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Recognition of inter-transmembrane regions of acetylcholine receptor α subunit by antibodies, T cells and neurotoxins

Implications for membrane-subunit organization

M. Zouhair Atassi, Taghi Manshouri and Tsuyoshi Yokoi

Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030, USA

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Three regions of the α chain of *Torpedo californica* acetylcholine receptor (AChR), corresponding to residues α262–276, α388–408 and α427–437 were synthesized, purified and characterized. The first two peptides have been proposed to occupy inter-transmembrane regions while the third represented the C-terminal segment, proposed by various models to be either extracellular or intracellular. Peptide α388–408 stimulated a good response in the AChR-primed T cells of H-2^s haplotype mice, a low response in the H-2^q haplotype and no response in the H-2^b haplotype. Peptide α427–437 stimulated AChR-primed T cells of the H-2^s haplotype, but caused no response in the q and b haplotypes. Peptide α262–276 evoked no in vitro stimulation in any of the s, q or b haplotypes. In antibody binding studies, peptide α388–408 bound antibodies raised against free AChR or against membrane-bound AChR. The other two peptides showed little or no binding activity. Further, peptide α388–408 bound specifically both ^{125}-I-labelled bungarotoxin and cobratoxin, while the other two peptides had no binding activity. These results were consistent with only one of the models for subunit organization within the membrane.

Acetylcholine receptor; Toxin binding; Synthetic peptide; Cobratoxin; α-Bungarotoxin; Antibody binding; T-cell activation

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) plays a central role in post-synaptic neuromuscular transmission. In response to binding of acetylcholine [1–4], the receptor mediates ion flux across the cell membrane. This regulatory activity is inhibited by binding of α-neurotoxin to the AChR α subunit [5] or by some anti-receptor antibodies. The receptor is a pentamer comprising four subunits ($\alpha_2\beta\gamma\delta$). The primary structures of all four AChR subunits of *Torpedo californica* have been deduced from the respective cDNA sequences [6–8]. It was possible to identify the extracellular part of the chain [8–10] and four transmembrane hydrophobic regions interconnected by two short peptide segments on the extracellular side of the membrane. In the present work, we have synthesized the two inter-transmembrane regions α262–276 and α388–408 as well as the C-terminal segment α427–437 and have determined their ability to bind α-neurotoxins (BgTX and CbTX) and antibodies against free AChR and membrane-bound AChR and to stimulate the in vitro proliferation of AChR-primed T lymphocytes.

Correspondence address: M.Z. Atassi, Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

Abbreviations: AChR, the acetylcholine receptor molecule; BgTX, α-bungarotoxin; CbTX, cobratoxin; PBS, 0.01 M sodium phosphate buffer, pH 7.2, in 0.15 M NaCl; Con A, concanavalin A; BSA, bovine serum albumin

2. MATERIALS AND METHODS

The preparation of AChR from the electric organ tissue of *T. californica* (Pacific Bio-Marine Laboratories) was carried out as described [11,12]. The AChR preparations had an α-bungarotoxin binding activity of 8.7–9.1 nmol/mg AChR.

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Table 1

Proliferative responses of lymph node cells from mice primed with AChR to in vitro challenge with peptides or with AChR

Mouse strain:	Proliferative response (Δ cpm ± SD) of different mouse strains[a]					
	SJL (H-2^s)		SWR (H-2^q)		C57BL/6 (H-2^b)	
	Δ cpm	Opt. dose[b] (μg/ml)	Δ cpm	Opt. dose[b] (μg/ml)	Δ cpm	Opt. dose[b] (μg/ml)
Challenge antigen						
Peptide α262–276	337 ± 90	40	447 ± 55	20	128 ± 30	20
Peptide α388–408	9854 ± 580	20	2490 ± 440	20	552 ± 66	80
Peptide α427–437	9029 ± 601	20	537 ± 15	20	408 ± 40	40
Controls						
AChR[c]	108972 ± 7100	2.5	77952 ± 5510	2.5	48985 ± 6063	2.5
Peptide α146–162[c]	72015 ± 3600	80	18478 ± 1130	80	35595 ± 3240	80
Con A	314136 ± 18850		409852 ± 13360		205270 ± 7431	
Ovalbumin	992 ± 365		0		nd	
Lysozyme	0		157 ± 80		0	

[a] Note that unchallenged T cells incorporated the following cpm of [^{3}H]thymidine (medium background): SJL, 2244 ± 135 cpm; SWR, 2700 ± 50 cpm; C57BL/6, 1105 ± 66 cpm

[b] Studies were carried out in the dose range 1–100 μg/ml. The table summarizes the maximum proliferative response obtained at the indicated optimum dose

[c] AChR and peptide α146–162, previously shown to stimulate AChR primed T cells in these strains [23], were used here as positive controls to confirm that the T cells were responding correctly

The peptide regions studied were: α262–276, Glu-Leu-Ile-Pro-Ser-Thr-Ser-Ser-Ala-Val-Pro-Leu-Ile-Gly-Lys(Gly); α388–408, Ser-Asp-Glu-Glu-Ser-Ser-Asn-Ala-Ala-Glu-Glu-Trp-Lys-Tyr-Val-Ala-Met-Val-Ile-Asp-His(Gly); α427–437, Ala-Gly-Arg-Leu-Ile-Glu-Leu-Ser-Gln-Glu-Gly. The peptides were synthesized on a Gly-resin by the Merrifield solid phase method [13] as described [14]. They were purified by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals) in 0.05 M triethylamine followed by ion exchange chromatography on CM-Sephadex or DEAE-Sephadex [15–17]. Peptide homogeneity and amino acid analysis were examined as described [18,19].

Rabbit and outbred mouse antibodies against soluble AChR or against membrane-bound AChR were prepared as described [12]. The antisera used in the present studies were obtained 57 days after the initial injection with free receptor immunization and at 42 days in the case of membrane-bound AChR. The IgG fraction of the antisera were prepared as described [17].

Quantitative adsorbent titrations of ^{125}I-labelled BgTX, CbTX or immune IgG were done as described [20]. Adsorbents

Table 2

Binding of anti-AChR antibodies to AChR and to the peptides

Adsorbent	Antibodies bound (Δ cpm)						
	Antibodies against free AChR				Antibodies against membrane-bound AChR		
	Rabbit antiserum	Mouse antiserum 43	Mouse antiserum 40	Mouse antiserum 41	Mouse antiserum 51	Mouse antiserum 54	Mouse antiserum 55
AChR	115980	189970	201230	212200	121040	110460	179670
Peptides							
α262–276	1910	480	2120	1112	474	170	1040
α388–408	10410	41233	28550	49811	20970	9680	25040
α427–437	1280	370	0	720	652	220	1150

Values were obtained by double antibody titrations as described in the text and in the legend of fig.1. Results represent the average of 6 replicate analyses (using 100 μl of a 1:1, v/v, adsorbent suspension) which varied ± 1.3% or less. The values have been corrected for non-specific binding (± 3% or less) to uncoupled Sepharose and adsorbents of BSA, sperm whale myoglobin, hen lysozyme, ovalbumin, a nonsense peptide [20] and hemoglobin α chain synthetic peptides 41–55, 51–65 and 45–60 [21]

of unrelated proteins (BSA, sperm whale myoglobin, hen lysozyme, ovalbumin) and unrelated synthetic peptides [20,21] were used as controls for non-specific binding. AChR was coupled as described recently [12]. Also, the binding to AChR peptides, of antibodies against unrelated proteins (myoglobins, lysozymes, ragweed allergen Ra3) served as additional controls. Antibody binding was also determined by double-antibody solid-phase titrations [22] using ^{125}I-labelled rabbit anti-mouse IgG and mouse anti-rabbit IgG to monitor the binding of the respective unlabelled anti-AChR antibodies. The specificity of binding of ^{125}I-labelled toxin to peptide adsorbents was confirmed by inhibition with unlabelled toxin.

The ability of the three peptides to stimulate the in vitro proliferation of T cells from AChR-primed mice was examined in four mouse strains. Two of the strains (SJL, H-2^s and SWR, H-2^q) are high responders to AChR, one strain (C57BL/6, H-2^b) is an intermediate responder and one strain (C3H/He, H-2^k) is a non-responder [23]. Peptide α146–162 was used as a control because all three strains respond to this peptide [23]. The optimum immunizing AChR dose and the procedures for immunization, cell harvesting, culture, in vitro challenge with AChR or peptides and pulsing with [^{3}H]thymidine were performed as described [23].

3. RESULTS

3.1. *Purification and characterization of the synthetic peptides*

After purification, the synthetic peptides were homogeneous by peptide mapping [18]. In amino acid analysis, the synthetic peptides had compositions which were in agreement with those expected from their sequence. Their amino acid compositions (with the values expected from the sequence in parentheses) were: peptide α262–276, Thr, 1.01(1); Ser, 3.00(3); Glu, 1.02(1); Pro, 1.94(2); Gly, 2.02(2); Ala, 1.02(1); Val, 0.99(1); Ile, 2.03(2); Leu, 2.04(2); Lys, 0.99(1); peptide α388–408, Asp, 3.07(3); Ser,2.96(3); Glu, 4.20(4); Gly, 1.12(1); Ala, 3.20(3); Val, 1.93(2); Met, 1.07(1); Ile, 0.99 (1); Tyr, 0.95(1); His, 0.88(1); Lys, 1.04(1); Trp, 0.86(1); peptide α427–437, Ser, 0.94(1); Glu/Gln, 3.2(3); Gly, 2.13(2); Ala, 1.18(1); Ile, 1.00(1); Leu, 2.08(2); Arg, 0.97(1).

3.2. *Proliferation of AChR-primed T cells in response to peptides*

The responses of T cells from three AChR-primed mouse strains are summarized in table 1 which gives the data at the optimum challenge doses for the respective proteins and peptides. Immunization of SJL with native AChR gave T cells which responded strongly to AChR, and to peptides α388–408 and α427–437, but mounted no

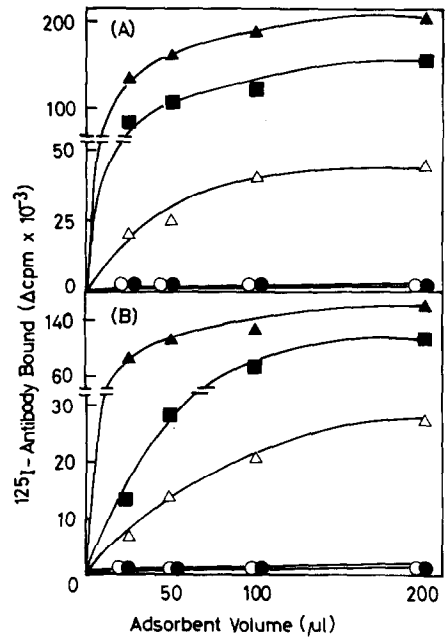

Fig.1. Radioimmunoadsorbent double-antibody titrations of anti-AChR antibodies with peptide adsorbents. (A) Binding of antibodies in mouse antiserum (No.43) against free AChR. (B) Binding of antibodies in mouse antiserum (No.51) against membrane-bound AChR. Constant amounts of mouse anti-AChR antisera (20 μl, pre-diluted 1:500, v/v) were incubated with varying amounts of peptide or protein adsorbents. After washing, the adsorbents were incubated with a fixed amount of ^{125}I-labelled rabbit anti-mouse IgG. Binding studies were done in 0.1% BSA in PBS at a constant reaction volume of 250 μl. The adsorbents were: (▲) AChR; (△) peptide α388–408; (○) peptide α262–276; (●) peptide α427–437; (■) peptide α182–198, previously shown to bind anti-AChR antibodies [12,14] and used here as a positive control. These antibodies did not bind to any unrelated control proteins and peptides and conversely antibodies against unrelated proteins (see text) did not bind to any of the AChR peptides. The binding curves of these controls superimposed with those of peptides α262–276 and α427–437.

significant response to peptide α262–276. Cells from AChR-primed SWR mice gave the expected [23] responses to AChR and to peptide α146–162, and also responded weakly to peptide α388–408 but did not respond to peptides α262–276 and α427–437 (table 1). T cells from AChR-primed mouse strains, C3H/He and C57BL/6, gave the expected responses [23] to AChR and to peptide α146–162, but did not respond to any of the three peptides α262–276, α388–408 and α427–437. The specificity of the stimulation by the AChR peptides was confirmed by absence of stimulation by

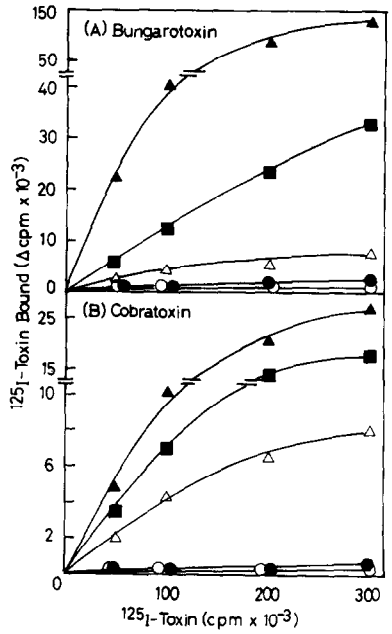

Fig.2. Binding of ^{125}I-labelled toxins to AChR and peptides. (A) Binding of BgTX; (B) binding of CbTX. Fixed volumes (25 μl of 1:1 suspension) of each adsorbent were reacted (16 h, room temperature) in triplicate, with increasing amounts of ^{125}I-labelled toxin in a final reaction volume of 60 μl of 0.1% BSA in PBS. After washing 5 times in the centrifuge with PBS, the tubes were counted. (▲) AChR; (△) peptide α388–408; (○) peptide α262–276; (●) peptide α427–437; (■) peptide α182–198, previously shown [35] to bind BgTX and CbTX and used here as a positive control. Note that adsorbents of several unrelated proteins and peptides (see text) did not bind toxins giving binding curves that superimposed with that of peptide α262–276.

unrelated proteins and peptides. Conversely, AChR or its peptides did not stimulate proliferation of T cells from mice that had been immunized with unrelated proteins (myoglobin and ovalbumin) and peptides (not shown). The viability of the cells in all these studies was indicated from their responses to Con A, PPD and LPS.

3.3. *Antibody binding studies*

Radioimmunoadsorbent titrations were performed both by direct binding of ^{125}I-labelled anti-AChR antibodies and by a double-antibody assay using a fixed amount of antibody and increasing amounts of adsorbents (see fig.1, for example). The results in table 2 summarize the mean binding values obtained by the double-antibody assay, and show that peptide α388–408 binds a considerable fraction of anti-AChR antibodies with antisera against free AChR as well as with antisera against membrane-bound AChR. The other two peptides showed little or no binding activity with these antisera.

3.4. *Binding of ^{125}I-labelled toxins to the peptides*

The binding of BgTX to adsorbents of the present synthetic peptides and appropriate controls [13] was determined by titrating a fixed amount of ^{125}I-labelled BgTX and CbTX with varying amounts of peptide adsorbents. Binding studies were also carried out using a fixed amount of adsorbent and increasing amounts of ^{125}I-labelled CbTX and BgTX. In titration of the present three peptides with radiolabelled CbTX and BgTX, only peptide α388–408 bound toxin (fig.2). The negative (i.e., no binding activity) and positive control proteins and peptides gave the expected binding results. The specificity of toxin binding to peptide α388–408 was confirmed by inhibition studies. The binding of labelled toxins to the peptide was inhibited almost completely by unlabelled toxin (fig.3), whereas other unrelated proteins had no inhibitory effect, even at much higher concen-

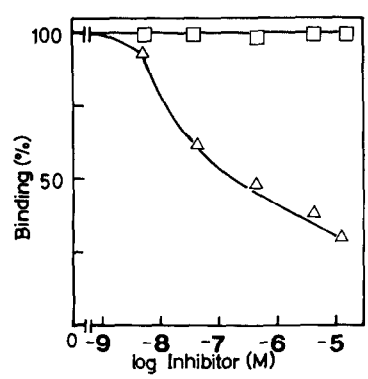

Fig.3. Inhibition by unlabelled BgTX of the binding of ^{125}I-labelled BgTX to peptide α388–408. Constant volumes of peptide adsorbent (10 μl of 1:1 suspension, v/v) were incubated (4 h, room temperature) with increasing concentrations of unlabelled BgTX or control proteins, followed by the addition of ^{125}I-labelled BgTX (2×10^5 cpm) and a further incubation for 14 h at room temperature. The reaction was done in triplicate in 0.1% BSA in PBS and a reaction volume of 35 μl. After reaction, the adsorbents were washed 5 times with PBS and counted. (△) Inhibition by unlabelled BgTX; (□) inhibition by unrelated control proteins (BSA, sperm whale myoglobin, lysozyme and ovalbumin). Note that the amount of ^{125}I-labelled BgTX bound to uninhibited peptide adsorbent was 4760 cpm.

trations than the toxin inhibitor. The IC_{50} for toxin binding to peptide α388–408 was 6.7×10^{-6} M.

4. DISCUSSION

Knowledge of the organization of the polypeptide chains of AChR in the cell membrane is valuable for the understanding, in molecular terms, of the mechanism of the biological function of AChR. Three models have been proposed for the folding of AChR subunits in the cell membrane. One model [8,9] proposed four transmembrane regions (M1 to M4). In the second model [24,25], a fifth transmembrane region (M5) was postulated, while the third model [26] envisioned seven transmembrane regions. In the first two models, the entire region 1–210 was proposed to be extracellular, while in the third model, the region comprising residues 142–192 was made into two transmembrane segments.

Recently, by applying a comprehensive synthetic strategy [27], uniform-sized overlapping synthetic peptides representing the entire region 1–210 of the α chain were studied for their ability to activate AChR-primed T cells [23] and to bind anti-AChR antibodies [12] (made against free AChR and against membrane-sequestered AChR) and neurotoxins [19,28]. These studies [14] helped to dismiss the third model [26], but were unable to differentiate between the first two models, because in both models residues 1–210 are extracellular. The two models were also in agreement by placing the region 262–276 on the extracellular side of the membrane interconnecting transmembrane regions M2–M3. The two models, however, differed in the placement of the C-terminal segment 427–437. Additionally, in the first model, the region 388–408 is intracellular while in the second model, this region is extracellular connecting transmembrane regions M4 (residues 409–425) and M5 (residues 365–387). Thus, it was decided to synthesize the regions α262–276 (linking extracellularly M2 and M3, in both models), α388–408 (linking M4 and M5 extracellularly in the second model, but is intracellular in the first model) and the C-terminal α427–437. Should one, or more, of these peptides activate AChR-primed T cells, bind antibody or, most importantly, bind α-neurotoxin it might then be possible to deduce the appropriate disposition of that region. Obviously, absence of activity will not be informative.

The finding that the peptide α388–408 bound antibodies against soluble AChR and against AChR bound to membrane vesicles indicated that this region is accessible to the immune system and is not buried in the membrane. Its accessibility to the immune system was further supported by its ability to stimulate the in vitro proliferation of AChR-primed T cells. The finding that the C-terminal region α427–437 was recognized by T cells and not by antibodies is not unusual and has been observed with other regions on the AChR α chain [23] and with other proteins [29–33]. The dependence of T-cell recognition on the mouse strain is indicative of Ir gene control operating at the antigenic site level [34]. At any rate, the immunological findings indicate that the regions α388–408 and α427–437 are not transmembrane, but do not permit the choice of whether they are extracellular or intracellular.

Although the binding activity of peptide α388–408 for BgTX and CbTX was considerably lower than that of peptide α182–198, it was nevertheless significant and specific. The ability of peptide α388–408 to bind α-neurotoxin will enable the localization of this region on the extracellular side of the cell membrane. It is not unreasonable to assume that intracellular parts of the α chain will have no functional requirement, and hence will not be expected, to bind α-neurotoxin. The localization of the region α388–408 on the extracellular side of the membrane would enable the choice between the two organizational models which postulate either four transmembrane regions [8,9] or five transmembrane regions [24,25]. Our results are consistent with the latter model [24,25] in which this region is depicted as extracellular.

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