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ANTIBODIES AGAINST AN α-BUNGAROTOXIN-BINDING PEPTIDE OF THE α-SUBUNIT OF THE ACETYLCHOLINE RECEPTOR

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SUMMARY. Polyclonal and monoclonal antibodies were raised against a peptide comprising residues 173-204 of the α-subunit of the acetylcholine receptor. The polyclonal and pooled monoclonal antibodies inhibited up to 50% of ^{125}I-α-bungarotoxin binding to peptide 173-204. Some of the antibodies recognized native receptor but did not significantly affect α-bungarotoxin binding. Epitope mapping revealed that the antibodies are directed against residues 183-194 indicating this region is a major determinant of toxin binding. This region is most likely conformationally constrained in the native receptor.

The nicotinic acetylcholine receptor (AChR) has been well characterized at a molecular level (1). Much current research on the receptor is directed towards localizing functional domains, such as the acetylcholine-binding site, on the primary sequence of the receptor subunits. Present evidence based on studies employing snake venom curaremimetic neurotoxins, affinity alkylating agents, peptide fragments, synthetic receptor peptides, and genetic engineering points most strongly to the sequence flanking Cys 192 and Cys 193 of the α-subunit of the AChR as the major site for the binding of cholinergic ligands (2). In previous studies, we demonstrated that a synthetic peptide comprising residues 173-204 of the Torpedo α-subunit (173-204 32-mer) bound α-bungarotoxin (α-Btx) with the same affinity as isolated, denatured α-subunit (3,4), indicating this region contains a major determinant of neurotoxin binding. Monoclonal antibodies have also proven useful as specific probes for functional sites on the receptor (5). In order to further characterize the α-Btx-binding site on the receptor, polyclonal and monoclonal antibodies were raised against the α173-204 32-mer. The epitopes of these antibodies were further localized by testing their binding to shorter, overlapping peptides comprising portions of the 32-mer. The ability of the antibodies to inhibit toxin binding to the 32-mer was tested. It was found that single antibodies only partially competed toxin binding and those that were most effective were reactive against peptides containing Cys 192 and Cys 193. Pooled antibodies competed up to 50% of toxin binding. These studies confirm that a major determinant of toxin binding is located in the vicinity of Cys 192 and Cys 193

Abbreviations used: AChR, acetylcholine receptor; α-Btx, α-bungarotoxin; BSA, bovine serum albumin; ELISA, enzyme-linked immunoabsorbent assay; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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and indicate that other α–Btx-binding determinants are distributed over the sequence of the 32-mer.

METHODS AND MATERIALS

Receptor preparation and α-bungarotoxin iodination. Affinity purification of AChR from Torpedo californica electric organ, preparation of receptor-rich membranes, and iodination of α-Btx were performed as described previously (4,6).

Synthetic peptides. A 32 amino acid region of the α-subunit corresponding to residues 173-204 (α173-204 32-mer) and several shorter peptides within this region (Table I) were synthesized by the Yale Protein and Nucleic Acid Chemistry Facility, Yale University, New Haven, CT. The peptides are designated by their position on the α-subunit sequence and number of residues. The integrity of the peptide sequences was determined by amino acid composition analysis and reverse-phase high performance liquid chromatography.

Production of polyclonal and monoclonal antibodies. New Zealand white female rabbits were immunized with 200 μg of uncoupled 32-mer emulsified in complete Freund's adjuvant and injected subcutaneously. A boost of 200 μg 32-mer emulsified with incomplete Freund's adjuvant was administered 14 days later. The rabbits were bled 2 weeks following booster injections and antibody titer against the 32-mer tested using an enzyme-linked immunoabsorbent assay (ELISA) (see below). Additional boosts were given every 11-15 days, if required.

Balb/c mice were injected subcutaneously with 50-100 μg of the α173-204 32-mer in complete Freund's adjuvant. After two weeks, mice were boosted with the α179-192 14-mer. After seven days, the mice were bled and antibody titers tested against peptides using an ELISA assay. Once a high titer was obtained against the α173-204 32-mer, the mice were boosted in the tail vein intravenously and three days later the spleens were fused with a nonsecreting mouse myeloma cell line SP2/0-Ag 14. Fusions and cloning were performed by the Hybridoma and Long Term Culture Facility, Department of Pathology, Yale University, New Haven, CT. Positive hybridomas were cultured and supernatants collected. The antibody fractions were isolated and concentrated by 40% ammonium sulfate precipitation followed by dialysis against phosphate-buffered saline (PBS).

Peptide mapping of the antibodies. Reaction of monoclonal and polyclonal antibodies with the synthetic peptides was tested using an ELISA assay. 96-well polystyrene microtiter plates were coated with either affinity purified AChR (1 μg), receptor-rich Torpedo membranes (15 μg) or peptides (5 μg) by incubating overnight at 4°C in 100 μl 0.05M $NaHCO_3$ buffer, pH=9.6 (coating buffer). Alternatively, the peptides (5 μg in 100 μl coating buffer) were evaporated onto the microtiter wells by incubating in an oven at 45°C overnight. Unbound antigen was removed and the wells were quenched with 2.5% bovine serum albumin (BSA) in PBS for one hr at room temperature to reduce nonspecific binding. The monoclonal antibodies or rabbit serum was added to each well and incubated for either 2 hrs at room temperature or overnight at 4°C. After four washes with PBS, alkaline-phosphatase labeled goat anti-mouse IgM (μ specific) or rabbit IgG (diluted 1:750) was added and similarly incubated. The wells were washed again five times followed by incubation with the substrate, p-nitrophenyl phosphate. The absorbance was read at 405 nm using a SLT Easy Reader (EAR 400).

Solid phase competition assay. A solid phase assay was used to measure the binding of ^{125}I-α-Btx to the AChR, Torpedo membranes and the peptides. The wells of a 96-well polystyrene microtiter plate were coated with either 1 μg purified AChR or 15 μg Torpedo membranes in coating buffer. Synthetic receptor peptides were evaporated onto the plates.

Table I. Amino acid sequences of *Torpedo* acetylcholine receptor α-subunit synthetic peptides

α-173-204	32-mer	SGEWVMKDYRGWKHWVYYTCCPDTPYLDITYH
α-173-180	8-mer	SGEWVMKD
α-179-192	14-mer	KDYRGWKHWVYYTC
α-185-196	12-mer	KHWVYYTCCPDT
α-186-196	11-mer	HWVYYTCCPDT
α-194-204	11-mer	PDTPYLDITYH

After aspiration of the antigen, the wells were quenched with 200 µl 5% BSA for 90 min at room temperature. After quenching, the wells were aspirated and incubated with the rabbit serum or monoclonal antibody overnight at 4°C (50 µl). Subsequently, 50 µl of 6nm ^{125}I-α-Btx in 10 mM phosphate buffer, pH=7.4 containing 0.2% BSA was added for 2 hrs at room temperature. Wells were quickly washed with 200 µl of phosphate buffer/0.2% BSA. Bound ^{125}I-α-Btx was removed from wells by adding 100 µl of 0.25N NaOH containing 2.5% sodium dodecyl sulfate (SDS) and swabbing each well twice with a cotton tipped applicator. These applicators were then monitored for radioactivity in a gamma counter. It has been previously determined in our laboratory that α-Btx binding to the synthetic peptides is linear up to 3 hrs.

Protein blotting of the 32-mer. The 32-mer (10 µg) was placed in lithium dodecyl sulfate sample buffer containing 0.1M dithiothreitol and boiled for 20 min. The sample was loaded onto a 12.5% SDS/polyacrylamide gel and electrophoresed for 5 hrs at 30 mA (3). After electrophoretic transfer onto nitrocellulose filter paper, blots were then quenched with 5% BSA and incubated with ^{125}I-α-Btx (6nM for 2 hrs) or rabbit serum (overnight at 4°C). The filter was washed four times and incubated with alkaline phosphatase labeled goat anti-rabbit IgM second antibody for 2 hrs at room temperature. The filter was developed with naphthol ASMX phosphate/fast-red TR salt solution in the dark.

RESULTS

Monoclonal antibodies were raised against the proposed neurotoxin-binding site by immunizing Balb/c mice with a 32 amino acid synthetic peptide of the Torpedo AChR α-subunit (α173-204 32-mer) followed by boosts with a 14 residue peptide (α179-192 14-mer). In addition, polyclonal antibodies were raised in rabbits by immunization with α173-204 32-mer. The antibodies were tested for reactivity against native AChR, the α173-204 32-mer, and shorter overlapping peptides within residues 173 and 204. Most of the antibodies recognized the α173-204 32-mer (Table II). With one exception (9B), the antibodies showed weaker reactivity with native receptor, either within membranes or detergent solubilized. Most of the antibodies bound to peptides located within residues 179-196 but none bound to peptides (α173-180 and α194-204) comprising the N and C terminal regions of the α173-204 32-mer. Binding of polyclonal antibody to α173-204 32-mer was also demonstrated by immunoblotting (Fig.1, lane A). The upper band represents a dimer of the 32-mer due to incomplete reduction. Isotyping to determine the class and subclass of the antibodies revealed them to be IgMs (data not shown).

Table II. Antibody binding to acetylcholine receptor and synthetic α-subunit peptides as determined by ELISA

	ANTIBODIES								
	Polyclonal		Monoclonal						
Antigen	# 1	# 2	3B	4B	5B	6B	9B	10A	16D
AChR (memb)	+ +	+ +	+ +	- -	+	- -	+ + +	+	- -
AChR (pur)	+	+	+ +	- -	+	- -	+ + +	- -	- -
α173 32mer	+ + + +	+ + + +	+ + +	- -	+ + +	+ + +	+ + + +	+ +	- -
α173 8mer	- -	- -	- -	- -	- -	- -	- -	- -	- -
α179 14mer	+ + +	+ + +	+	+ + +	+	- -	+ +	- -	+ +
α185 12mer	+ +	+ +	+ +	+	+ +	+	+ + +	+	- -
α186 11mer	+	+	+ +	+	+ +	- -	+ +	+	- -
α194 11mer	- -	- -	- -	- -	- -	- -	- -	- -	- -

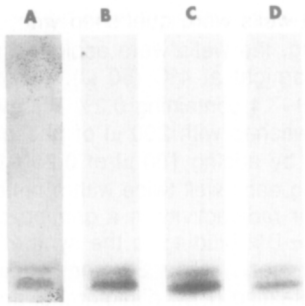

Figure 1. Electrophoresis and protein blotting of the 32-mer with ^{125}I-α-BTX. The 32-mer (10 μg) was solubilized in sample buffer containing 0.1M dithiothreitol and loaded on a 12.5% SDS/polyacrylamide gel, electrophoresed, and then transferred onto a nitrocellulose filter. Lane A is an immunoblot of the transferred 32-mer peptide incubated with rabbit #2 anti-32-mer serum followed by alkaline phosphatase labeled second antibody. Lane B shows an autoradiograph of the transferred 32-mer after overlay with ^{125}-I-α-BTX. A competition assay was performed by incubating the transferred 32-mer first with either rabbit preimmune serum (Lane C) or rabbit #2 anti-32-mer serum (Lane D) overnight before overlaying with^{125}-I-α-BTX.

The antibodies were tested for their ability to inhibit ^{125}I-α-Btx binding to the α173-204 32-mer using a solid phase assay. Three of the monoclonal antibodies (3B, 4B, 9B) partially competed toxin binding to the 32-mer (28-37%) (Table III). Pooled monoclonal antibodies and the polyclonal antibodies inhibited up to half of α-Btx binding. Competition of toxin binding by the polyclonal antibodies could also be demonstrated on Western blots. After electrophoresis and transfer of the 32-mer to nitrocellulose filters, the polyclonal antiserum inhibited approximately half of the ^{125}I-α-Btx binding (Fig.1, lane D). Antibodies were also tested for their ability to inhibit α–Btx binding to native receptor in a solid phase assay. One monoclonal antibody (9B) showed partial inhibition (25%) of toxin binding while the others were ineffective (data not shown).

Table III. Inhibition of ^{125}I-α-BTX binding to α-173-204 32-mer by antibodies

Fusion C378 Monoclonal Antibody	% of Btx Inhibition
3B	27.5%
4B	30.4%
5A	- -
6B	- -
9B	37.2%
15C	- -
16D	12.0%
Pool	48.0%
Polyclonals	
Rabbit #1 Serum	42.0%
Rabbit #2 Serum	54.0%
Preimmune Serum	11.0%

Peptide mapping of the antibodies that partially inhibited α-Btx binding reveals the approximate location of their epitopes. Antibody 3B recognizes residues 189-194 based on its greater reactivity with α185-196 and α186-196 than α173-192 and lack of binding to α194-205. Antibody 4B shows much stronger reactivity with α179-192 than with α185-196 and α186-196 indicating it binds within residues α183-188. Antibody 9B shows a stronger reaction with α185-196 than α186-196 indicating it recognizes residues α185-190. The polyclonal antibodies bind strongly to α179-196 and moderately to α185-196 and α186-196 so that the major epitope appears located between residues α184-189.

DISCUSSION

Polyclonal and monoclonal antibodies were raised against a synthetic peptide (α173-204) previously shown to bind α-Btx with the same affinity as isolated α-subunit (3,4,6). Some of the antibodies recognized the native receptor and partially inhibited α-Btx binding to the synthetic peptide. Although several laboratories have reported α-Btx binding to synthetic peptides located within residues 172 and 205 of the AChR α-subunit (3,4,6-10) there have been few reports regarding antibodies against this region. Ralston et al. (9) reported that attempts to produce antisera against peptide α172-205 which bind to the native receptor were unsuccessful. Gotti et al. (10) obtained monoclonal antibodies against α188-201 which recognized the native receptor, but it was not reported whether they blocked toxin binding.

Some antibodies in myasthenic patients or raised against intact receptor are directed against the toxin-binding site and inhibit binding of α–Btx (11-14). However, most evidence suggests that the region of the α-subunit encompassing the cholinergic-binding site is not highly immunogenic. The majority of antibodies produced in animals by immunization with AChR or in myasthenic patients are directed against a region of the receptor termed the main immunogenic region (MIR) (5). The MIR has been localized to residues 61-76 of the mouse α-subunit (15). An exception to these conclusions is the report by Mulac-Jericevic et al. (16) that polyclonal anti-AChR antibodies from mice and rabbits are directed against eight regions of the extracellular portion of the α-subunit (residues 1-14, 25-36, 41-53, 63-75, 102-114, 128-138, 172-182, and 188-198).

The apparent low degree of antigenicity of the neurotoxin-binding site on the AChR may be related to the conformation of this region and to the high proportion of hydrophobic residues. In addition, the cholinergic-binding site may not be fully accessible to the binding regions of antibodies. The receptor binding site of human rhinovirus 14 (17) and influenza virus hemagglutinin (18) are located within a canyon or pocket of highly conserved amino acids. The pocket is surrounded by antigenically variable antibody-binding sites. Although the canyon or pocket is exposed on the surface of the virus, the floor of the binding site may not be accessible to antibody because of the large binding surface of the latter. These residues would not be under immune selection and could remain constant (17). It is conceivable that the ligand-binding sites of receptors may lie within surface clefts, accessible to small ligands but not to large antibodies. Although the three-dimensional structure of the AChR is not known, the region considered to bind acetylcholine is highly conserved among muscle α–subunits of different

species and, based on weak binding of antibodies raised against synthetic peptides, is conformationally highly constrained.

Polyclonal antibodies and pooled monoclonal antibodies against α173-204 inhibited up to half of α-Btx binding to the peptide. The failure of these antibodies to completely inhibit toxin binding may be because they are IgMs and bind with low affinity. Alternatively, not all of the α-Btx-binding determinants on the 32-mer are contained within epitopes recognized by the antibodies. The epitopes of antibodies producing partial inhibition of α-Btx binding were mapped using overlapping peptides within residues 173-204. This study revealed that the epitopes of the antibodies were located within residues α183-194, suggesting that this region is an important determinant of neurotoxin binding. This conclusion is in agreement with a study in which α-Btx binding to the shorter overlapping peptides was measured (6). The latter study showed that toxin-binding determinants are distributed over the entire length of the 32-mer but that those with the highest affinity are located between residues 179 and 196.

Residues 183-194 of the α173-204 32-mer peptide appear to be an immunodominant region for this peptide, although apparently not for the native conformation of the whole protein (9). Synthetic peptides have been shown to bind to the major-histocompatibility complex class II molecules due to the fact that they mimic a processed antigen (19). The localization of epitopes to residues 183-194 of the α173-204 32-mer suggests that the amino and carboxy termini of the peptide are processed and trimmed upon binding to the class II molecules before presentation to the helper T-cells.

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REFERENCES

1. Changeux, J.-P., Giraudat, J. and Dennis, M. (1987) Trends in Pharm. Sci. 8, 459-465.
2. Lentz, T. L. and Wilson, P. T. (1988) Int. Rev. Neurobiol. 29, 117-160.
3. Wilson, P. T., Lentz, T. L. and Hawrot, E. (1985) Proc. Natl. Acad. Sci. USA 82, 8790-94.
4. Wilson, P. T. and Lentz, T. L. (1988) Biochemistry 27, 6667-74.
5. Lindstrom, J. (1986) Trends in Neurosci. 9, 401-407.
6. Wilson, P. T., Hawrot, E. and Lentz, T. L. (1988) Mol. Pharm. 34, 643-650.
7. Mulac-Jericevic, B. and Atassi, M. Z. (1986) FEBS Lett. 199, 68-74.
8. Neumann, D., Barchan, D., Fridkin, M. and Fuchs, S. (1986) Proc. Natl. Acad. Sci. USA 83, 9250-53.
9. Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, J. L. and Lindstron, J. (1987) Biochemistry 26, 3261-3266.
10. Gotti, C., Frigerio, F., Bolognesi, M., Longhi, R., Racchetti, G. and Clementi, F. (1988) FEBS Lett. 228, 118-122.
11. Mochly-Rosen, D. and Fuchs, S. (1981) Biochemistry 20, 5920-24.
12. Watters, D. and Maelicke, A. (1983) Biochemistry 22, 1811-19.
13. Dowding, A. J. and Hall, Z. W. (1987) Biochemistry 26, 6372-81.
14. Mihovilovic, M. and Richman, D. P. (1987) J. Biol. Chem. 262, 4978-86.
15. Barkas, T., Gabriel, J.-M., Mauron, A., Hughes, G. J., Roth, B., Alliod, C., Tzartos, S. J. and Ballivet, M. (1988) J. Biol. Chem. 263, 5916-20.

10. Ronnett, G.V., Knutson, V.P., Kohanski, R.A., Simpson, T.L., and Lane, M.D. (1984) J. Biol. Chem. 259, 4566-4575
11. Forsayeth, J., Maddux, B., and Goldfine, I.D. (1986) Diabetes 35, 837-846
12. Hedo, J.A., Collier, E., and Watkinson, A. (1987) J. Biol. Chem. 262, 954-957
13. Arakaki, R.F., Hedo, J.A., Collier, E., and Gorden, P. (1987) J. Biol. Chem. 262, 11886-11892
14. Duronio, V., Jacobs, S., Romero, P.A., and Herscovics, A. (1988) J. Biol. Chem. 263, 5436-5445
15. Salzman, A., Wan, C.F., and Rubin, C.S. (1984) Biochemistry 23, 6555-6565
16. Olson, T.S., and Lane, M.D. (1987) J. Biol. Chem. 262, 6816-6822
17. Olson, T.S., Bamberger, M.J., and Lane, M.D. (1988) J. Biol. Chem. 263, 7342-7351
18. Hedo, J.A., Harrison, L.C., and Roth, J. (1981) Biochemistry 20, 3385-3393
19. Cama, A., Marcus-Samuels, B., and Taylor, S.I. (1988) Diabetes 37, 982-988
20. Herrera, R., Petruzzelli, L., Thomas, N., Bramson, H.N., Kaiser, E.T., and Rosen, O.M. (1985) Proc. Natl. Acad. Sci., USA 82, 7899-7903
21. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., and Kahn, C.R. (1983) Proc. Natl. Acad. Sci., USA 80, 2137-2141
22. Zick, Y., Kasuga, M., Kahn, C.R., and Roth, J. (1983) J. Biol. Chem. 258, 75-80
23. Hedo, J.A., Kasuga, M., Van Obberghen, E., Roth, J., and Kahn, R.R. (1981) Proc. Natl. Acad. Sci., USA 78, 4791-4795
24. Laemmli, U.K. (1970) Nature 277, 680-685
25. Rees-Jones, R.W., Hedo, J.A., Zick, Y., and Roth, J.R. (1983) Biochem. Biophys. Res. Comm. 116, 417-422
26. Blackshear, P.J., Nemenoff, R.A., and Avruch, J. (1983) FEBS 158, 243-246
27. Yoshimasa, Y., Seino, S., Whittaker, J., Kakehi, T., Kosaki, A., Kuzuya, H., Imura, H., Bell, G.I., and Steiner, D.F. (1988) Science 240, 784-787
28. Kobayashi, M., Sasaoka, T., Takata, Y., Hisatomi, A., and Shigeta, Y. (1988) Diabetes 37, 653-656
29. Kobayashi, M., Sasaoka, T., Takata, Y, Ishibashi, O., Sugibayashi, M., Shigeta, Y., Hisatomi, A., Nakamura, E., Tamaki, M., and Teraoka, H. (1988) Biochem. Biophys. Res. Comm. 153, 657-663
30. Kakehi, T., Hisatomi, A., Kuzuya, H., Yoshimasa, Y., Okamoto, M., Yamada, K., Nishimura, H., Kosaki, A., Nawata, H., Umeda, F., Ibayashi, H., and Imura, H. (1988) J. Clin. Invest. 81, 2020-2022