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Analysis of Specificity of Antibodies against Synthetic Fragments of Different Neuronal Nicotinic Acetylcholine Receptor Subunits

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Abstract—We have compared specificity of a panel of polyclonal antibodies against synthetic fragments of the α7 subunit of homooligomeric acetylcholine receptor (AChR) and some subunits of heteromeric AChRs. The antibody interaction with extracellular domain of α7 subunit of rat AChR (residues 7-208) produced by heterologous expression in *E. coli* and rat adrenal membranes was investigated by the ELISA method. For comparison, membranes from the *Torpedo californica* ray electric organ enriched in muscle-type AChR and polyclonal antibodies raised against the extracellular domain (residues 1-209) of the *T. californica* AChR α1 subunit were also used. Antibody specificity was also characterized by Western blot analysis using rat AChR extracellular domain α7 (7-208) and the membrane-bound *T. californica* AChR. Epitope localization was analyzed within the framework of AChR extracellular domain model based on the crystal structure of acetylcholine-binding protein available in the literature. According to this analysis, the 179-190 epitope is located on loop C, which is exposed and mobile. Use of antibodies against α7 (179-190) revealed the presence of α7 AChR in rat adrenal membranes.

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Nicotinic acetylcholine receptors (AChRs) exhibit various functions in the body; they are involved in regulation of muscle contraction and in processes of higher nervous activity. Thus, they are reasonably subdivided into two main groups: muscle-type AChR and neuronal AChR [1-3]. The former consists of four types of subunits: two α1, one β1, one γ (ε in adult organisms), and one δ. The assembly of these subunits into a pentamer complex forms the central ion channel [4]. Information on spatial organization of various AChRs is mainly based on results of studies of *T. californica* electric organ AChR, which is referred to the muscle-type receptors. Neuronal AChRs are composed of two types of subunits, α (α2-α10) and β (β2-β4). The homopentamer receptors consist of α-subunits only (e.g., AChR composed of five α7-subunits or five α8-subunits). Heteromer AChRs are various combinations of α2-α6 and β2-β4 subunits [1].

Impairments of AChR functioning are associated with various diseases. For example, formation of autoantibodies against muscle-type AChRs or mutations in these receptors can cause *myasthenia gravis* [5, 6]. Mutations in neuronal α4-subunit are associated with one type of epilepsy. Changes in patterns of neuronal AChR distribution in the brain are associated with some psychiatric diseases, and impairments of cholinergic neurotransmission and cognitive capacities are seen in such neurodegenerative diseases as Alzheimer's and Parkinson's diseases [7]. Results of some studies suggest that changes in AChR in various pathological conditions may be detected on the surface of blood cells [8].

So detection of various AChRs requires certain tools for detection of both individual AChR subunits and their combinations. Antibodies are widely used for these pur-

Abbreviations: AChBP) acetylcholine binding protein; AChR) acetylcholine receptor; mAB) monoclonal antibodies; ELISA) enzyme linked immunosorbent assay.

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poses. For example, AChR subunits were detected in brain sections using commercially available antibodies obtained against the cytoplasmic domain of neuronal subunits expressed in bacteria (mAB 318-320) [9, 10] or antibodies against native neuronal receptor (mAB 306) [11]. These antibodies specifically stained α7-subunit in immunoblotting of rat brain homogenate [12, 13]. However, a large number of neuronal AChRs are not available as antigens. In these cases synthetic fragments corresponding to certain stretches of polypeptide chain of AChR subunits [14, 15] or extracellular domains of AChR subunits obtained by heterologous expression [16] are used as the antigens.

In spite of the development of a representative list of antibodies against various AChR subunits (see for example [17-19]), the problem still exists. Commercially available monoclonal antibodies stained cells on brain sections from non-transgenic and α7-knockout mice; however, immunoblotting of mouse brain cortex homogenates with these antibodies revealed many nonspecific bands [20]. Due to high homology (up to 70%) of amino acid sequences of neuronal subunits belonging to one class (e.g., α), the problem of elaboration of highly specific antibodies may be solved by immunization with synthetic fragments of AChR subunits [14, 15, 22].

The other aspect is evaluation of antibody specificity. In this study, the specificity has been evaluated using "model systems" from biological sources characterized by high content of whole AChRs of certain type or their functional domains. We have compared a panel of antibodies against synthetic fragments of AChR α7-subunit and also against fragments of other neuronal subunits of rat AChR and their interaction with the extracellular domain of rat AChR α7-subunit and rat adrenal membranes. The presence of α7 and other neuronal AChR (α2, α3, α4, α5, and β2) can be expected on the basis of literature data [23-25]. In control experiments, we have analyzed antibody interaction with *T. californica* electric organ AChR and used antibodies against α-subunit extracellular domain of this receptor obtained by heterologous expression.

MATERIALS AND METHODS

Synthetic peptides. Peptides corresponding to fragments of neuronal subunits of rat AChR (Table 1) were synthesized by the solid-phase method using *p*-alkoxybenzyl polymer with the use of the Fmoc protecting group for α-amino protection [26].

Peptides were desalinated by chromatography on Sephadex G-15 (Pharmacia, Sweden). The peptides were then purified by reverse-phase HPLC using a Reprosil-Pur column and a gradient of acetonitrile with 0.1% TFA (from 10 to 40% during 30 min) at flow rate 3 ml/min. (Absorbance was registered at 226 nm.) Analytical HPLC employed the same conditions chosen for preparative chromatography but at flow rate of 1 ml/min. MALDI mass spectrometry was carried out using VISION 2000 instrument (Bioanalysis, UK) (Table 1).

Preparation of polyclonal antibodies. Selection of peptides for immunization was based on lack of homology between subunits in corresponding fragments of polypeptide chain (α4 (residues 432-371), α7 (8-25), α7 (322-338), β2 (6-22), β2 (300-346)) and on putative exposure degree of peptides corresponding to toxin binding sites of AChR α-subunits. According to computational analysis the peptides α2 (11-27), α3 (75-91), α4 (8-22), α4 (104-121), α5 (63-81) would serve as the immunogenic epitopes.

The synthetic peptides were conjugated with snail hemocyanin as described in [27] with some modifications. Briefly, 2 mg of peptide and 2 mg hemocyanin (Calbiochem, USA) were mixed in 0.5 ml PBS. After mixing, 0.5 ml of 0.25% glutaraldehyde (Sigma-Aldrich, Germany) in PBS was slowly added (within 30 min) under stirring. The mixture was incubated at room temperature for 18 h and dialyzed against PBS.

Female rabbits (1.5-2 kg) were subcutaneously immunized with 1 mg of conjugate (or peptide) in complete Freund's adjuvant (Sigma-Aldrich). The peptide corresponding to α4 (342-371) and domain α1 (1-209) obtained using heterologous expression in *E. coli* [28] were used without conjugation. The immunization was repeated after 40 days in Freund's incomplete adjuvant using the same amount of antigen. Antiserum was obtained after seven days using blood from the marginal ear vein.

Immunoglobulin fraction obtained by ammonium sulfate fractionation was dissolved in PBS, dialyzed, lyophilized, and stored at −70°C.

Polyclonal affinity purified antibodies against α3 (181-192), α4 (181-192), α5 (180-191), α7 (179-190) were prepared as described earlier [15].

Antigens. The recombinant fragment (7-208) of AChR α7-subunit was obtained as described in [29]. Membranes isolated from *T. californica* electric organ were kindly presented by F. Hucho (Institut fur Chemie-Biochemie, Freie Universitat, Germany).

Rat adrenal membranes were isolated using a modification of the method described in [30]. All procedures (except centrifugation) were carried out in a cold room. All plastic tubes, vials, and also nitrocellulose were treated with 1 mM EDTA (pH 7.0) and soaked with bidistilled water. Animals were killed by cervical dislocation; organs taken immediately were kept prior to homogenization in ice–salt bath in ten-fold excess of the homogenization buffer (v/v) containing 1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiothreitol, 0.005 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, and several micrograms of DNase I in 10 mM Na-phosphate buffer, pH 7.4. Samples were homogenized in two volumes of the buffer using a glass–glass Potter's homogenizer (B. Braun, Germany) at

Table 1. Synthetic fragments of AChR used for antibody elaboration

Receptor fragment	Amino acid sequence	Molecular mass, daltons	
		theoretical	MALDI
α2(11-27)	RLFKHLFGGYNRWARPV	2117.5	2117.3
α3(75-91)	VEFMRVPAEKIWKPDIV	2057.5	2059.2
α4(8-22)	AEERLLKRLFSGYNK	1824.13	1824.0
α4(104-121)	DFAVTHLTKAHLFYDGRV	2090.4	2091.0
α4(342-371)	RRLIESMHKMANAPRFWPEPVGEPGILSDI	3448.1	3448.3
α5(63-81)	DVKLRWNPDDYGGIKIIRV	2257.6	2260.2
α7(8-25)	YKELVKNYNPLERPVAND	2162.5	2164.5
α7(322-338)	RMKRPGEDKVRPACQHK	2035.4	2036.4
β2(6-22)	RLVEHLLDPSRYNKLIR	2122.5	2123.1
β2(330-346)	RCARQRLRLRRRQRERE	2337.8	2339.4

700 to 1500 rpm; the glass cylinder was cooled with tap water. The resulting homogenate was layered onto 41% sucrose in the homogenization buffer and centrifuged at 95,000*g* for 1 h using a Beckman SW27 rotor (Beckman Instruments, USA). The resulting membrane fraction was diluted with the homogenization buffer and centrifuged under the same conditions for 20 min. The resulting membrane preparations were kept in a small volume of the homogenization buffer at −70°C. Protein content was determined by the method of Lowry as modified by Peterson [31].

Solid phase ELISA. The following preparations were used as antigens: 1.8 μl rat adrenal membrane suspension (protein content 7-9 mg/ml), 3 μl *T. californica* membrane suspension (protein content 1.1 mg/ml), 100 μl recombinant protein (0.05 mg/ml) in 0.1 M $NaHCO_3$ (pH 8.0). Each antigen was added in 100 μl into wells of a 96-well EIA/RIA flat bottom plate (Costar, USA), and after antigen addition the plate was incubated at 37°C for 60 min. (Pilot experiments revealed that this method of membrane suspension sorption onto the solid phase gave satisfactory results.)

After removal of the solution, 200 μl of 2% BSA in PBS was added to each well to prevent nonspecific sorption. After incubation at room temperature for 40 min, the BSA solution was removed. Wells were washed with 200 μl PBS-T (0.1% Tween-20 в PBS, pH 7.5), and after addition of 100 μl of increasing concentrations of polyclonal antibodies in PBS-T the plates were incubated at 37°C for 60 min. The solution of unbound antibodies was removed, wells were washed, and after addition of 100 μl of peroxidase labeled anti-rabbit antibodies (Amersham Biosciences, Ltd., England), antibody dilution 1 : 5000, the plates were incubated at 37°C for 60 min. The wells were washed three times with 200 μl of PBS-T and 100 μl of the substrate solution (4 μg *o*-phenylenediamine, 30 μl 30% H_2O_2, 10 μl 50 mM Na-citrate buffer, pH 4.5) was added. Color developed during 15 min incubation was measured using a Titertek Multiscan Plus MKII photometer (Labsystems, Finland) at 492 nm after addition of 100 μl 3 M HCl to each well.

Nonspecific rabbit immunoglobulins were used as a negative control.

Immunoblotting. Proteins from denaturing 8% polyacrylamide gels were electrophoretically transferred onto nitrocellulose membrane (Schleicher & Schuell, Germany) using the following buffer: 192 mM glycine, 25 mM Tris-HCl, 15% C_2H_5OH, pH 8.3. The amount of transferred protein was evaluated by staining with Ponceau S (DiaM, Russia), and the stain was then removed with bidistilled water. Nonspecific sorption was prevented by overnight incubation with 2% BSA in PBS-T at 4°C. BSA solution was then removed and the membranes were washed three times with PBS-T. The membranes were then incubated with polyclonal antibody solution (20-30 μg/ml) at room temperature for 120 min. Solution with non-bound antibodies was removed, and the membranes were washed three times with the same buffer (each time for 15 min) and incubated at room temperature for 90 min with peroxidase-labeled anti-rabbit antibodies (Amersham Biosciences, Ltd.), antibody dilution 1 : 5000. After the above described washing, the bound antibodies were detected using 0.1% 4-chloro-1-naphthol (Fluka Chemie, Switzerland) in PBS-T containing 10% C_2H_5OH and 0.05% H_2O_2.

Mathematical treatment of data. Results were treated using OriginPro 7.5, which was also used for histogram presentation of solid phase ELISA data.

Homology analysis of the synthesized peptides was carried out using Swiss-Prot and TrEMBL databases.

Modeling of spatial structure of AChR extracellular domain. Models of the extracellular domains of nicotinic receptors were built using the crystal structure of acetylcholine binding protein (AChBP) (Protein Data Bank II9B, IUX2) and cryoelectron microscopy structure of AChBP from *Torpedo marmorata* electric organ (PDB 2BG9). Amino acid sequences were aligned as in LGIC database (http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php).

The modeling employed the program MODELLER 7v7 program (http://www.salilab.org/modeller) and multiple modeling requests to SwissModel server (http://swissmodel.expasy.org). Structures were verified using the program WHAT_CHECK (http://swift.cnbi.nl/gv/whatcheck); structures were relaxed by means of GROMOS'96 tools (http://www.igc.ethz.ch/gromos) built into SPDBViewer 3.7 sp5 (http://swissmodel.expasy.org/spdbv/). The generated structures were studied using the program SPDBViewer.

The exposure degree was calculated as a relative value [32]. The exposure of amino acid X of the pentapeptide GGXGG in fully extended conformation was defined as 100%. The calculation was carried out for the van der Waals surface of the molecule. Amino acids characterized by the value of 75% were considered as the exposed ones.

Illustrations were created using PyMOL, version 0.97 (http://www.pymol.org).

RESULTS

Immunization of rabbits with synthetic fragments of various neuronal subunits of AChR, extracellular domain (1-209) of α1-subunit of *T. californica* electric organ AChR produced by heterologous expression in *E. coli* yielded immune sera. These sera were used for isolation of immunoglobulin fractions of polyclonal antibodies by the sedimentation method. Antisera titers obtained following rabbit immunization with fragments of AChR subunits varied from 1/800 to 1/6400. Antisera to β2 (6-22) and α4 (8-22) were characterized by the minimal titer of 1/800. If titers of antisera obtained during immunization of a couple of animals did not exceed one dilution factor, such sera were pooled. In the case of larger differences (e.g. 1/400 and 1/6400 for α7 (8-25) and 1/1600 and 1/6400 for α4 (104-121)), we used the sera with higher titer. Antibodies against α7 (179-190), α5 (180-191), α4 (181-192), and α3 (181-192) were purified by affinity chromatography on corresponding peptides immobilized on aminohexyl-Sepharose (AH-Sepharose) using glutaraldehyde as described in [15]. These antibodies were obtained against peptides corresponding to ligand-binding sites of these subunits. Since we planned to use these antibodies in competition with toxins exhibiting high affinity to receptor, additional antibody purification was necessary.

In the first stage, antibodies elaborated against various fragments of amino acid sequences of rat AChR were compared in the solid phase ELISA test using α7 (7-208) domain (Fig. 1).

The affinity purified antibodies exhibited the highest selectivity to the α7 (179-190) fragment (Fig. 1b). It represents a fragment of the ligand-binding site of AChR, which also includes vicinal disulfide 192-193 [33, 34]; peptides derived from this region of the α1- and α7-subunits can bind α-bungarotoxin [35, 36], the effective blocker of α7-AChR and muscle-type AChR. The antibodies to the N-terminal fragment of α7 (8-25) were characterized by significantly lower effectiveness. So it was plausible to suggest that antibodies produced against the cytoplasmic fragment 322-328 would not interact with the extracellular domain. However, in reality they did, and the effectiveness was close to that of antibodies against fragment 8-25. Antibodies against various sites of extracellular domains of AChR α4-subunit exhibited similar effectiveness (Fig. 1a). It should be noted that effectiveness of the interaction between antibodies against the AChR α3 (75-91) fragment and the α7 (7-208) domain was nearly the same as in the case of antibodies against α7 (179-190). Such high affinity interaction was also typical for polyclonal antibodies elaborated against the whole domain (1-209) of the AChR α-subunit.

We have also investigated antibody binding to natural objects: *T. californica* electric organ membranes containing muscle-type AChR and rat adrenal membranes. Antibodies against the extracellular domain (1-209) of *T. californica* AChR α-subunit exhibited high affinity to natural membrane receptor (Fig. 2a). There was also high level of response in the case of antibodies against the α7-subunit cytoplasmic domain (322-338) and antibodies against fragments of other neuronal subunits: α4 (104-121) and α2 (11-27). Interestingly, the antibodies against α7 (179-190) (Fig. 2b) exhibited the lowest level of interaction with *T. californica* AChR, the muscle type receptor. The latter indicates specificity of these antibodies to neuronal α7-AChR.

Rat adrenal membranes are an attractive research object because adrenal medulla chromaffin cells express various types of native AChR. Studies [23, 24] revealed expression of α2, α3, α4, α5, and β2-subunits on the surface of these cells. These cells also contain AChR α7-subunit mRNA, but literature data on the expression of functionally active pentamer are contradictory [23-25]. Thus, employing adrenal membrane we would expect interaction of all antibodies prepared except antibodies against the extracellular domain (1-209) of *T. californica* AChR α-subunit.

Affinity purified antibodies against α3 (181-192) exhibited the highest effectiveness in the interaction with these membranes (Fig. 3b). All antibodies against various α7 fragments did bind to rat adrenal membranes. Antibodies against both cytoplasmic and extracellular

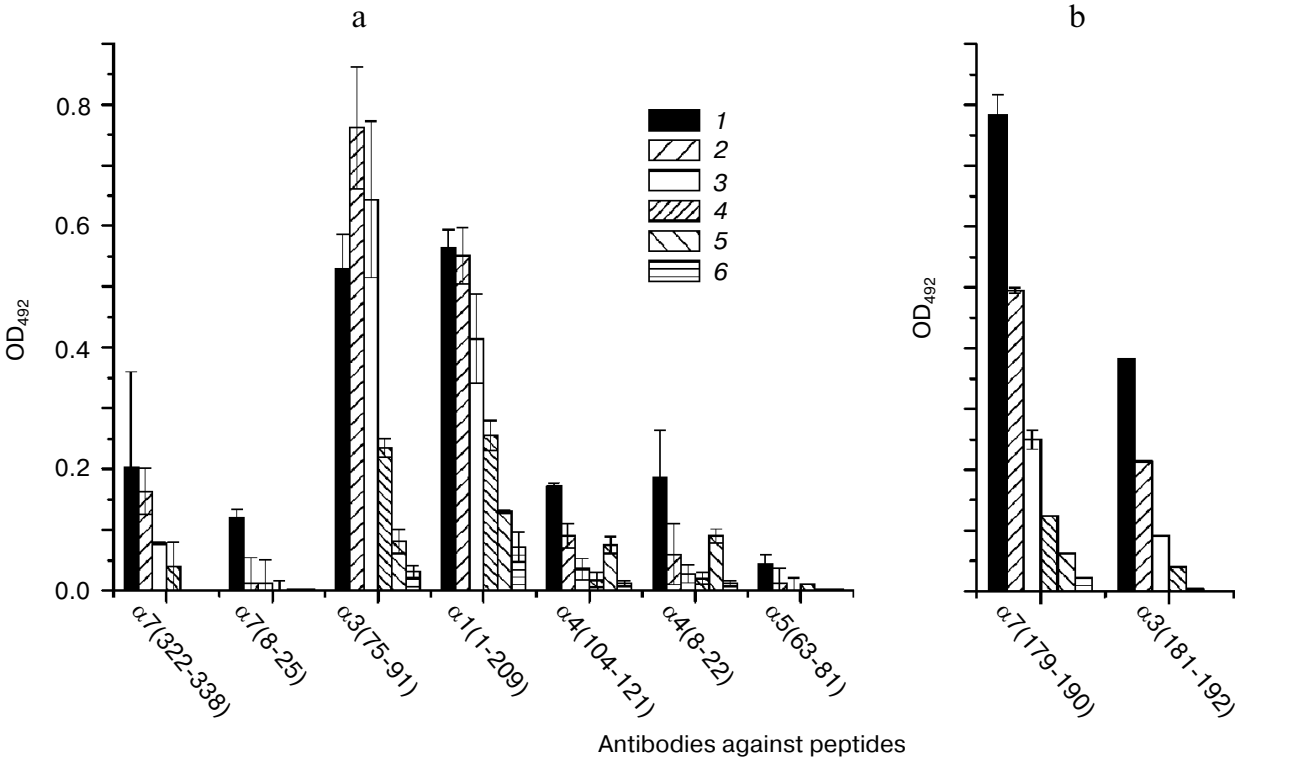

Fig. 1. ELISA analysis of polyclonal antibody interaction with α7 (7-208) extracellular domain: a) immunoglobulin fraction; b) affinity purified antibodies. *1-6*) Antibody concentrations 30, 7.5, 1.9, 0.47, 0.12, and 0.08 μg/ml, respectively. Here and in subsequent figures data represent mean ± SEM of 2-3 independent experiments.

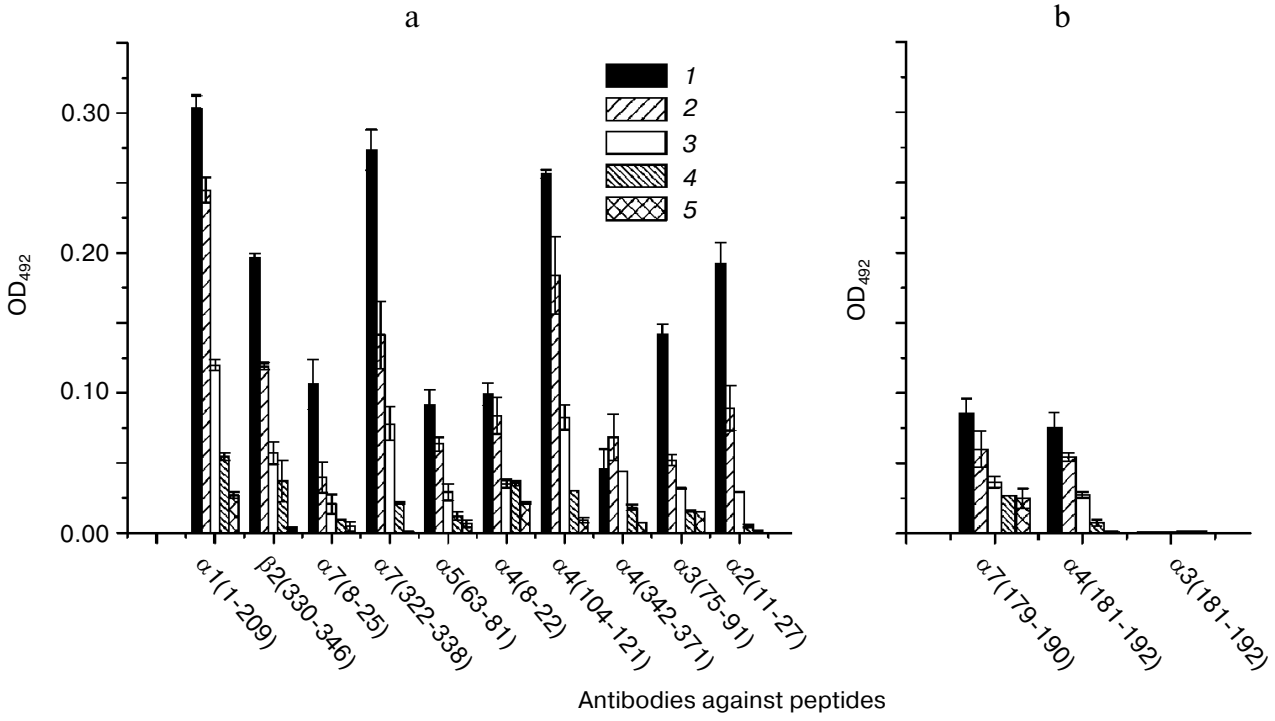

Fig. 2. ELISA analysis of polyclonal antibody interaction with *T. californica* membrane-bound AChR: a) immunoglobulin fraction; b) affinity purified antibodies. *1-5*) Antibody concentrations 25, 15, 3.75, 0.94, and 0.23 μg/ml, respectively.

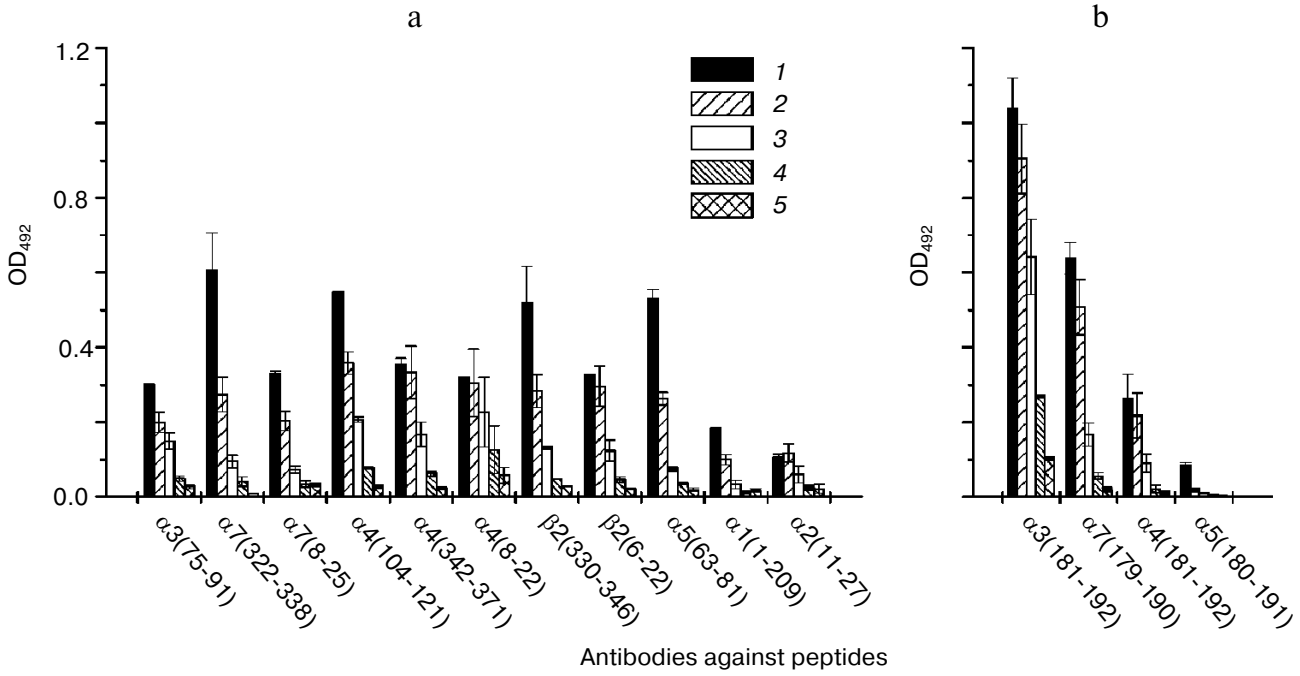

Fig. 3. ELISA analysis of polyclonal antibody interaction with rat adrenal membranes: a) immunoglobulin fraction; b) affinity purified antibodies. *1-5*) Antibody concentrations 25, 15, 3.75, 0.94, and 0.23 μg/ml, respectively.

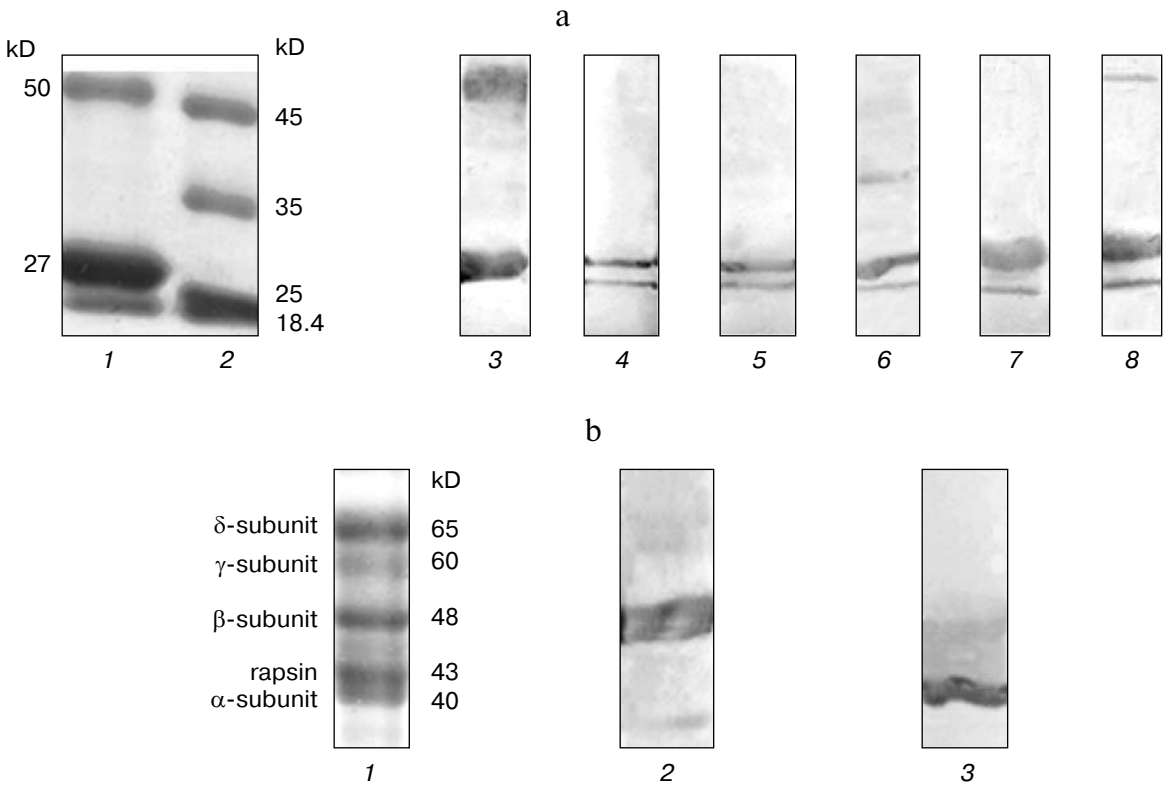

Fig. 4. Results of immunoblot analysis. a) Extracellular domain α7 (7-208): *1*) PAGE of the expressed extracellular domain α7 (7-208); *2*) protein molecular weight markers, staining with Coomassie; *3-8*) interaction of the domain with antibodies against α7 (179-190), α3 (75-91), α1 (1-209), α4 (8-22), β2 (6-22), and α5 (63-81), respectively. b) *T. californica* membranes: *1*) PAGE of *T. californica* membranes, staining with Coomassie; *2*, *3*) interaction of the membranes with antibodies against α3 (75-91) and α1 (1-209), respectively.

domains of β2-subunits also demonstrated binding to these membranes. Antibodies against α5 (63-81) fragment exhibited reasonably effective interaction with adrenal membranes, whereas antibodies against α5 (180-191) fragment nearly failed to react with these membranes. Antibodies against α2 (11-27) and as expected antibodies against α1 (1-209) domain demonstrated insignificant interaction with rat adrenal membrane preparations.

For subsequent characterization of the antibodies obtained, we have employed the method of immunoblotting. Figure 4 shows results of immunoblotting with the extracellular domain α7 (7-208) and *T. californica* membranes. The most intensive staining of ~27 kD band corresponding to domain α7 (7-208) was observed in the case of antibodies against α7 (179-190) fragment. (This was also accompanied by staining of ~50 kD band, which became more evident after protein storage, and so it would be related to aggregation and denaturation.)

Figure 1 shows that these antibodies as well as antibodies against α3 (75-91) and α1 (1-209) exhibited the highest reactivity to α7 (7-208) fragment in the ELISA test. However, staining of the domain band after immunoblotting with the antibodies against α3 (75-91) and α1 (1-209) was less intensive (Fig. 4a). The opposite situation was observed in the case of antibodies against α5 (63-81): they exhibited weaker interaction in the ELISA test but behaved similarly to the antibodies against α7 (179-190) during immunoblotting. No binding was detected in the case of antibodies elaborated against the cytoplasmic fragment of α7-subunit.

Immunoblots with *T. californica* membranes (Fig. 4b) revealed specificity of antibodies against α1 (1-209). Unexpectedly, we found cross-reactivity of antibodies against α3 (75-91) with *T. californica* AChR β-subunit. However, other antibodies exhibiting reasonably high effectiveness in the ELISA test, α4 (104-121), α2 (11-27), and α7 (322-338) did not react with *T. californica* AChR membranes during immunoblotting (data not shown).

DISCUSSION

The cross-reactivity of the extracellular domain of α7 (7-208) expressed in *E. coli* with antibodies against α3 (75-91) and against *T. californica* extracellular domain α1 (1-208) can be attributed to homologies recognized between fragments (Table 2).

Binding of antibodies against α4 (104-121) and α2 (11-27) with *T. californica* membrane AChR can also be attributed to homology in primary structures (Table 2). However, these antibodies did not exhibit specific interactions during immunoblotting. Antibodies against α3 (75-91) stained AChR β-subunit in spite of lack of marked homology between this fragment and amino acid sequences of AChR β- and γ-subunits from *T. californica* electric organ. (Comparison with amino acid sequence of the γ-subunit was included due to known proteolytic cleavage of this subunit yielding large fragments, which could mimic the position of α- and β-subunits on elution profiles.)

Table 2. Homology of the synthesized peptides with fragments of extracellular domains of *T. californica* α1 (1-209) and rat α7 (7-208) subunits of AChR

Peptides	Sequence fragments	Identity
α1(1-209)	6 16 ..RLVANLLENYN..	5/11 (45.5%)
α2(11-27)	RLFKHLFGGYNRWARPV	
α1(1-209)	99 111 .. DFAIVHMTKLLL..	7/13 (53.8%)
α4(104-121)	DFAVTHLTKAHLFYDGRV	
α7(7-208)	75 91 ..VKNVRFPDGQIWKPDIL..	9/17 (53%)
α3(75-91)	VEFMRVPAEKIWKPDIV	

Note: Sequences of the synthesized peptides are indicated in bold.

Antibodies against various sites of extracellular domain of α7-subunit reacted with extracellular domain of α7-AChR obtained by heterologous expression (Fig. 1) and did not exhibit cross-reactivity with *T. californica* membranes (Fig. 2). This suggests specificity of α7-subunit recognition by these antibodies.

It should be noted that selection of potential epitopes and synthesis of corresponding peptide fragments for elaboration of the panel of antibodies obtained in the study was made before publications on AChBP spatial structure appeared [37, 38]. These publications demonstrated that AChBP is a model of ligand-binding extracellular domains of various AChRs. This conclusion was confirmed by studies of functional characteristics of AChBP [39] and chimera obtained by fusion of AChBP with a domain of 5HT3 receptor, a member of ligand-gated ion channels, containing all transmembrane fragments [40, 41], and also by data of X-ray analysis of complexes of various AChBP with agonists [42] and AChR antagonists [43]. In some studies, interpretations of interactions between AChR with agonists and antagonists also employed models of extracellular domains of corresponding AChR generated on the basis of AChBP crystal structures. We have also generated a computer model of the extracellular domains of rat AChR neuronal subunits for elucidation of putative regions of the spatial structure which would correspond to peptide fragments employed for antibody production. Since the extracellular domain of any AChR represents a pentamer, it is important to take into consideration not only localization of the selected peptide fragment inside or outside each particular subunit, but also its possible exposure to the contact regions

Table 3. Comparison of antibody binding and epitope exposure

Epitope	Immune response			Exposure*
	α7(7-208)	*T. californica* AChR	rat adrenal membranes	
α2(11-27)	–	–	±	epitopes are totally exposed in both single subunit and pentamer complex
α4(8-22)	–	–	+	
α7(8-25)	±	–	±	
β2(6-22)	–	±	+	
α3(181-192)	–	–	+	
α4(181-192)	–	–	–	
α5(180-191)	–	–	–	
α7(179-190)	++	–	++	
α3 (75-91)	++	–	+	epitopes are partially exposed, they are located in the contact area of adjacent (neighboring) subunits
α4(104-121)	+	–	+	

* Within the spatial model of the extracellular domain.

with other neighboring subunits. Table 3 shows data on antibody binding and epitope exposure.

All the peptides can be subdivided into three groups by their exposure degree. This first group includes α2 (11-27), α4 (8-22), α7 (8-25), and β2 (6-22); they should be fully exposed because they cover N-terminal α-helix and a part of the following loop exposed into the synaptic cleft (Fig. 5). The fragments α3 (181-192), α4 (181-192), α5 (180-191), and α7 (179-190) of C-loop constitute the second group. This region is exposed in both single subunit and the pentamer complex. Amino acid residues belonging to C-loop of one subunit are involved in formation of the major surface responsible for agonist and antagonist binding, whereas residues of a neighboring subunit are a part of additional surface for ligand binding [37, 42, 43]. X-Ray analysis revealed mobility of C-loop, which changes its positioning depending on complex formation of AChBP with agonist or antagonist [42, 43]. Thus, selection of the peptide fragment of this loop for antibody elaboration was quite lucky due to positioning and properties of C-loop and also due to relatively low homology with the corresponding subunit fragments of neuronal AChR. This suggests that antibodies against similar fragments of various subunits of neuronal AChR will exhibit high reactivity and selectivity towards corresponding receptors.

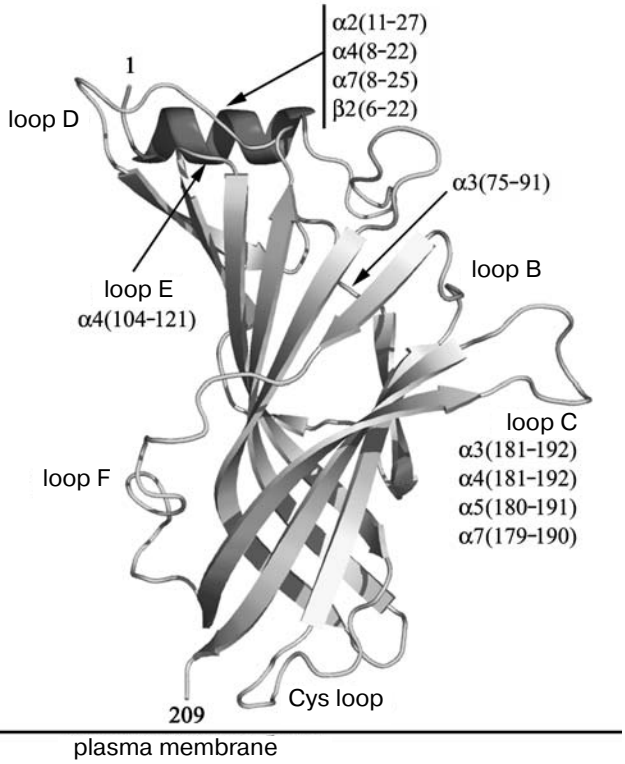

Fig. 5. Scheme of location of the synthesized peptides within the model of AChR extracellular domain.

The third group consists of antibodies against α3 (75-91) and α4 (104-121). Corresponding fragments should be partially exposed, as they are located within contact sites of adjacent subunits. Within this framework it is difficult to explain why antibodies against α3 (75-91) effectively react with α7 (7-208) domain (Fig. 1a) and antibodies against α4 (104-121) bind membrane-bound AChR from *T. californica* (Fig. 2a) and rat adrenal membranes (Fig. 3a). We may just suggest that antibody binding causes restricted conformational changes in AChR molecule similar to those that occur during agonist or antagonist binding.

Results of immunoblotting (Fig. 4) confirm high specificity of some antibodies, but they do not clarify reasons for the detected interactions. As expected, the most intensive staining of α1-subunit of *T. californica* AChR was observed in the case of antibodies against α1 (1-209) domain, whereas antibodies α3 (75-91) unexpectedly stained β-subunit of *T. californica* AChR (Fig. 4).

Use of extracellular domain α7 (7-208) obtained by heterologous expression in *E. coli* cells as the antigen in the ELISA test revealed high effectiveness of antibodies against α7 (179-190), representing the toxin binding site, which was used before in some tests [15, 46]. Results of ELISA tests on the interaction of these antibodies with rat adrenal membranes and *T. californica* electric organ membranes (enriched with the muscle-type AChR) together with results of immunoblotting suggest applicability of the antibodies against α7 (179-190) for detection of α7-AChR level under normal and pathological conditions. Results of the present study also indicate that certain precautions are required for correct interpretation of data obtained with one antigen. Lack of reactivity in the ELISA test evidently suggests lack of detectable levels of a given AChR receptor subtype, whereas immunoblot staining not necessarily means the presence of a certain type receptor reacting with antibody generated against its peptide fragment. For example, immunoblot revealed staining of α7 domain band with antibodies against α5 (63-81). Cross-interaction during immunoblotting was noted earlier for antibody interaction with recombinant extracellular domains. For example, antibodies against α2 did bind to α5, β3, and β4, whereas antibodies against α3 reacted with α5 and β3 [16]. Antibodies elaborated against synthetic peptides corresponding to unique sequences of cytoplasmic loops of various subunits are effectively employed for staining procedures during immunoblotting, but they are inapplicable for staining of intact cell receptors [14, 22].

Summarizing all the results obtained in the present study, the necessity of using an antibody panel becomes evident. Results may be more reliable if neurotoxins from snake venoms (α-neurotoxins) or *Conus* snail neurotoxins (α-conotoxins), which are specific ligands for certain subtypes of AChR [47], are used in combination with antibodies for identification and quantification of AChR subtypes. Such approach was successfully used for demonstration of expression of α7-AChR on the surface of B-lymphocyte cell line [46]. The conclusion on the presence of α7-AChR in rat adrenal membranes made on the basis of effective binding of antibodies against α7 (179-190) is consistent with our preliminary data on the binding of radioactive iodine-labeled α-cobratoxin to these membranes.

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