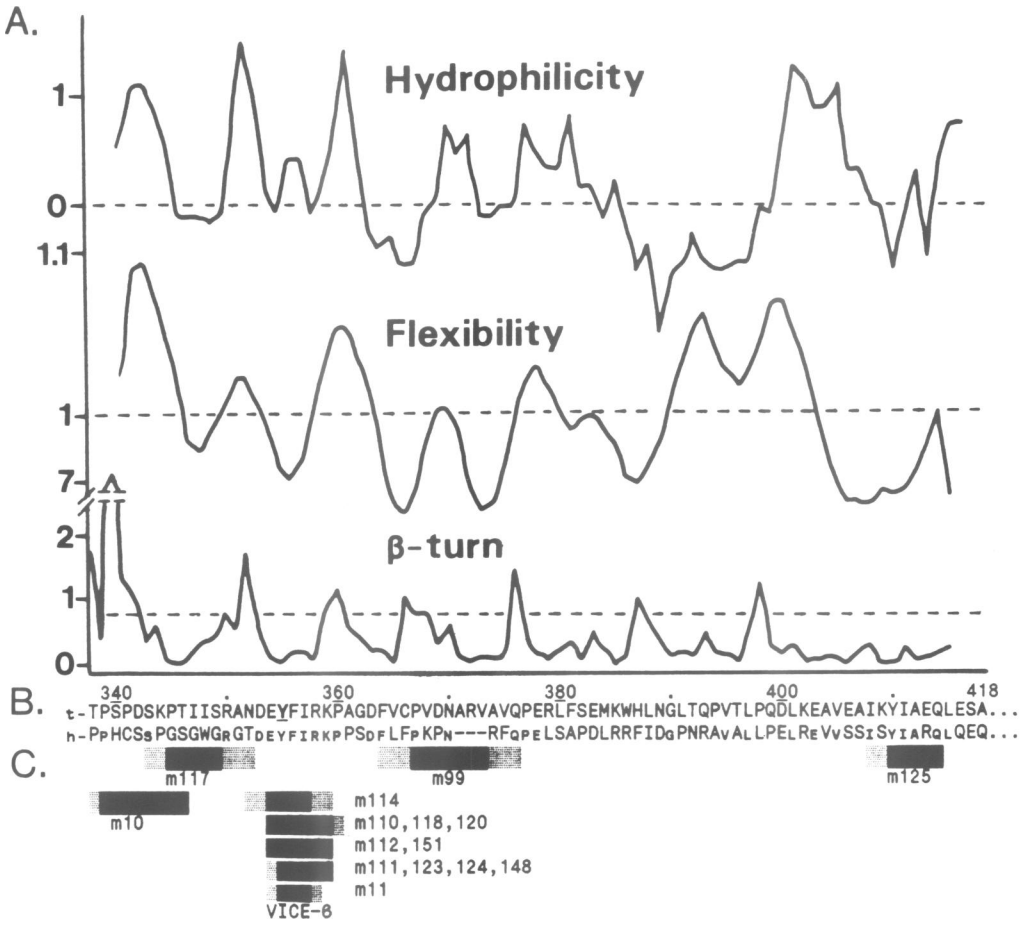

Fig. 4. Summary of the determined epitope locations and comparison with structural profiles of the corresponding β subunit sequence. (**A**) Hydrophilicity, flexibility and β-turn propensity profiles of the *Torpedo* β338−418 sequence. Segments of the three plots above the dashed lines represent hydrophilic, flexible or probable β-turn sites, respectively. (**B**) Amino acid sequence of the *Torpedo* β338−418 and the corresponding human segment (Numa, 1987; Beeson *et al.*, 1989). (**C**) The presently determined epitopes are represented by horizontal bars that correspond to the above sequences in panel B; dotted parts of the bars denote that the corresponding residues enhanced but were not necessary for mAb binding. The VICE-β mAbs are classified into five subgroups. m10, m117 etc. represent mAb numbers.

residues β354, β358 and β359. These differences prove that the antigen binding sites of the VICE-β mAbs are not identical.

Tyrosine phosphorylation of AChR reduces binding of VICE-β mAbs

Since VICE-β contains the tyrosine phosphorylation site of the β subunit, Tyr355 (Wagner *et al.*, 1991), we proceeded to test whether tyrosine phosphorylation of the AChR affects binding of VICE-β or other mAbs. Initially we compared mAb binding to [^{125}I]α-bungarotoxin labelled endogenously phosphorylated AChR with their binding to AChR dephosphorylated by exogenous alkaline phosphatase. *Torpedo* AChR preparations contain 0.1−0.5 phosphates per mol AChR (Hopfield *et al.*, 1988). Table II shows a representative experiment in which binding of the VICE-β mAbs 114, 151 and, to a lesser extent, 148 was distinctly increased in the case of the dephosphorylated AChR. This suggests that phosphorylation interferes with VICE-β mAb binding. It also suggests that the three VICE-β mAbs were raised against dephosphorylated rather than phosphorylated β subunit. Binding of other tested VICE-β and non-VICE-β mAbs was not affected by AChR dephosphorylation.

In order to determine directly whether the above effect was indeed due to tyrosine phosphorylation of the β subunit epitope, we subsequently performed *in vitro* labelling of the AChR by [γ-^{32}P]ATP. The AChR on *Torpedo* electric organ membranes was partially phosphorylated by the endogenous protein tyrosine kinase and [γ-^{32}]ATP (Huganir *et al.*, 1984). The membranes were then solubilized by SDS and/or Triton X-100, and their ^{32}P-labelled AChR molecules were immunoprecipitated by various anti-AChR mAbs. Control samples, in which $MnCl_2$ was omitted resulted in very weak labelling. Selective ^{32}P-labelling of the tyrosine residues rather than serine residues was confirmed by alkali treatment of the SDS−polyacrylamide gels (Cooper and Hunter, 1981).

Two approaches were used for testing mAb binding to the ^{32}P-labelled products. First, we tested mAb binding to SDS-denatured AChR subunits. The mAb/^{32}P-labelled subunit immunoprecipitates were subjected to SDS−PAGE analysis and autoradiography. The VICE-β mAb114 was apparently inhibited from binding to the ^{32}P-occupied epitope since it bound very weakly, if at all, to the ^{32}P-labelled β subunit (not shown). The remaining anti-β subunit mAbs precipitated various amounts of the ^{32}P-labelled β subunit. However, it was difficult to evaluate the significance of the relative differences among mAbs since such differences could simply reflect possible differences in their affinity for the β subunit.

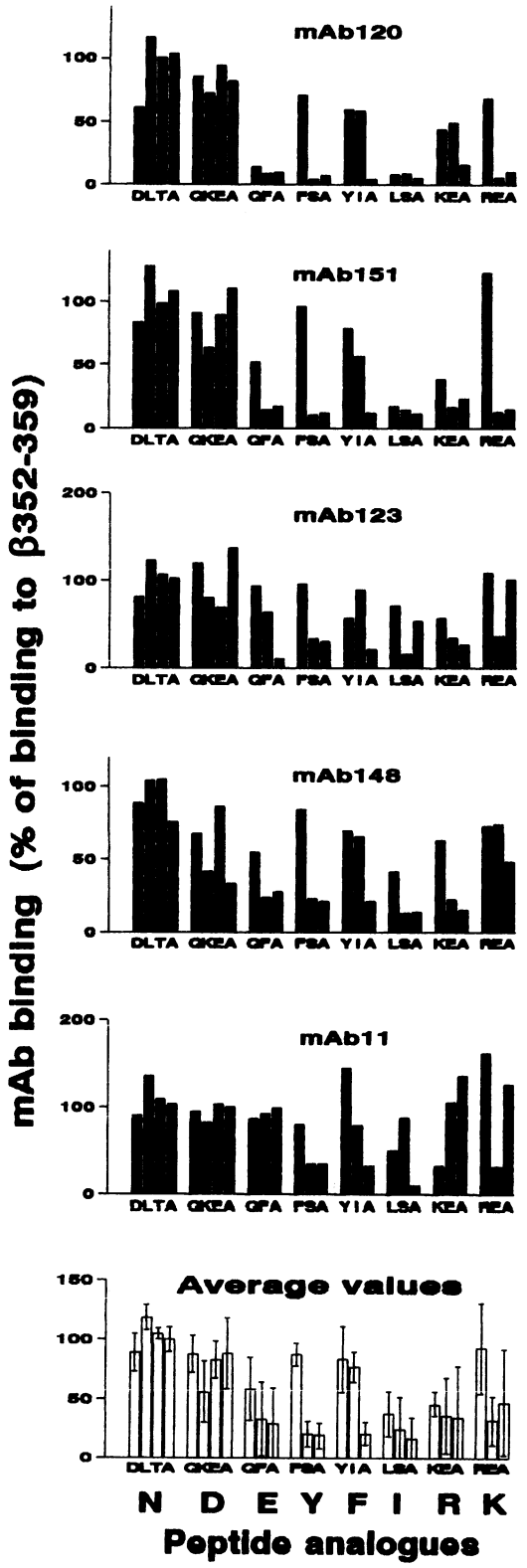

Fig. 5. Binding of representative VICE-β mAbs to single residue analogues of the β352–359 octapeptide. Letters just below the bars represent the amino acids used for each substitution. Each filled bar represents binding of a specific mAb as percentage of its binding value to the original peptide. Last row of empty bars represents the average binding value (± SD) of the eight tested mAbs (Nos 110, 118, 120, 151, 111, 123, 148 and 11).

In order to determine unequivocally the effect of AChR tyrosine phosphorylation on mAb binding, we subsequently used a system which includes reliable internal controls. Here, tests of mAb binding had the additional advantage of being performed by using intact Triton-X-100-sobulized AChR rather than its SDS-dissociated subunits. Since the fraction of the ^{32}P-labelled AChR was ~0.1–0.2, most labelled molecules would have only one ^{32}P-labelled subunit (β, γ and δ) each. Therefore, a mAb unable to bind to the phosphorylated β subunit would still bind to the AChR molecules with unphosphorylated β subunit (but possibly with their γ and/or δ subunits labelled).

Although differences among the mAbs in their affinity for AChR would result in significant differences in the precipitated radioactivity among them, the proportions of precipitated radioactivity among the three subunits, δ, γ and β, should be constant for all mAbs whose binding is not affected by phosphorylation. On the contrary, for mAbs whose binding is affected by tyrosine phosphorylation these proportions should be different depending on the effect of phosphorylation on mAb binding and on the epitope-carrying subunit.

Figure 6 shows the autoradiograms that correspond to some of the mAbs tested by the above technique. Each tested mAb (including the VICE-β mAbs) precipitated to some extent all three labelled subunits (β, γ and δ). However, three VICE-β mAbs (Nos 114, 151 and, to a lesser extent, 148) precipitated significantly less labelled β subunit than the other mAbs. In particular, while in the case of the control mAbs 5 and 155 (anti-α), 166 (anti-γ) and 139 (anti-δ) the β subunit band was of similar density to that of the γ subunit, its intensity was much weaker for mAbs 114 and 151 and significantly weaker for mAb148. The other VICE-β mAbs did not unequivocally reduce binding to AChRs with phosphorylated β subunit, although some seemed to exhibit somewhat weaker binding (Figure 6). Other mAbs, including the non-VICE-β anti-β mAbs 117 and 125 did not exhibit reduced binding to AChRs with ^{32}P-labelled β subunit (not shown). Similar results were obtained when instead of Sepharose-bound mAbs, soluble mAbs were used and precipitated by the addition of rabbit anti-rat γ globulin antibodies immobilized on Sepharose–protein A (not shown).

Overall, these experiments showed that the AChR binding efficiency of some mAbs, which are specific for epitopes carrying the β subunit tyrosine phosphorylation site (including the major β subunit mAb blocker), was weaker when their corresponding epitope was phosphorylated.

Discussion

In the present study we precisely determined the sites of the sequential epitopes for several mAbs to the AChR β subunit. The epitope that contains the single verified β subunit phosphorylation site and the site of a mAb that blocks AChR function proved highly antigenic (VICE-β); this epitope was characterized in depth in order to provide the basis for subsequent phosphorylation studies. At the same time, anti-β subunit mAb tools for such studies were also identified.

The validity of the present mapping could be confirmed when correlated with earlier mapping approaches. (i) Our anti-AChR mAbs have been classified into ~30 groups

Table II. mAb binding to phosphorylated and dephosphorylated AChR

mAb	Untreated AChR (endogenously phosphorylated)	Dephosphorylated AChR	
	Percent (bound versus total AChR)		Ratio
6 (anti-MIR)	20.5	20.3	0.99
5 (anti-α)	19.9	19.7	0.99
155 (anti-α)	25.6	27.8	1.09
114 (VICE-β)	20.6	44.5	2.16
151 (VICE-β)	17.1	31.5	1.84
148 (VICE-β)	21.3	28.6	1.34

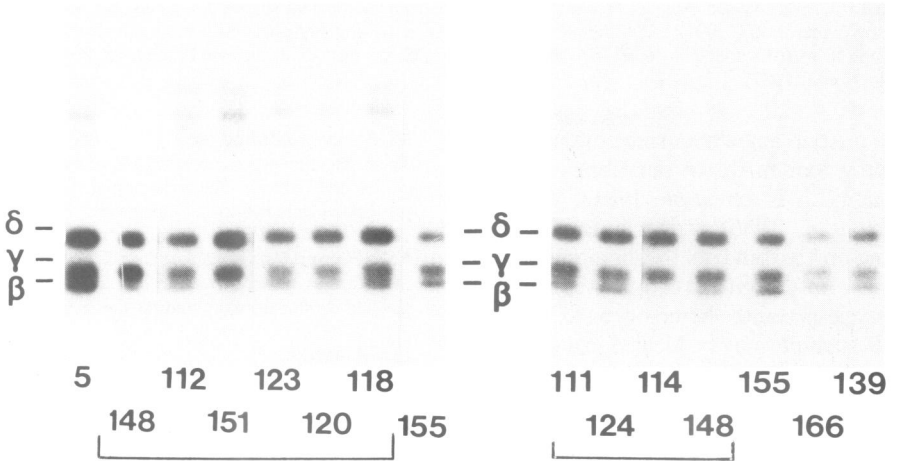

Fig. 6. Monoclonal antibody binding to [^{32}P]tyrosine-phosphorylated *Torpedo* AChR. AChR-rich *Torpedo* membranes were partially ^{32}P-labelled by the endogenous tyrosine kinase using [γ-^{32}P]ATP. The subsequently Triton-X-100-solubilized extracts were incubated with the test mAbs immobilized on CNBr-activated Sepharose. The pellets were analysed by SDS−PAGE and autoradiography. mAbs whose binding was not affected by phosphorylation precipitated AChRs having any of their β, γ or δ subunits ^{32}P-labelled. mAbs whose binding was inhibited by the phosphorylation of the corresponding epitope would selectively precipitate AChRs with the corresponding subunit unlabelled; thus the relative intensities of the precipitated subunits corresponding to such mAbs would be different from the control mAbs. Note that mAbs 114, 148 and 151 precipitated proportionally much lower amounts of labelled β subunit.

according to their binding to overlapping proteolytic *Torpedo* AChR peptides (Gullick and Lindstrom, 1983). All VICE-β mAbs (except mAb114 which was not characterized), but not the other four presently mapped mAbs, were classified in the almost identical β2 and β3 groups (see Table I, column 9). (ii) The indirectly estimated locations of some epitopes by the use of large proteolytic peptides (Ratnam *et al.*, 1986a) differed only by ~20 residues from those presently described (compare last two columns in Table I). (iii) All tested VICE-β mAbs have been shown to bind on the cytoplasmic side of the AChR (Sargent *et al.*, 1984; see Table I, column 7). (iv) All tested VICE-β mAbs completely inhibited each other (but not the non-VICE-β mAbs 117 and 125) from binding to the intact AChR (Kordossi and Tzartos, 1987; see Table I, column 8). These correlations strongly suggest that the presently identified epitopes represent at least parts of the actual epitopes on the intact AChR.

The great majority (14/15) of the mapped mAbs bound within a small (35 residues) segment, β339−373, located in the centre of the β subunit's cytoplasmic domain; furthermore, 11 of them bound to almost a single epitope (VICE-β) between β352 and 359. The VICE-β mAbs have been derived from five different rats of two strains immunized with various protocols and belong to various Ig subclasses (Table I). We thus conclude that independently of the immunization conditions this β subunit segment has a very high immunogenic tendency, being probably the most immunogenic epitope of the cytoplasmic side of the AChR. Its high immunogenicity could not be explained when the hydrophilicity, flexibility and β-turn propensity patterns of the *Torpedo* β subunit were computed (Figure 4A). Nevertheless, its high immunogenicity is suppressed when immunizations are performed with intact rather than denatured AChR (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1981, 1986).

The peptide analogue studies showed that overall, each of the six residues between β354 and 359 is crucial for VICE-β mAb binding. For some residues (Glu354, Ile357, Arg358) even conservative substitutions abolished binding of most mAbs. Despite the overall similarities in the binding patterns among most mAbs to these analogues, significant differences could also be observed. These differences apparently form the basis that explains the earlier noted differences in their AChR binding behaviour, in terms of binding efficiency, species cross-reactivity and other characteristics (Tzartos and Lindstrom, 1980; Tzartos *et al.*, 1986; Kordossi and Tzartos, 1987) as well as their presently described differential sensitivity in AChR phosphorylation.

The region containing VICE-β seems to play a critical role in AChR function and phosphorylation. Thus, a VICE-β

Table III. Epitope locations of the four anti-AChR mAbs known to inhibit carbamoylcholine-induced ion channel function

mAb	Sequence numbering		Epitope residues		
	Primary	Consensus			
			370	380	390
mAb13	α332–350	α380–399		AS KEKQENKI FADDI DI SD	
mAb148 (VICE-β)	β354–359	β383–388		EY FI RK	
mAb10	β339–346	β368–375	PSPDSKPT		
mAb168	γ363–372	γ383–393		EY I LKKP–RSE	

Functional studies are from Lindstrom *et al.* (1981a), Wan and Lindstrom (1985) and Blatt *et al.* (1986). Epitope localization is from Tzartos and Remoundos (1992) for mAb13, from Nelson *et al.* (1992) for mAb168 and from the present studies for mAbs 148 and 10. Underlined Y denotes the Tyr phosphorylation sites (Wagner *et al.*, 1991) and S denotes the postulated α subunit Ser phosphorylation site (Huganir and Miles, 1989). The larger peptide corresponding to mAb13 does not necessarily denote that the epitope extends to the whole segment. Primary and consensus sequence numbering is according to Numa (1987).

mAb (No. 148) and a mAb to a neighbouring epitope (mAb 10) are two of the only four mAbs of our library known to inhibit AChR function (Lindstrom *et al.*, 1981a; Wan and Lindstrom, 1985; Blatt *et al.*, 1986); furthermore, the other two function blocking mAbs bind to the homologous to VICE-β regions on the γ (mAb168) and α (mAb13) subunits (see Table III). This suggests that the homologous to VICE-β segments on all AChR subunits may be located in a strategic cytoplasmic 'ring' that surrounds the ion channel. Interestingly, this putative 'ring' also contains all three tyrosine phosphorylation sites of the AChR, on β, γ and δ subunits (Wagner *et al.*, 1991). Specifically, VICE-β contains Tyr355 (i.e. the only known phosphorylation site of the β subunit), while the epitope for the anti-γ blocker mAb168 contains the tyrosine phosphorylation site γTyr364. It may also be of relevance that the epitope of the anti-α function blocking mAb13 seems to include the single α subunit putative phosphorylation site αSer333 (Table III).

Tyrosine phosphorylation of the *Torpedo* AChR affects its desensitization rate (Hopfield *et al.*, 1988), while tyrosine phosphorylation specifically of the β subunit (at least in chick AChR) seems to be involved in AChR clustering (Wallace *et al.*, 1991). We showed that tyrosine phosphorylation of the β subunit on the intact AChR remarkably reduced binding to the AChR of at least three VICE-β mAbs (including the blocker of AChR function mAb 148), whereas it did not affect binding of mAbs that are directed to other AChR sites (Figure 6 and Table II). Similarly, substitution of Tyr355 by the small amino acids Ser and Ala essentially blocked binding of all VICE-β mAbs to the VICE-β peptide analogues (Figure 5).

The identification of mAbs to a critical site on the AChR β subunit, whose binding affects AChR function and is affected by AChR tyrosine phosphorylation, offers valuable tools for the study of AChR phosphorylation.

Materials and methods

Peptide synthesis

Peptides were synthesized on the tips of small polyethylene rods on which polymers of acrylic acid had been formed by radiation grafting (Geysen *et al.*, 1984, 1987a). Rods with attached *N*-(9-fluorenylmethoxycarbonyl) (Fmoc)-protected β-alanine, Fmoc-amino acids and hydroxybenzotriazole were obtained from Cambridge Research Biochemicals (Cambridge, UK); piperidine was from Fluka; all other reagents used for peptide synthesis were from Sigma. Peptide synthesis was performed in 96-well microtitre plates according to Geysen *et al.* (1984, 1987a), the instructions of the manufacturer and small modifications as described (Tzartos and Remoundos, 1990).

Monoclonal antibodies

The production and characterization of the mAbs used have been described earlier (see Table I). The preparations used were 50% ammonium sulfate precipitates from hybridoma supernatants, dialysed against phosphate buffered saline solution (PBS: 145 mM NaCl, 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, pH 7.4) containing 0.05% NaN_3. Apparent antibody titres were measured as previously described and were expressed as mols of ^{125}I-labelled α-bungarotoxin binding sites per litre (Lindstrom *et al.*, 1981b).

ELISA assays

ELISAs were performed as described (Geysen *et al.*, 1987a; Tzartos and Remoundos, 1992). In brief, each peptide-carrying rod was preincubated with 200 μl of PBS containing 0.1% Tween-20, 0.8% bovine serum albumin and 0.2% ovalbumin for 1 h followed by overnight incubation with 200 μl of a dilution of the test mAb (1/1000 to 1/5000 dilution) in the above solution. Then the rods were washed four times with PBS containing 0.05% Tween-20, followed by incubation with peroxidase-labelled rabbit anti-rat γ-globulin (DACO, 1/1000 to 1/2000 dilution) for 1 h and washed again three times. The presence of bound mAbs was 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, and measured at 405 nm. Peptides retained their antibody binding capacity for several assays. For each sequence, 2 or 3 copies were synthesized, i.e. 2 or 3 rods with the same sequence, in 1 or 2 independent cycles.

Structural profiles

Hydrophilicity, flexibility and β-turn propensity profiles of the *Torpedo* β338–418 sequence were analysed according to the methods of Hopp and Woods (1981), Karplus and Schulz (1985), and Chou and Fasman (1979) respectively using the PC-Gene program.

AChR preparation, dephosphorylation, tyrosine phosphorylation and mAb binding

AChR-rich membranes were prepared from frozen *Torpedo californica* electric organs (Marinus, Long Beach, CA) according to Saito *et al.* (1980) or Huganir *et al.* (1984). The latter results in the production of not tightly sealed vesicles (Huganir *et al.*, 1984). The former had to be subjected to hypotonic lysis by adding 0.4 M NaCl to concentrated vesicles and then diluting them ~10 times just before the ^{32}P labelling in principle according to Davis *et al.* (1982). Dephosphorylation of endogenously phosphorylated AChR by alkaline phosphatase (Minotech, Crete) was performed according to Hopfield *et al.* (1988).

^{32}P-labelled tyrosine phosphorylated AChR was prepared in principle according to Wagner *et al.* (1991) with a few modifications: frozen AChR-rich *Torpedo* membranes (100 pmol α-bungarotoxin binding sites) were incubated in 100 μM [γ-^{32}P]ATP (~20 000 c.p.m./pmol) in a total volume of 20 μl of 20 mM Tris–HCl, pH 7.8, containing 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EGTA, 0.5 mM EDTA, 1 mM Na_2VO_4, 1 mM ouabain, 1 μM staurosporine, 0.1 μM protein kinase inhibitor, 100 μM dithiothreitol, 20 μg/ml antipain, 20 μg/ml leupeptin and 10 units/ml aprotinin (all from Sigma). In a control sample, $MnCl_2$ was omitted. After 2–40 min at 30°C, the reaction was stopped by placing the samples on ice. The membranes were washed with PBS and the AChRs of the pelleted membranes were either SDS-dissociated or Triton-solubilized: (i) SDS dissociation: the membranes were treated with 1% SDS in PBS for 30 min followed by 20 times dilution in 0.5% Triton X-100 in PBS. (ii) Triton solubilization: the membranes were treated with 0.5% Triton X-100, 2 mM dithiothreitol (to

obtain AChR monomers) in PBS, for 60 min at 4°C followed by the addition of 2 mM iodoacetamide. After centrifugation to eliminate any debris the extracts were subjected to immunoprecipitation: 1–2 pmol ^{32}P-labelled AChR (or subunits) were incubated with 0.5–2 pmol test Sepharose-immobilized mAb for 2 h at 4°C in 50–500 μl PBS containing 0.5% Triton X-100, 0.05% NaN_3. The mAbs were immobilized on Sepharose–CNBr activated beads (Pharmacia), according to the manufacturer's instructions. The Sepharose beads with the bound radioactivity were washed three times with 1 ml PBS supplemented with 0.4 M NaCl, 0.5 M sucrose, 0.5% Triton X-100 and 0.05% NaN_3, and once with plain PBS. The labelled AChR subunits were eluted by SDS–mercaptoethanol sample buffer and subjected to SDS–PAGE (10% w/v polyacrylamide) in principle according to Laemmli (1970), without boiling. After electrophoresis the gels were fixed, dried and subjected to autoradiography. In order to confirm that phosphorylation involved only tyrosines, SDS–polyacrylamide gels were occasionally subjected to alkaline treatment (Cooper and Hunter, 1981).

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