CONTRACT NO: DAMD17-88-C-8120

TITLE: STRUCTURE AND CHARACTERIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS FROM PERIPHERAL AND CENTRAL NERVOUS SYSTEMS

PRINCIPAL INVESTIGATOR: Michael A. Raftery, Ph.D.
Bianca M. Conti-Tronconi

CONTRACTING ORGANIZATION: University of Minnesota
Department of Chemistry
Small Hall 29
207 Pleasant St., S.E.
Minneapolis, Minnesota 55455

REPORT DATE: November 7, 1991

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 7, 1991	Final Report (7/1/88 - 6/30/90)

4. TITLE AND SUBTITLE
Structure and Characterization of Nicotinic Acetylcholine Receptors From Peripheral and Central Nervous Systems

5. FUNDING NUMBERS
Contract No.
DAMD17-88-C-8120

61102A
3M161102BS11.AA.007
WUDA314936

6. AUTHOR(S)
Michael A. Raftery, Ph.D.
Bianca M. Conti-Tronconi

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Department of Chemistry, Small Hall 29
207 Pleasant St., S.E.
Minneapolis, Minnesota 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS
RAV; Lab Animals; Rats; Mice; Skeletal Muscles; Nicotinic Receptors; Acetylcholine; Brain

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified		

PUBLICATIONS

Conti-Tronconi, B.M., Tzartos, S., Spencer, S.R., Kokla, A., Tang, F. and Maelicke, A. "Identification of surface domains on the nicotinic receptor molecule by the use of toxins and antibodies". In: "Nicotinic acetylcholine receptors in the nervous system", NATO-ASI, series H, Vol. 25, pp. 119-136 (F. Clementi, C. Gotti and E. Sher, Eds.), Springer-Verlag, Berlin-Heidelberg-New York, 1988.

Conti-Tronconi, B.M., Tang, F., Walgrave, S. and Gallagher, W. "Nonequivalence of α-bungarotoxin binding sites in the native nicotinic receptor molecule". Biochemistry 29:1046-1054, 1990.

Conti-Tronconi, B.M., Tang, F., Diethelm, B., Spencer, S., Reinhardt-Maelicke, S. and Maelicke, A. "Elucidation of the structure of a cholinergic binding site by the use of monoclonal antibodies and synthetic peptides". Biochemistry 29:6221-6230, 1990.

McLane, K.E., Wu, X.D., Diethelm, B. and Conti-Tronconi, B.M. "Structural determinants of α-bungarotoxin binding to the sequence segment 181-200 of the muscle acetylcholine receptor α subunit: effect of cysteine/cystine modification and species-specific amino acid substitutions". Biochemistry 30:4925-4934, 1991.

It is expected that several other publications will result from the work described in the report. Some of these are already submitted or in press.

PERSONNEL

M.A. Raftery

B.M. Conti-Tronconi

L. Waddell

B. Carlson

S-J. Tine

M. Arriola

M. Humble

S. Jones

F. Tang

K. McLane

B. Diethelm

S. Nelson

S. Spencer

Degrees

Ph.D.	S. Nelson K. McLane
M.Sc.	M. Arriola

Table of Contents

STUDIES OF LONG-TERM INCUBATION OF ACETYLCHOLINE RECEPTOR-ENRICHED MEMBRANES WITH AN AGONIST

Abbreviations:

AcChR, acetylcholine receptor; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; MCA, methylcoumarylamide.

Introduction:

In order to study the effects of long-term preincubation of the AcChR with cholinergic ligands, especially to detect any functional changes we first conducted experiments on the long-term stability of the membranes in the absence of any added ligands. The stability was monitored by analytical SDS-gel electrophoresis. During the early years of study of these receptors we had noted that proteolysis was a great problem during purification of detergent solubilized preparations. The use of EDTA in particular was very important (1). In addition PMSF and iodoacetamide allowed protection from proteolytic fragmentation. The first isolation studies of receptor-enriched membranes from Torpedo californica (2) demonstrated that proteolysis was not as grave a concern as for detergent solubilized preparations and the initial recognition that the receptor was composed of four subunits came from studies where the purified molecule was produced by solubilization of already enriched membranes (3). In this section we describe studies of the long-term stability of the receptor subunits in highly enriched membrane fractions purified by sucrose density gradient centrifugation followed by brief treatment at pH 11.0 to extract peripheral membrane proteins. This preparation represents the most highly purified membrane preparation available from any source. In addition we describe the purification of a proteolytic enzyme which is present in both unfractionated membranes. in sucrose density gradient fractions enriched in the AcChR and to a lesser extent in the pH 11.0 treated membranes. The last part of this section deals with studies designed to determine whether long-term incubation with cholinergic agonists affects the functional states of the AcChR. For this purpose we describe experiments designed to determine whether the receptors ability to reversibly undergo changes between the resting state and the densensitized state is affected by incubation with agonist.

Experimental:

Materials - Electric tissue was obtained from Pacific BioMarine Labs, Venice, CA. ^{125}I-α-BTX was purchasd from New England Nuclear. All materials for gel electrophoresis were obtained from Bio-Rad, Richmond, CA. DEAE-cellulose was from Sigma Co.

General:

Methods - Membrane fractions were obtained as previously described (2,4) and were assayed for ^{125}I-α-BTX binding by a DEAE-disc assay (5). SDS gel electrophoresis was conducted by a standard procedure (6). Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue and destaining by known procedures (6).

Enzyme purification - Extracts of membranes were obtained using buffer (10 mM Tris-HCl, pH 7.4) containing 1.0% Lubrol-px, with stirring for fifteen minutes at -4°C followed by centrifugation at 100,000 g for forty five minutes. The initial fractionation procedure involved DEAE-52 ion exchange chromatography using a column (2.5 x 17 cm) equilibrated with 10 mM Tris-HCl, pH 8.0 and 0.2% Lubrol. Following application of the sample the column was developed with the equilibration buffer and fractiosn of 3 ml were collected. After forty fractions a linear gradient formed from 50 ml each of (A) 10 mM Tris-HCl pH 8, 0.05 M NaCl and (B) 10 mM Tris-HCl pH 8, 0.5 M NaCl was put through the column. The eluted fractions were routinely assayed for total protein (absorbance at 280 nm), protease activity (see below) and for ^{125}I-α-BTX binding (5).

Assay of Enzymatic Activity:

BOC-Gln-Arg-Arg-MCA and related peptides were obtained from Sigma. Assay of releas of 7-amino-4-methyl coumarin was performed as previously described (7) by monitoring generation of fluorescence. Excitation and emission maxima were 380 and 460 nm respectively.

Kinetics of ^{125}I-α-BTX binding. The kinetics of ^{125}I-α-BTX binding to AcChR enriched membranes was conducted exactly as described by Lee et al. (8), Quast et al. (9) and Moore and Raftery (10) with or without preincubation with cholinergic agonist for varying periods of time (see figure legends).

Kinetics of Thallium transport. This was conducted as described earlier (15) using an Applied photophysics stopped-flow spectrofluorometer.

RESULTS AND DISCUSSION

Membrane degradation as a function of incubation

The structural stability of AcChR enriched membranes incubated at 4°C for varying periods of time was studied by observation of changes in subunit integrity as determined by SDS gel electrophoresis. A typical example is shown in Fig. 1. The example shown is one for membranes which had been treated at pH 11.0 to remove peripheral membrane proteins to facilitate observation of any change in subunit structure. These membranes were then left at 4°C for varying periods of time up to forty eight hours. A progressive change in the polypeptide pattern was consistently observed, with the appearance of new polypeptides indicative of a slow degradation of the receptor system. The gel shown in Fig. 1 depicts the degradation pattern after forty eight hours and it clearly demonstrates new polypeptide species (a) one between the α- and β-subunits (->) and three of Mr lower than the α-subunit (Δ). Since the amount of AcChR protein applied to the gel in lanes 1 and 2 (degraded sample) was the same as the control in lane 7 (lanes 5,6 were lesser amounts of the control) it appears that clearly the γ- and β-subunits had been degraded since the intensity of their staining is diminished compared with lane 7. It is not possible on this basis to state whether the δ- and α-subunits were also degraded. It should be possible in the future to determine, by amino acid microsequencing, the exact locations of the four new species in terms of the precise peptide bonds cleaved in order to generate them. Thus the specificity of the proteolytic enzyme(s) that generate the new polypeptide species can be determined.

The effects of acetylcholine and carbamoylcholine on the degradation were also investigated. Neither agonist (Ach at 10^{-4}M and carb. at 10^{-3}M) had any reproducible effect on the degradation patterns either in terms of the Mr of the new species generated or on the amounts of their abundance as determined by the staining intensities observed on the SDS gel electrophoresis polypeptide patterns.

Protease purification:

Assay of various electroplax fractions following homogenization and fractionation revealed that protease activity was detectable in the crude homogenate, in unfractionated membranes, in membranes highly enriched in AcChR following sucrose density gradient centrifugation and even in these latter membranes after treatment at pH 11.0 to remove peripheral membrane proteins.

Such assays involved the use of a variety of peptide substrates capable of generation of a fluorescent product. These peptide substrates were of the general formula t-BOC-Gln-XX-MCA (7) where MCA is 4-methylcoumaryl-7 amide. Assay of crude or enriched membranes and cell fractions including pH 11.0 treated membranes revealed that the best substrate was t-BOC-Gln-Arg-Arg-MCA, indicating that the major protease had a recognition site for paired basic residues. The protease which was membrane bound was remarkably stable since it survived the treatment at pH 11.0 for thirty minutes at 4°C plus an additional forty five minutes during centrifugation of these membranes. Such treatment resulted in complete inactivation of residual acetylcholinesterase activity associated with the highly enriched membrane fraction from sucrose density gradient centrifugation (11).

For initial purification of the enzyme the fraction highly enriched in AcChR following sucrose density centrifugation was chosen since the proteolytic enzyme there present should represent that enzyme species in closest proximity to the AcChR. The activity could readily be solubilized from these membranes by several detergents at 1% (v/v) concentration such as Triton X-100, Lubrol-px and Nouidet. Since Lubrol was the most effective of these it was used throughout the following purification steps. However, it was also possible to use membranes which had not been treated at pH 11.0 for purification purposes since the results obtained were identical and higher yields were obtained.

When such an extract, following solubilization in 1% Lubrol and centrifugation as described in the experimental section, was chromatographed on DE-52 (see experimental) the profile depicted in Fig. 2 was obtained. The bulk of the applied protein was eluted with the starting buffer while the protease activity remained absorbed to the column. Following application of a salt gradient the activity was eluted coincident with a smaller protein peak (Fig. 2). The most active fractions (50-54) were pooled and a portion of this was applied to a Sephadex G-100 gel filtration column (0.9 x 60 cm). All of the protease activity and the majority of the protein emerged from this column in the void volume (data not shown), indicating that the enzyme is of high molecular weight (≥ Mr 100 kD). Although the protein and enzymatic activity co-eluted both from

the DE-52 and Sephadex G-100 columns it was still not clear how highly purified a preparation had been obtained. Therefore a further purification step was introduced, namely an affinity chromatography step. For this purpose a column with arginine covalently coupled to Sepharose via the cyanogen bromide method (12) was used. The results obtained from an affinity purification step of the protease fraction from pooled DE-52 chromatographed material is shown in Fig. 3. Clearly an additional further purification of modest level was obtained. A comparison is made in Fig. 4 of the protein species from the initial homogenate, the crude membrane fraction, a Lubrol extract of these membranes, the pooled DE-52 protease fractions and the fractions emerging from the void volume of the G-100 column and the affinity column, using SDS-gel electrophoresis. It is clear that the steps involved in extraction, ion-exchange chromatography and gel filtration resulted in progresive purification. The major protein component present after these stages of purification had an Mr of approx. 100 kD, with other minor components of both lower and higher Mr being present.

Since there were always traces of the AcChR subunits present in our most highly purified fractions it was conceivable that protease activity was actually associated with the AcChR molecule itself. We therefore assayed a DE-52 column eluate for protease activity and for ^{125}I-α-BTX binding activity. The results of these assays are shown in Fig. 5 (with the fractions numbered followed application of the salt gradient rather than the initial buffer wash). It is clear that the two activities are not coincident in their elution positions and we can safely conclude that the protease activity does not reside on the AcChR molecule. One further aspect of this activity will be investigated in the future. It is possible that the enzyme activity is associaed with acetylcholinesterase. An approach similar to that described above, using acetylthiocholine as substrate should clarify this issue.

The identity or not of this highly purified protease fraction associated with electroplax membranes with the activity that promotes AcChR degradation, described at the beginning of the Results section also remains to be elucidated.

<u>Functional states of the AcChR after long-term exposure to a cholinergic agonist</u>:

In order to assess any effects of long-term exposure of the AcChR to agonists with respect to its functional properties we conducted experiments to determine whether the reversible transition between the resting state and the desensitized state was affected by such exposure. The most straightforward manner in which to do this is by monitoring the rate of ^{125}I-α-BTX-receptor complex formation in the presence and absence of a cholinergic agonist and with short-term preincubation with the agonist in order to induce the desensitized state (8,13,14). The control experiments for this study were conducted as previously described (8-10) using pH 11.0 treated membranes which are devoid of acetylcholinesterase activity. The results of these control experiments are depicted in Fig. 6. The rate of ^{125}I-α-BTX-AcChR complex formation is only slightly faster than that in the presence of 1 μM carbamoylcholine (-Δ-) when the agonist and the toxin are added to the membranes together at time t=0, thus demonstrating that the AcChR has a low affinity for the AcChR has a low affinity for the agonist [K = 30 μM for carb (9)]. Following preincubation of the membranes with the same carb concentration for ten minutes prior to addition of the toxin, however, has a dramatic effect on the kinetics (-O-) since the receptor has been converted to the desensitized state which has a high affinity for the agonist [K ~ 50-100 nM (9)]. Preincubation of the membranes with carbamoylcholine (10^{-3}M) for twenty four hours was then conducted to determine whether such treatment had any effect on the properties described above for the control. Following the preincubation with the agonist the membranes were diluted by one hundred fold and centrifuged and this step was then repeated twice to bring the nominal concentration of the agonist to 10^{-9}M. Following this the kinetics of ^{125}I-α-BTX were repeated as in the control, i.e. with and without short-term preincubation with the agonist. The data in Fig. 7 were obtained. It was clear that within the precision of the method no discernible affects that differed significantly from those of the control were obtained. Thus long-term preincubation with a high concentration of carbamoylcholine has no measurable effect on the ability of the AcChR to interconvert between the initial resting state of low affinity for agonist to a state of high affinity (the desensitized state).

Stopped flow kinetics of thallium influx into vesicles that had been preincubated with 10^{-3}M carb for 24 hours (Fig. 9) were identical to the kinetics observed for the control (Fig. 8) demonstrating that the long term exposure to the agonist had no deleterious effect on the functional properties of the AcChR.

References:

1. Vandlen, R., Schmidt, J. and Raftery, M.A. (1976) J. Macromolec. Sci. A10, 73-109.
2. Duguid, J. and Raftery, M.A. (1973) Biochemistry 12, 3593-3597.
3. Raftery, M.A., Vandlen, R., Michaelson, D., Bode, J., Chao, Y., Reed, K., Deutsch, J. and Duguid, J. (1974) J. Supramolec. Str. 2, 252-260.
4. Elliot, J., Blanchard, S., Wu, W., Miller, J., Strader, C., Hartig, P., Moore, H.-P., Racs, J. and Raftery, M.A. (1980) Biochem. J. 185, 667-677.
5. Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349.
6. Laemmli, U.K. (1970) Nature 227, 159-212.
7. Mizuno, K., Nakamura, T., Takada, K., Sakakibara, S. and Matzu, H. (1987) Biochem. Biophys. Res. Commun. 144, 807-814.
8. Lee, T., Witzemann, V., Schimerlik, M. and Rafter, M.A. (1977) Arch. Biochem. Biophys. 183, 57-63.
9. Quast, U., Schimerlik, M., Lee, T., Witzemann, V., Blanchard, S. and Raftery, M.A. (1978) Biochemistry 17, 2045.
10. Moore, H.-P. and Raftery, M.A. (1979) Biochem. 18, 1907.
11. Dunn, S.M., Blanchard, S. and Raftery, M.A. (1980) Biochem. 19, 5645-5653.
12. Porath, J. (1973) J. Chromatcg. 86, 53-56.
13. Weber, M., David-Pfenty, T. and Changeux, J-P. (1975) Proc. Natl. Acad. Sci. USA 72, 3443-3448.
14. Weiland, G., Georgia, B., Lappi, S., Chignell, C. and Taylor, P. (1976) Molec. Pharmacol. 12, 1091-1100.
15. Moore, H-P. and Raftery, M.A. (1980) Proc. Natl. Acad. Sci. USA 77, 7409-7414.

Fig. 1. SDS-gel electrophoresis of AcChR incubated at 4°C for 48 hours. Lane 1,2 incubated AcChR; lanes 3,4 low and high molecular weight standards; lanes 5-7 increasing concentrations of control AcChR membranes.

Fig. 2. Chromatography of Lubrol extract of AcChR-enriched membranes on DEAE-cellulose (DE-52). Open symbols denote absorbance at 280 nm, filled symbols denote enzymatic activity.

Fig. 3. Affinity chromatography on arginine-Sepharose column, pooled fractions from the void volume of a Sephadex G-100 column were adsorbed onto the affinity column, washed extensively with buffer A (see experimental) and eluted with a linear gradient of buffer B (starting at Fraction 1 in this figure Open symbols denote protein (absorbance at 280 nm; closed symbols denote enzymatic activity).

Fig. 4. SDS-gel electrophoresis of protease through various purification steps. Lanes 1,2 low and high molecular weight standards; lane 3 membranes from crude membrane homogenate; lane 4, Lubrol extract of these membranes; lane 5, pooled protease active fractions from DE-52 chromatography; lane 6 AcChR-enriched membranes; lane 7, fractions emerging from the void volume of a Sephadex G-100 column; lanes 8,9, increasing concentrations of active fractions from an affinity column (see text).

Fig. 5. DEAE-52 column chromatograhy of a Lubrol extract of AcChR enriched membranes, using conditions described in Fig. 2 for elution of protease. Open symbols, ^{125}I-α-BTX binding; closed symbols, protease activity.

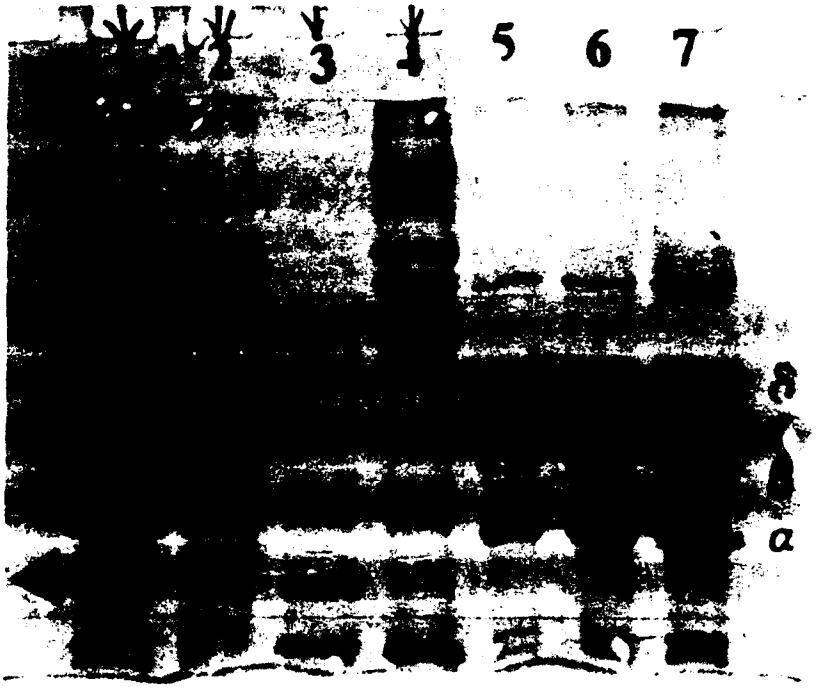
Fig. 6. Time dependent desensitization of AcChR-enriched membranes in the presence of carbamoylcholine (10^{06}M) in Ringers solution at 25°C. Receptor-[^{125}I]α-BTX complex formation was measured as a function of time. - - AcChR and [^{125}I]α-BTX only; -Δ- AcChR with carb and radiotoxin added simultaneously at t=0, -O- AcChR preincubated with carb for 10 min before addition of ^{125}I-α-BTX.

Fig. 7. Time-dependent desensitization of AcChR-enriched membranes after long-term incubation with carbamoylcholine (10^{-3}M, 24 hours). The carb was removed as described in the

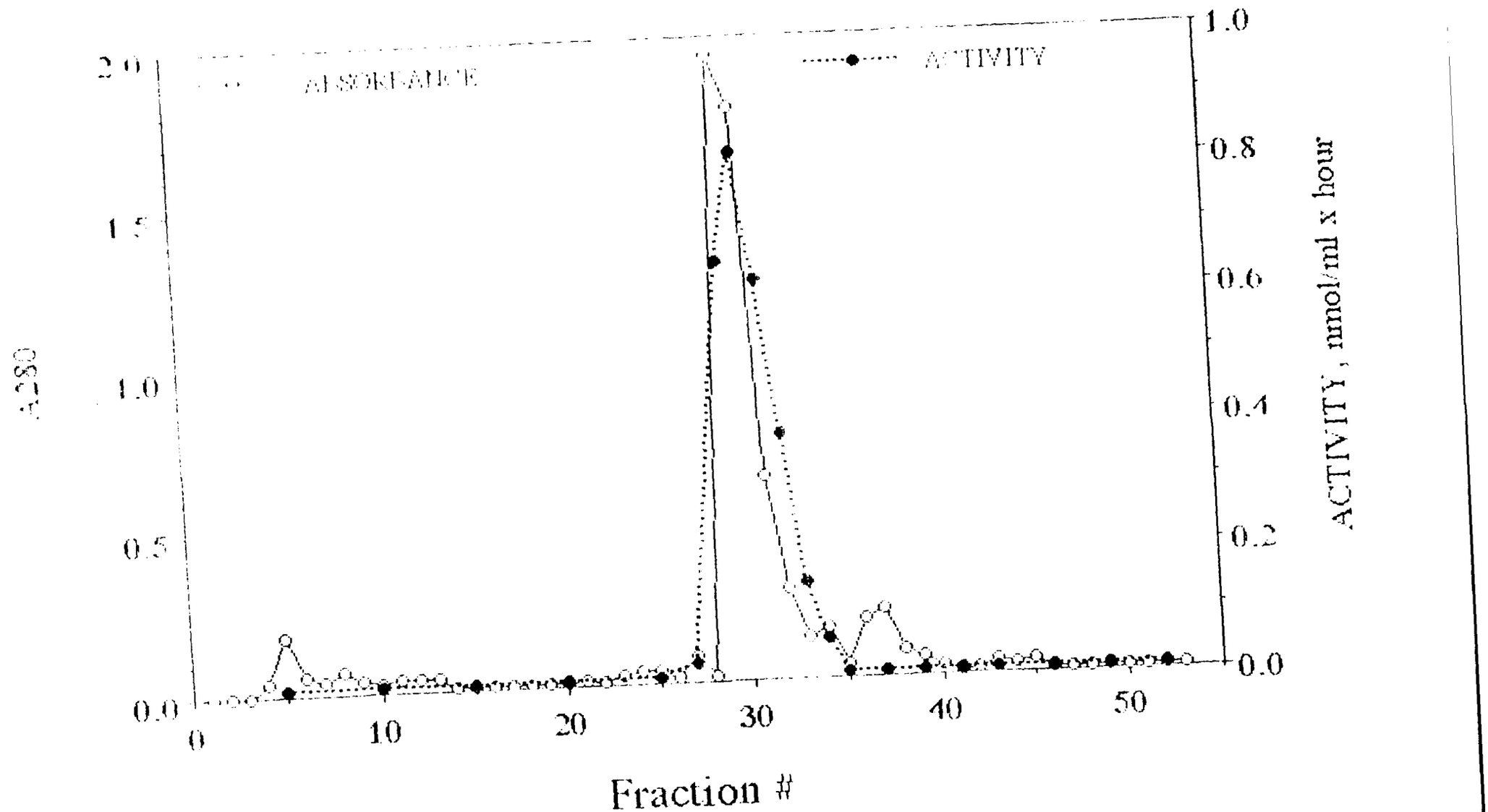
experimental section and the desensitization was performed exactly as for the control (Fig. 6). The symbols have the same meaning as in Fig. 6.

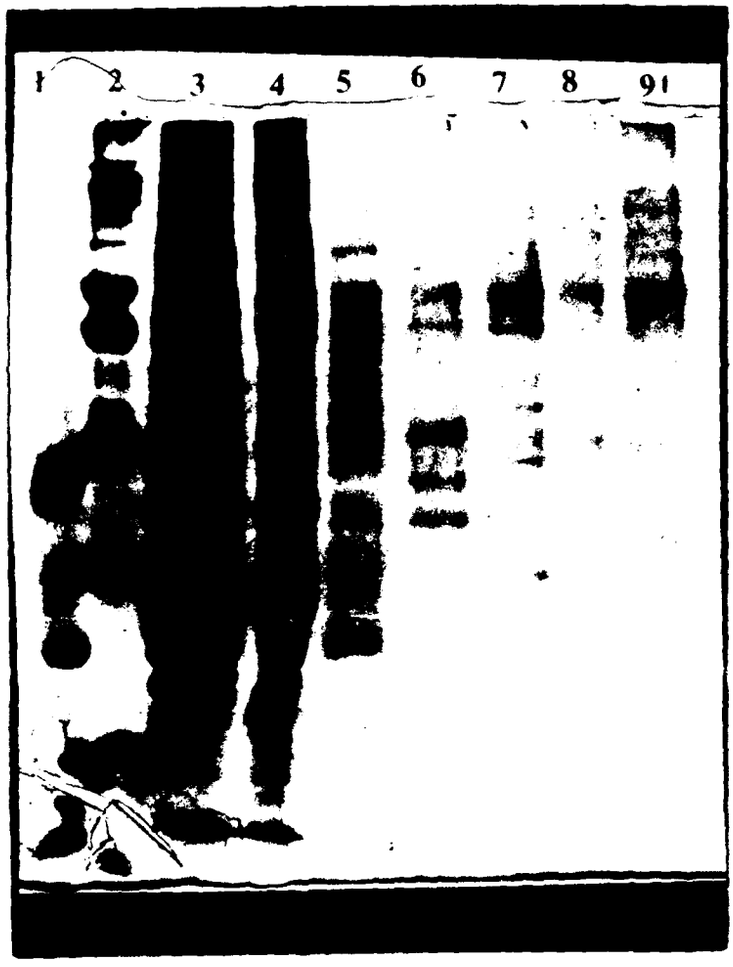
Fig. 8. Stopped-flow study of thallium influx into AcChR-enriched vesicles loaded with pyrenetetrasulfonic acid (tetra sodium salt). [AcChR] = 2 x 10^{-6}M, [carb] = 0.25 x 10^{-3}M. The observed rate (rate 1) = 46.94 sec^{-1} and the total amplitude of the fluorescence quench was 464 mV.

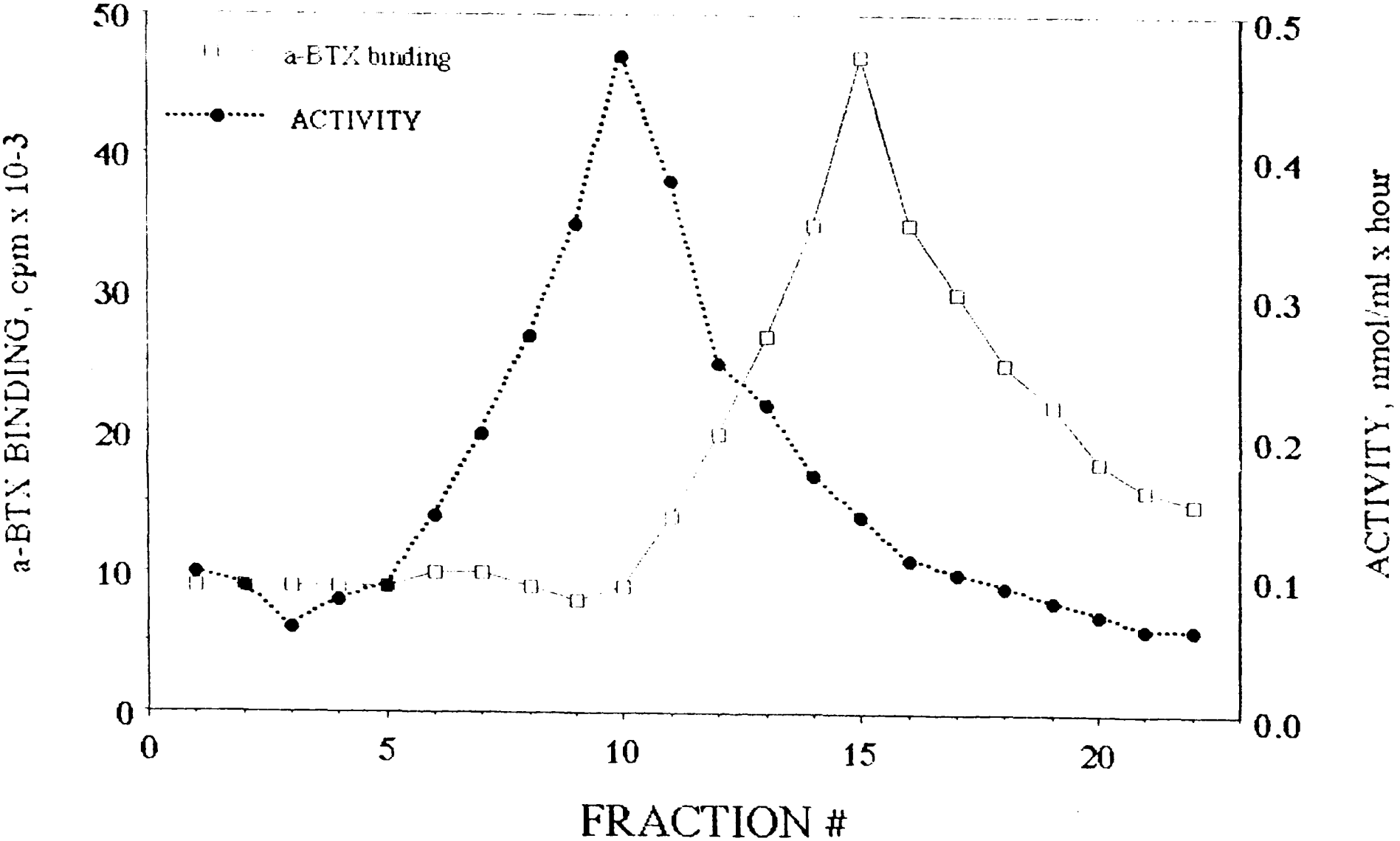
Fig. 9. Stopped flow study of thallium influx into AcChR-enriched vesicles loaded with pyrenetetrasulfonic acid (tetra sodium salt, following long-term incubation with carb (10^{-3}M) and its removal as described under experimental. Conditions were identical to those for the control (Fig. 8). The observed rate was 44.58 sec^{-1} and the total amplitude was 449 mV.

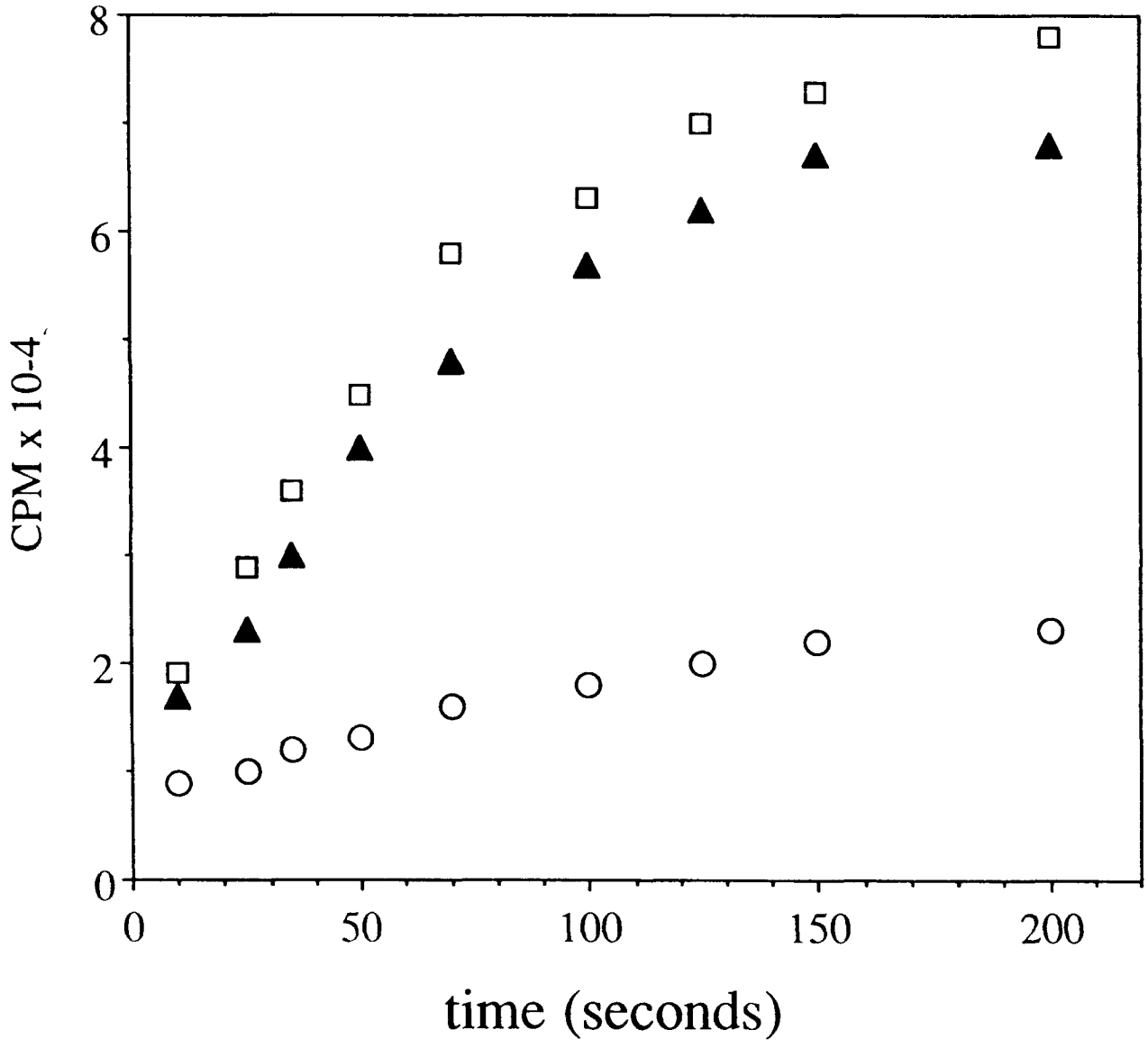


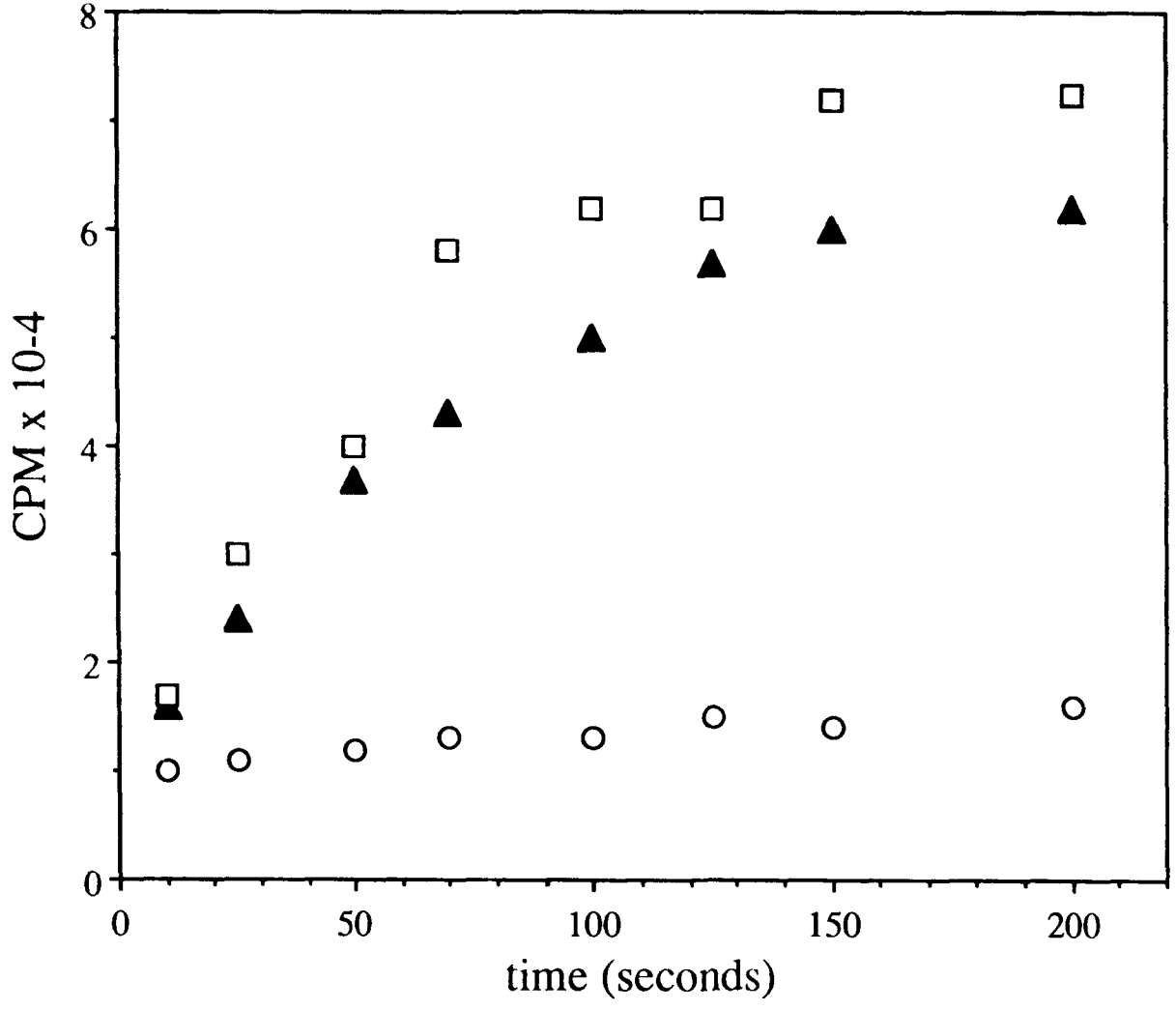
A280
0
50
100
150
ABSORBANCE
ACTIVITY
Fraction #
0
20
40
60
0
20
40
60
80
ACTIVITY, nmol/ml x hour

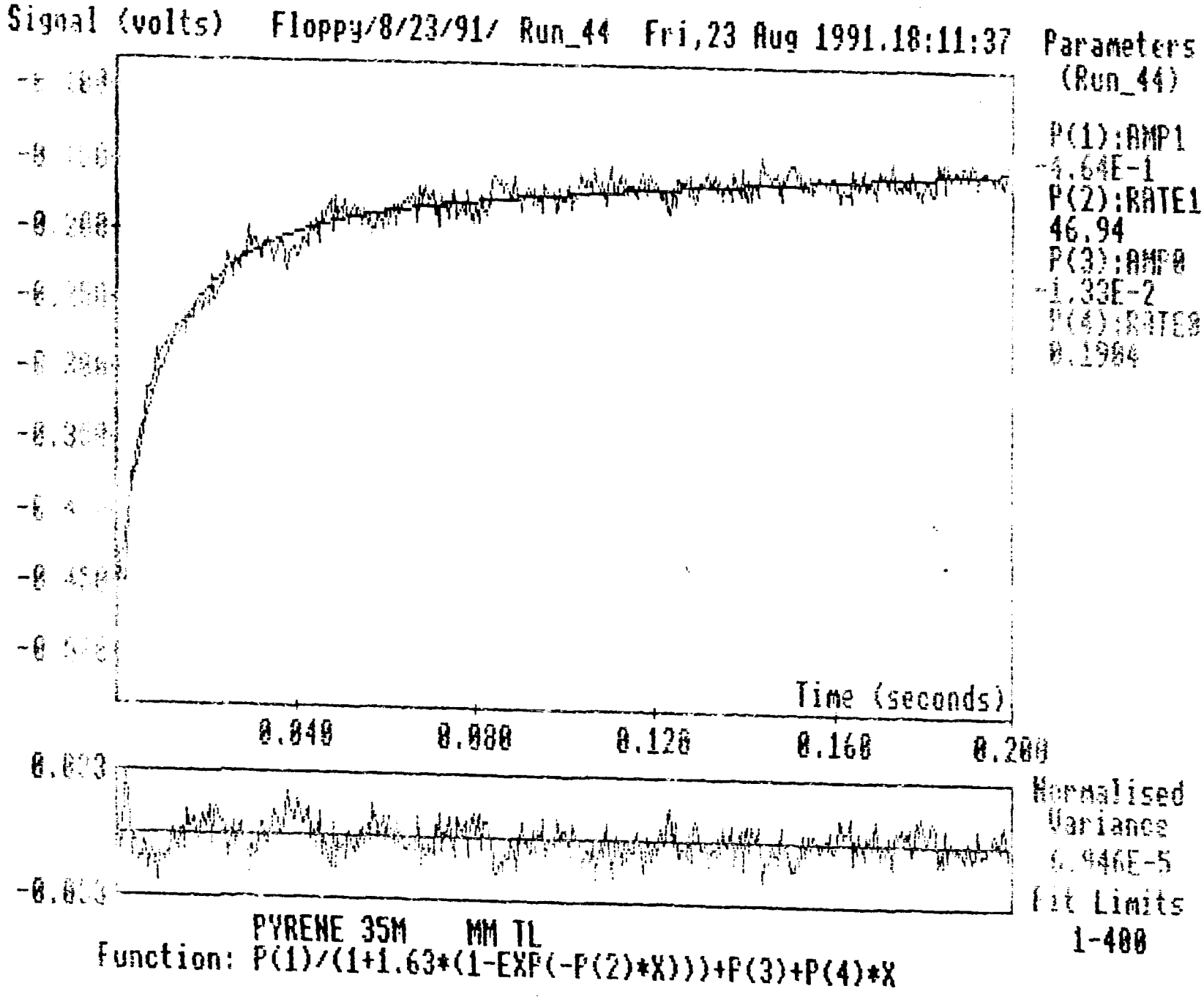

ABSORBANCE
ACTIVITY
A280
ACTIVITY, nmol/ml x hour
Fraction #
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50

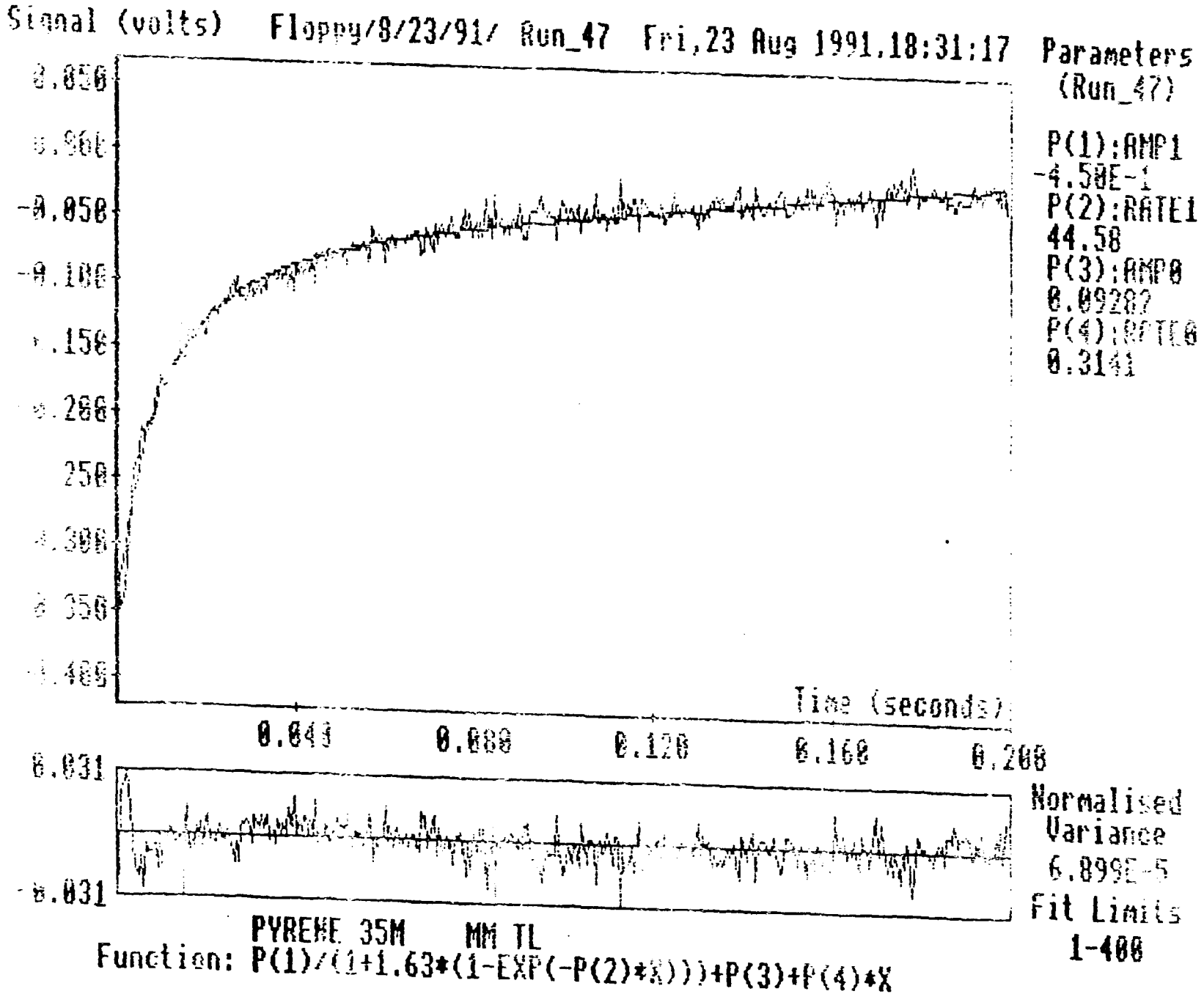



a-BTX binding
ACTIVITY
a-BTX BINDING, cpm x 10-3
ACTIVITY, nmol/ml x hour
FRACTION #
0
10
20
30
40
50
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20


8
6
4
2
0
CPM x 10-4
0
50
100
150
200
time (seconds)


8
6
4
2
0
CPM x 10-4
0
50
100
150
200
time (seconds)


Signal (volts) Floppy/8/23/91/ Run_44 Fri,23 Aug 1991.18:11:37
Parameters (Run_44)
P(1):AMP1
-9.64E-1
P(2):RATE1
46.94
P(3):AMP0
-1.33E-2
P(4):RATE0
0.1984
Time (seconds)
0.040
0.080
0.120
0.160
0.200
Normalised Variance
6.946E-5
Fit Limits
1-400
PYRENE 35M MM TL
Function: P(1)/(1+1.63*(1-EXP(-P(2)*X)))+P(3)+P(4)*X


Signal (volts) Floppy/8/23/91/ Run_47 Fri,23 Aug 1991.18:31:17
Parameters (Run_47)
P(1):AMP1
-4.50E-1
P(2):RATE1
44.58
P(3):AMP0
0.09287
P(4):RATE0
0.3141
-0.050
-0.100
-0.150
-0.200
-0.250
-0.300
-0.350
-0.400
Time (seconds)
0.040
0.080
0.120
0.160
0.200
0.031
-0.031
Normalised Variance
6.899E-5
Fit Limits
1-400
PYRENE 35M MM TL
Function: P(1)/(1+1.63*(1-EXP(-P(2)*X)))+P(3)+P(4)*X

NON EQUIVALENCE OF α-BUNGAROTOXIN BINDING SITES IN THE NATIVE NICOTINIC RECEPTOR MOLECULE+.

ABSTRACT

In the native, membrane bound form of the nicotinic acetylcholine receptor (M-AcChR) the two sites for the cholinergic antagonist α-bungarotoxin (α-BGT) have different binding properties. One of them has high affinity, and the M-AcChR/α-BGT complexes thus formed dissociate very slowly, similar to the complexes formed with detergent-solubilized AcChR (S-AcChR). The second site has much lower affinity ($K_D \approx 59 \pm 35$ nM) and forms quickly reversible complexes. The nondenaturing detergent Triton X-100 is known to solubilize the AcChR in a form unable, upon binding of cholinergic ligands, to open the ion channel and to become desensitized. Solubilization of the AcChR in Triton X-100 affects the binding properties of this second site and converts it to a high affinity, slowly reversible site. Prolonged incubation of M-AcChR at 4°C converts the low-affinity site to a high-affinity site similar to those observed in the presence of Triton X-100. Although the two sites have similar properties when the AcChR is solubilized in Triton X-100, their nonequivalence can be demonstrated by the effect on α-BGT binding of concanavalin A, which strongly reduces the association rate of one site only. The B_{max} of α-BGT to either Triton-solubilized AcChR or M-AcChR is not affected by the presence of concanavalin A. Occupancy of the high affinity, slowly reversible site in M-AcChR inhibits the Triton X-100 induced conversion to irreversibility of the second site. At difference with α- BGT, the long α-neurotoxin from Naja naja siamensis venom (α–NTX) binds with high affinity and in a very slowly reversible fashion to two sites in the M-AcChR (Conti-Tronconi & Raftery, 1986). We confirm here that Triton-solubilized AcChR or M-AcChR bind in a very slowly reversible fashion the same amount of α-NTX. In support of the contention that α-BGT binds "irreversibly" to one site only in the native M-AcChR, we found that when M-AcChR is saturated with radiolabelled α-BGT, addition of α-NTX markedly accelerates the dissociation of the bound α-BTX, presumably because occupancy of the second site by tightly bound α-NTX influences and decreases the affinity for α-BGT of the other site. The different properties of the two α-BGT binding sites in the native AcChR molecule support the possibility that these sites have different structural properties and that a sugar moiety is in close proximity to at least one such site.

INTRODUCTION

The nicotinic acetylcholine receptor (AcChR) of peripheral tissues such as Torpedo electroplax and mammalian muscle is a transmembrane protein formed by homologous subunits in a stoichiometry $\alpha_2\beta\gamma\delta$ [reviewed in Conti-Tronconi & Raftery (1982), McCarthy et al. (1986), and Maelicke (1987)]. Binding of cholinergic agonists to two low affinity sites, which operate in a somehow cooperative fashion, results in a fast conformational change and in activation of a cation channel contained in the AcChR molecule (Conti-Tronconi & Raftery, 1982; McCarthy et al., 1986). Ligand binding to two high-affinity sites causes a slow conformational change that results in a desensitized state, characterized by inability of the channel to open (Katz & Thesleff, 1957; Rang & Ritter, 1970 a,b). A major goal in the elucidation of AcChR function has been to define the ligand binding events leading to activation and inactivation of the cation channel. Two models for ligand binding mechanisms have been proposed.

In linear models [reviewed in Sine & Taylor (1980), Conti-Tronconi & Raftery (1982), Maelicke (1987)] activation and desensitization occur upon binding to the same two sites which undergo multiple changes in affinity as a consequence of ligand binding. Strong support to this model is the demonstration that each of the two α-subunits present in the AcChR molecule contains a high-affinity binding site for the affinity label bromoacetylcholine (Wolosin et al., 1980) and that the peptide cholinergic antagonist α-bungarotoxin (α-BGT), which also binds to two sites (reviewed in Conti-Tronconi & Raftery, 1982), recognizes the denatured α-subunit (Haggerty & Froehner 1981, Gershoni et al. 1983, Wilson et al. 1984). It has been therefore concluded that also the two sites for α-BGT are on the two α-subunits, and because α-BGT irreversibly inhibits AcChR activation, this has been construed as proof that activation and desensitization are controlled by the same two sites on the α-subunits.

A second model, a multiple site model, argues that separate low and high affinity sites regulate channel activation and receptor desensitization (Dunn et al. 1983, Dunn & Raftery 1982 a,b, Raftery et al. 1983). Binding of acetylcholine and carbamoylcholine cause a conformational change of the AcChR molecule which results in enhancement of the fluorescence of the fluorescent

probe, 4-[N-(iodoacetoxy) ethyl-N-methyl] amino-7-nitrobenz-2-oxa-1,3 diazole (IANBD) covalent attached to the AcChR molecule itself. This process occurs on a timescale compatible with the fast process leading to channel opening, (Dunn & Raftery 1982 a,b, Dunn et al. 1983) and the apparent dissociation constants for the cholinegic binding for different cholinergic agonists of the site monitored by the changes in IANBD fluorescence are identical to the apparent equilibrium constants for activation of monovalent cation flux (Dunn & Raftery 1982 a,b). It was therefore concluded that the enhancement in IANBD fluorescence monitored binding to cholinergic site(s) involved with activation of the ion channel, because it had the affinity for cholinergic ligands characteristic of activation of the resting state of the AcChR, and triggered a conformational change fast enough to be within the chain of events ultimately leading to channel opening (Dunn & Raftery, 1982a,b). The agonist-induced enhancement of IANBD fluorescence was seen under equilibrium conditions when, according to the linear model, the receptor should be desensitized. These observations suggest that low affinity binding sites exist that are separate from the high affinity site that regulates receptor desensitization. Further evidence to support this possibility comes from the observation that enhancement of IANBD fluorescence-induced by cholinergic agonists is also observed when both binding sites on the α-subunits are covalently modified by bromoacetylcholine (Conti-Tronconi et al. 1982; Dunn et al, 1983).

α-BGT interaction with the AcChR has been crucial for constructing models of its function. The long α-neurotoxins from the venom of Elapid snakes, like α-BGT from Bungarus multicinctus venom, and α-cobratoxin (α-NTX) from the venom of different Naja species form a family of peptide cholinergic antagonists which have been very useful for isolation and characterization of AcChRs from different peripheral tissues, like mammalian muscle and fish electric organ (rev. in Klett et al., 1973, Blanchard et al, 1979, Sine and Taylor, 1980, Wang and Schmidt, 198, Kang and Maelicke, 1980, Chang et al., 1984). These toxins bind specifically and with high affinity to the AcChR. They form complexes which dissociate very slowly (and cause an irreversible block the AcChR in functional assays. For these reasons, although their binding to the AcChR is actually not irreversible nor covalent, the binding of α-BGT and α-NTX to AcChR

is sometimes referred to as "irreversible". The kinetic studies focused on α-BGT binding to membrane bound AcChR yielded conflicting results, and the association kinetics of this toxin to M-AcChR have been reported to be monophasic (Blanchard et al. 1979; Lukas et al. 1981) or multiphasic (Leprince et al. 1981), and its dissociation has been shown to occur over several hours (Lukas et al. 1981) or days (Blanchard et al. 1979). Most studies on the properties of α-BGT binding to Torpedo AcChR have assumed the linear model and they have been carried out with detergent solubilized AcChR (reviewed in Conti-Tronconi & Raftery 1982 and Chang et al. 1984) or with AcChR-rich membranes isolated using protocols in which proteases were not inhibited (Lukas et al. 1981, Leprince et al. 1981). Under these circumstances, the two α-BGT sites have very similar binding properties and this has lent further credence to the notion of two identical binding sites, on identical domains. However, interaction with non-denaturing detergents drastically affects the binding properties of AcChR for cholinergic agonists (rev. in Conti-Tronconi & Raftery 1982) and (at least in mammalian brain) proteolysis affects the affinity of the AcChR for nicotine (Lippiello & Fernandes 1986). Because most studies on the interaction between α-BGT and Torpedo AcChR have been carried out under circumstances when artifactual changes in the binding properties may have been present because of the effects of detergents and proteases, we have investigated some binding properties of the two α-BGT binding sites in the non proteolized, membrane bound Torpedo AcChR (M-AcChR) and compared them with those of AcChR solubilized in the non denaturing detergent Triton X-100 (S-AcChR).

MATERIALS AND METHODS

Preparation of AcChR-rich membrane fragments. AcChR-rich membrane fragments were prepared from Torpedo californica electric organs (Elliot et al. 1980) and extracted at pH 11 to remove non receptor proteins (Neubig et al. 1979, Elliot et al. 1979). In this method Ca^{++} dependent proteases and proteases having sulphydryl going in the active site are fully inhibited, and the AcChR present in the membrane fragments thus obtained is intact. Upon SDS gel electrophoresis (Laemmli 1970) these preparations (M-AcChR) showed the four AcChR subunits

as the major protein components (Figure 1). The specific activity of these preparations (expressed as nmol of α-BGT binding sites/mg of protein), measured in the presence of Triton X-100, was 4-7 nmol/mg of protein, which is close to the maximum theoretical activity of pure AcChR (7.2 nmol/mg). The AcChR-rich membrane fragments isolated from Torpedo electric organ can spontaneously reseal to form microsacs. When this occurs, more than 95% of the closed vesicles thus formed are right-side-out (see below and Hartig and Raftery, 1979; Strader & Raftery, 1980; Froener, 1981; St. John et al., 1982; Conti-Tronconi et al., 1982). The M-AcChR preparations were stored at 4°C and used within one week. For some experiments, M-AcChR was left at 4°C for up to three weeks.

Purification and radiolabelling of snake toxins and calibration of ^{125}I-α-BGT. α-BGT was purified from Bungarus multicinctus venom (Biotoxins Inc., Fla.) and α-cobratoxin (α-NTX) was purified from Naja naja siamensis venom (Biotoxins Inc., Fla.) following the procedure of Ong & Brady (1974). Toxin purity was assessed by SDS gel electrophoresis (Laemmli 1970) using an exponential gradient of polyacrylamide (8-20%) and by amino-terminal amino acid sequencing using a gas-phase sequenator (Applied Biosystems). Only the known sequences of α-BGT or of α-NTX were found.The purified toxins were radiolabelled with ^{125}I using cloramine-T according to the procedure of Lindstrom et al. (1981), which yields monoiodinated toxins (Lindstrom et al., 198). The only modification was that after completion of the iodination reaction, any remaining cloramine T was reduced by adding an excess of sodium metabisulfite, and 25 µl of a saturated solution of potassium iodide were added. The iodinated protein was separated from the unreacted iodine and the other low molecular weight components by the use of a desalting column (Pierce, 5 ml of settled bed). The specific activity of ^{125}I-α-BGT was calibrated as described in Blanchard et al. (1979).

Assay of ^{125}I-α-BGT and ^{125}I-α-NTX binding. DEAE disk assay. The concentration of irreversible ^{125}I -α-BGT and ^{125}I-α-NTXbinding sites was measured by the DEAE disk assay of Schmidt & Raftery (1973). The disk assay was carried out either in the presence of 0.1% Triton X-100 during both the incubation of AcChR with the radiolabelled toxin and the washings, or without

any detergent. Previous experiments (Conti-Tronconi & Raftery 1986) showed that M-AcChR binds quantitatively to DEAE disks. In some experiments, after the incubation was carried out without detergent, the last washings were done with buffer containing 0.1% Triton X-100.

Assay of ^{125}I-α-BGT binding: Centrifugation assay. The total irreversible plus reversible binding site concentration was measured by a centrifugation assay. A constant amount of M-AcChR (32-50 nM) was incubated with increasing concentrations of ^{125}I-α-BGT (up to 300 nM) at room temperature for 60 minutes in 10 mM phosphate buffer, 50 nM NaCl, pH 7.2, in a total volume of 250 µl. Parallel control aliquots for nonspecific ^{125}I-α-BGT binding were prepared, preincubated with a 10-fold excess of unlabelled α-BGT, which was left in the mixture throughout the whole experiment. After incubation, 10 µl aliquots from each tube were counted for radioactivity and the remainder was centrifuged for at least 30 minutes at 8000 rpm in a Sorvall SHMT rotor. Three 10 µl aliquots of supernatant were counted for radioactivity. The amount of bound toxin was calculated from the difference between the control and the sample aliquots after centrifugation.

In another set of experiments, a centrifugation assay was carried out using the same conditions used for the disk assays, which were also carried out with or without Triton X-100 on the same membrane preparation. Increasing volumes of M-AcChR suspension (50 -70 nM) were incubated for 60 minutes at room temperature with a constant concentration of ^{125}I-α-BGT (500 µM in a final volume of 250µl). Parallel control aliquots for nonspecific ^{125}I-α-BGT were preincubated with 10-fold excess of unlabelled α-BGT, which was left in the mixture throughout the whole experiment. Both the sample and the control aliquots were processed as described above, and the α-BGT binding site concentration was obtained from the difference in the radioactivity remaining in the supernatant between the control and the sample.

Assessment of the effect of irreversibly bound α-BGT on the further binding to the reversible site. Aliquots of M-AcChR (approx. 100 µl of a 5 µM suspension) were preincubated with a 10-fold excess unlabelled α-BGT for 60 minutes at room temperature. The membrane fragments were washed free of unbound α-BGT by dilution to 30 ml and centrifugation in a

Sorvall SA 600 rotor at maximum speed for 45 minutes at 6°C. The pelleted membranes were resuspended and the wash was repeated a second time. A control aliquot of M-AcChR was incubated without α-BGT, then diluted and centrifuged as described above. The M-AcChR pellets were resuspended in the centrifugation assay buffer, diluted to the necessary concentrations in the appropriate buffer (see above) and used for either DEAE disk assays, carried out with and without Triton X-100, or for a centrifugation assay. In the centrifugation assay, 500 µl of a suspension of 50 nM M-AcChR were incubated with increasing amounts of ^{125}I- α -BGT for one hour at room temperature. The membranes containing the AcChR were pelleted by centrifugation for 45 minutes at 8000 rpm in a Sorvall SHMT rotor and counted for bound radioactivity. Control aliquots for nonspecific ^{125}I-α-BGT binding were preincubated with a 100-fold excess unlabelled α-BGT, which was left in the mixture throughout the experiment, and processed as described above.

Assessment of the effect of bound Concanavalin A on ^{125}I-α-BGT binding. The influence of bound Concanavalin A (Con A) on ^{125}I-α-BGT binding was assessed as follows. The effect on total ^{125}I-α-BGT binding to either S-AcChR or M-AcChR was tested by incubating for one hour at room temperature aliquots of M-AcChR (approximately 0.1 µM, measured as α-BGT binding sites) in 10mM Na phosphate buffer, pH 7.0, with a two-fold and 10-fold molar excess of Con A over the AcChR subunits (Con A concentration: 0.5 µM and 2.5 µM respectively) or without Con A. The α-BGT binding sites present in the Con A treated sample and on the untreated control sample were measured by DEAE disk assay, in the presence or in the absence of Triton X-100. In the case of the Con A treated samples the buffers used in the disk assays contained the same Con A concentration as during the preincubation. The final concentrations in the assay were 20-80 µM AcChR, 150 µM ^{125}I-α-BGT and 0.5 µM or 2.5 µM or 0 Con A.

To test the effect of bound Con A on the time course of ^{125}-α-BGT binding to S-AcChR, 20 µl aliquots of 3 µM M-AcChR (measured as α-BGT binding sites) in 10 mM Na phosphate buffer, pH 7.0, were incubated for 30 minutes at room temperature with a 10-fold molar excess of Con A, or without any Con A . The M-AcChR was solubilized and diluted to 1.25 nM using 10 nM Na phosphate buffer, pH 7.0, containing 0.1% Triton X-100 ("wash buffer") and 12.5 µM

Con A. A proper volume of a solution of ^{125}I-α-BGT in the same buffer was added. The final concentrations were: 1 nM S-AcChR, 2 nM ^{125}I-α-BGT, 10 μM or 0 Con A. The mixture was incubated at room temperature for two hours. In some experiments the incubation was prolonged up to twenty one hours. At different time intervals during the incubation 100 μl aliquots were pipetted in triplicate onto DEAE disks and the disks were washed three times in a large volume of wash buffer. In some experiments the wash buffer used for the washings contained 10 μM Con A. The amount of bound ^{125}I-α-BGT was measured in a Beckman gamma counter. The data obtained in the absence of Con A were fitted to a simple rate equation

$$\text{fmole bound} = \text{Amp}\ (1-\exp^{-k1_{appt}}) \qquad (1)$$

where Amp is the maximum-bound α-BGT.

The data obtained in the presence of Con A were fitted to the double exponential equation

$$\text{fmole bound} = 1/2\ \text{Amp}\ [(1-\exp^{-k1_{appt}}) + (1-\exp^{-k2_{appt}})] \qquad (2)$$

where Amp is the maximum-bound α-BGT.

Effect of α-NTX on the dissociation rate of α-BGT/AcChR complexes. Complexes between ^{125}I-α-BGT and M-AcChR were formed by incubating MAcChR (1-14 μM, measured as α-BGT binding sites, in 10mM phosphate buffer, pH 7.0) for 1 hour at 6° C with a two-fold excess of radiolabeled ^{125}I-α-BGT . The unreacted toxin was washed away by diluting the reaction mixture with 30 ml of the same buffer and pelleting the membranes at 19,000 rpm for 20 minutes in a SS-34 Sorvall rotor The washed complexes were resuspended in approximately 300 μl of the same buffer and aliquots of 0.1-0.8 nmol of α-BGT/M-AcChR complexes (measured as α-BGT binding sites) were incubated at room temperature with a 10-fold excess of either unlabelled α-BGT or α-NTX. The final M-AcChR concentration (measured as α-BGT binding sites) was 1 μM Aliquots of the mixtures were taken at time intervals and pipetted onto DEAE disks. The disks were washed as described by Schmidt & Raftery (1973) and the ^{125}I-α-BGT still bound was measured in a Beckman gamma counter. Because the dissociation rate of α-BGT/AcChR is very slow (reviewed in Conti-Tronconi & Raftery 1982), in preliminary experiments the half life of complexes between M-AcChR or S-AcChR and ^{125}I-α-BGT was determined by measuring the remaining ^{125}I-α-

BGT/AcChR complexes at several time intervals up to 40 hours. For the experiments aimed at determining the half life of the complexes in the presence of α-NTX which, as will be described below, greatly accelerates the dissociation of the complexes data points were collected from time 0 up to five hours. The dissociation rate of the ^{125}I-α-BGT/AcChR complexes was calculated from the amount of ^{125}I-α-BGT still bound to the AcChR at the various time intervals, using an exponential fitting program.

RESULTS

Measurements of irre :rsible ^{125}I-α-BGT binding sites by DEAE disk assay. In the disk assay of Schmidt & Raftery (1973), as well as in all the other assays which involve slow washing, gradient centrifugation or chromatographic separation of the bound from the free toxin, only irreversible or slowly reversible complexes of α-BGT with AcChR are measured. Table 1 reports the results obtained with seven typical preparations in the presence and in the absence of Triton X-100. Consistently M-AcChR irreversibly bound approximately 50% of the toxin bound by S-AcChR.

Occasionally (2 out of 13 experiments) similar numbers of sites were present in the presence or absence of Triton. SDS-PAGE analysis of the membranes used for these experiments revealed a peptide pattern consistent with subunit proteolysis. Therefore we tested the effects of endogenous proteolysis. M-AcChR which, when freshly prepared, had half as many irreversible binding sites as in the presence of Triton X-100, was left at 6°C for eighteen days and tested again. The number of irreversible ^{125}I-α-BGT binding sites of M-AcChR increased to 70% of those found in the presence of Triton X-100.

Measurement of irreversible ^{125}I-α-NTX binding sites by DEAE disk assay. The amount of ^{125}I-α-NTX bound by M-AcChR and S-AcChR, measured by DEAE disk assay was the same, irrespective of the presence of Triton X-100 during the incubation and the washings. Table 2 reports the results obtained with five different AcChR preparations. For one preparation (#4) the assays were carried out in two different experiments, with identical results. The values are expressed as cpm/μl of M-AcChR suspension (or S-AcChR solution) instead of μmoles/ml

because of difficulties encountered in the calibration of radiolabelled α-NTX. The difficulties to assess the exact specific activity of ^{125}I-α-NTX, and as a consequence to measure the molar ratio of its binding to M-AcChR and S-AcChR, were not a problem because the stoichiometry of the very slowly reversible binding of α-NTX to M-AcChR, is known, from direct measurements by quantitative aminoterminal sequencing of the stoichiometry of the peptides present in the α-NTX/M-AcChR complexes after extensive washings, and it is two molecules of α-NTX for one molecule of AcChR (Conti-Tronconi & Raftery 1986). α-NTX therefore binds to both S-AcChR and M-AcChR with the same stoichiometry, i.e. two sites on each AcChR molecule, and binding to both sites is "irreversible".

Measurement of total ^{125}I-α-BGT binding sites by centrifugation assay. Figure 2 reports the results of toxin binding assays where increasing volumes of 66 nM AcChR dissolved in Triton X-100 (S-AcChR, curve A) or not (M-AcChR, curves B and C) were incubated for 60 minutes at room temperature with an excess (approximately 500 nM) ^{125}I-α-BGT. For samples A and B the amount of bound ^{125}I-α-BGT was measured by DEAE disks, for samples C by centrifugation assay. The values obtained for S-AcChR by disk assay, and for M-AcChR by centrifugation assay are identical while only half as many α-BGT binding sites were detected for M-AcChR by the disk assay (curve B). Therefore, one of the two α-BGT sites present on M-AcChR is not irreversible and can be detected only by the centrifugation assay.

The dose-dependency of α-BGT binding to this reversible site was investigated. Samples containing a constant concentration of M-AcChR (30-50nM) were incubated with increasing concentrations of ^{125}I-α-BGT. The amount of bound ^{125}I-α-BGT was measured by centrifugation assay. The results of a typical experiment are reported in Figure 3. In this experiment 10.74 pmol of M-AcChR (measured as α-BGT binding sites in the presence of Triton X-100) in 250 µl were incubated for 60 minutes at room temperature with up to 225 nM α-BGT. The number of binding sites of this membrane preparation measured by disk assay without Triton X-100 was 50% of those measured in the presence of the detergent (10 nmol/ml and 19.3 nmol/ml respectively). In the centrifugation assay, the amount of bound ^{125}I-α-BGT increases linearly up

to the point where the ^{125}I-α-BGT concentration is approximately equivalent to half of the total concentration of binding sites (50% of 10.74 pmoles in 250 µl, i.e. 21.5 nM), yielding a titration curve of the high affinity sites. The slope then decreases, producing a plateau approximately at the expected concentration of the irreversible sites, and the binding reaches saturation at the concentration of the sites measured for S-AcChR. The biphasic behavior shown in Figure 3 was evident in most but not all experiments and it is probably due to the different association rates of the two sites. The reversible site seems to have a slower association rate and one hour incubation may not always be enough to reach equilibrium at the lower toxin concentrations.

An approximate estimate of the affinity of the reversible site was obtained by subtracting from both the total and the bound ^{125}I-α-BGT the component due to binding to the first irreversible site, and calculating from the plots thus obtained the concentration of ^{125}I-α-BGT necessary to achieve half-saturation. The values obtained in six different experiments were 80nM, 75 nM, 50 nM, 18 nM, 108 nM and 23 nM (average 59 ± 35 nM).

Effects of occupancy of the high affinity site on binding to the second site. M-AcChR was preincubated either in the 10-fold excess of unlabelled α-BGT or without any toxin and, after removal of the unreacted toxin, disk assays were performed on both samples, in the absence and in the presence of Triton X-100. The disk assay carried out on the untreated sample in the absence of any detergent revealed 50% of the sites measured after Triton X-100 solubilization. No ^{125}I-α-BGT bound to the unlabelled toxin-treated samples, even after Triton X-100 solubilization. When the presence of residual reversible binding sites was measured by centrifugation assay, the unlabelled toxin treated samples still bound approximately 50% of the ^{125}I-toxin bound by the control, untreated samples (Figure 4).

Effects of bound Con A on α-BGT binding. In order to differentiate between the two α-BGT sites on S-AcChR, we investigated the effect of bound ConA on ^{125}I-α-BGT binding. In four different experiments, preincubation with up to 2.5 nM Con A did not change the total amount of ^{125}I-α-BGT bound to S-AcChR (Table 3). Bound ConA did not reduce the amount of ^{125}I-α-BGT irreversibly bound to M-AcChR (Table 3). The effect on the rate of association of ^{125}I-α-

BGT to S-AcChR was then investigated using much lower concentrations of both S-AcChR and ^{125}I-α-BGT (1nM and 2nM respectively, compared with 80 nM and 150 nM in the disk assay), so that the binding reaction was complete after approximately 60 minutes. In Figure 5 the results of a typical experiment are reported, and fitted, using an exponential fitting program, to a single exponential in the case of the binding isotherm obtained in the absence of Con A (triangles in Figure 6), and to a double exponential equation in the case of the binding isotherm obtained in the presence of 10 μM Con A (circles in Figure 6), as described in the "Methods" section. In the absence of Con A the calculated maximum amount of bound α-BGT was 37.5 ± 0.58 fmoles, with a rate of association of 0.114 ± 0.005 min^{-1}. In the presence of Con A the calculated maximum amount of bound α-BGT was 30.3 ± 6.69 fmoles and the association rates were 0.130± 0.040 min^{-1} for a fast associating site accounting for 50% of the binding and 0.004± 0.005 min^{-1} for a slowly associating site, accounting for the remaining binding. In two experiments the incubation was continued up to twenty one hours, and as expected it was found that the sample incubated with ConA had reached 97% and 105% of the values obtained in the absence of ConA respectively.

Effect of α-NTX on the dissociation rate of α-BGT/AcChR complexes. The basal dissociation rate of the irreversible complexes between M-AcChR and ^{125}I-α-BGT was measured in the presence of an excess of unlabelled α-BGT for incubation periods of up to 48 hours. The half life of the complexes was found to be > 150 hours, in good agreement with the accepted notion that this toxin forms very slowly reversible complexes (Blanchard et al. 1979). When unlabelled α-NTX was present, the dissociation rate was markedly enhanced, and the half life was reduced to 9.06 ± 1.60 hours (n = 3). Figure 7 reports the results of a typical experiment.

DISCUSSION

In this study we demonstrate that the two α-BGT binding sites on the native AcChR molecule have substantially different binding properties. One site has properties similar to those found in detergent solubilized AcChR and it can be measured by assays which use slow

procedures to separate the bound from the free toxin. The second site binds α-BGT with much lower affinity (half-saturating concentration approximately 60 nM) and in a reversible fashion and can be revealed by centrifugation binding assays. Triton X-100 influences the binding properties of this second site and converts it to high affinity and irreversibility. The occurrence in the native AcChR molecule of only one binding site for α-BGT binds with high affinity and irreversibility does not conflict with the irreversible block caused by α-BGT on electroplax and muscle AcChR, because it has been demonstrated that occupancy of one site only by α-NTX (Sine & Taylor 1980,1981) or by the cyclic diterpenoid coral toxin lophotoxin (Culver et al. 1984) is sufficient to render the receptor nonfunctional.

The present data confirm and explain a previous report of Chang et al. (1984) in which twice as many sites for α-BGT were found when the binding assay was carried out in the presence of Lubrol. These authors proposed an "unmasking" effect of the detergent. In view of our findings, their results should be explained by inability of their assay to reveal reversible binding.

An effect of non-ionic detergent treatment on the binding properties of AcChR for small cholinergic ligands (acetylcholine, carbamoylcholine, etc.), consisting of an increase in the affinity similar to that induced by desensitization, has been described (reviewed in Sugiyama & Changeux 1975, Raftery et al. 1976). Also the interconversion of affinity states induced by agonists is lost upon dissolution of the membranes in neutral detergents or in sodium cholate (Sugiyama & Changeux 1975), unless exogenous lipids are present (Heidmann et al. 1980). Furthermore, disruption of AcChR-membrane lipid interactions by general anesthetics such as halothane, chloroform, or diethyl ether results in an enhanced rate of conversion of the AcChR to a state(s) of high affinity for carbamoylcholine (Young et al. 1978). Also the effect of aliphatic alcohols as non-competitive blockers of the AcChR seems to be due to a stabilization of the AcChR in a high affinity, desensitized state similar to that stabilized by agonists, probably caused by nonspecific partitioning of the alcohols into membrane lipid or by their interactions with the receptor/lipid interface (Heidmann et al. 1983, Boyd & Cohen 1984). It is therefore not surprising that the

disruption of the AcChR/membrane interaction induced by Triton X-100 may have a similar effect of inducing and stabilizing a high affinity conformation.

When membrane fragments were subjected to a long incubation at 4°-6°C and occasionally in freshly purified membranes, the number of high affinity binding sites was the same as in the presence of detergent. This could be due either to a detergent-like effect of the lysophospholipids produced by the saponifying effect of alkaline treatment, or to the action of an unknown endogenous enzymatic system, able to influence the affinity of α-BGT binding site(s). The finding that, on the rare occasions when newly prepared membranes had the same number of sites as in the presence of detergent, the SDS-PAGE pattern of their constituent subunits showed evidence of proteolysis argues in favor of the latter possibility. In addition, Chang et al. (1984) reported that from the same tissue they could obtain different membrane fractions, some of which displayed a different amount of binding sites for α-BGT in the presence or absence of Lubrol while other fractions did not, or they did so to a lesser extent. It is therefore conceivable that different binding states of native AchChR could exist, possibly due to endogenous proteolytic sytems able to modify the properties of the low affinity sites. Although exogenous proteolytic treatment does not cause obvious changes in AcChR function (Conti-Tronconi et al. 1982), proteases affect several structural properties of the native AcChR, including i) conversion of dimers to monomers (Conti-Tronconi et al. 1982), ii) modest but significant changes of the sedimentation coefficient of the monomers (Conti-Tronconi et al. 1982), iii) subtle morphological changes of negatively stained AcChR molecules (Lindstrom et al. 1980) and iv) induction of a higher degree of symmetry of the arrangement of AcChR molecules in membrane fragments (Bon et al. 1984).

In spite of the wealth of information on the AcChR yielded by the use of snake α-neurotoxins, and α-BGT in particular, no general agreement exists on their mechanisms of interaction with the AcChR. In addition to the discrepancies which may arise from different forms of iodinated α-BGT which may bind to AcChR with different kinetics (Wang & Schmidt 1980, Lukas et al. 1981), several of the reported disagreements may be reconciled by the results reported

here. For example, Blanchard et al. (1979) who used non proteolyzed M-AcChR and a DEAE disk assay found only one class of non-interacting sites. They probably measured only the one irreversible site present on intact M-AcChR. These authors found for purified S-AcChR binding kinetics of α-BGT faster than M-AcChR and biphasic, consistent with the presence of two different high affinity sites. On the other hand, Leprince et al. (1981) used assays able to detect irreversible α-BGT binding to membranes enriched in AcChR of unknown specific activity, which therefore may contain different, uncontrolled cellular and enzymatic components. The membranes they used were prepared following a protocol (Sobel et al. 1977) which does not inhibit protease activity, and which is known to yield proteolyzed AcChR. These authors may therefore have detected the binding to a mixture of partially proteolyzed AcChR forms, some AcChR molecules having acquired irreversibility of the second binding site because of the protease action. In this respect, Chang et al. (1984) found heterogeneity for α-BGT binding of different AcChR-rich membrane fractions prepared by the same method (Sobel et al. 1977). Some such fractions displayed a doubling of α-BGT binding sites after detergent treatment, while other fractions had the same number of binding sites before and after detergent treatment. In the same vein, the study of Lukas et al. (1981) was also carried out with AcChR rich membranes of low specific activity (100 pmol of α-toxin sites/mg of protein, i.e. only 1.39% of the specific activity of pure AcChR), prepared by a method (Hazelbauer & Changeux 1974) which does not inhibit proteases. These authors used a centrifugation assay able to detect both irreversible and reversible site, and they found monophasic kinetics. In view of the non-purified, proteolyzed M-AcChR they used it is difficult to interpret their results. In favor of a major proteolytic degradation of the AcChR, able to drastically affect the AcChR binding properties, is the unusually fast dissociation of the α-BGT/AcChR complexes, which occurred over hours and which was accelerated by the presence of unlabelled α-BGT (Lukas et al. 1981). In several other studies (reviewed in Conti-Tronconi & Raftery 1986 and Maelicke 1987), including the present one, the measured half-life of the irreversible complexes between ^{125}I-α-BGT was at least one order of magnitude longer, and was not obviously influenced by the presence of unlabelled α-BGT.

After saturation of the irreversible site of M-AcChR and washing away the unreacted toxin, one might expect that, upon Triton X-100 solubilization, α-BGT would bind irreversibly to the second site, which should still be free. On the other hand, it is known that after preincubation with unlabelled toxin and washing, no further irreversible binding of ^{125}I-α-BGT can be detected after Triton X-100 solubilization, a finding which has been used to demonstrate that Torpedo membrane fragments which spontaneously reseal form in more than 95% of cases right-side out microsacs (Hartig & Raftery 1979; Strader & Raftery, 1980; St. John et al., 1981). This dilemma is reconciled by the finding that occupancy by α-BGT of the irreversible site of M-AcChR inhibits the conversion of the second site to irreversibility upon Triton X-100 solubilization. The second site is however free and available for reversible ^{125}I-α-BGT binding, as shown in Figure 4.

Triton X-100 solubilization makes the binding properties of the two sites for α-BTX more similar, but they can still be distinguished by their slightly different kinetic properties in both Torpedo electric tissue (Blanchard et al. 1979) and skeletal muscle (Wang & Schmidt 1980). The presence of bound Con A further differentiates between the two sites on S-AcChR, because it drastically affects the association rate of α-BGT to one site (Figure 5), without affecting the total bound α-BTX. Con A has been shown to inhibit 40% of the α-BTX binding to S-AcChR from Torpedo marmorata (Wonnacott et al. 1980). The results of that study are in excellent agreement with our data, because Wonnacott et al. (1980) used low concentrations of α-BGT and AcChR (below 1.4 μM) and a relatively short incubation (90 minutes). Under these circumstances only one site is fully saturated. Under similar circumstances, (e.g. 90 minute incubation, 2 μM α-BGT, see Figure 5) we also had only approximately 60% of the binding obtained in the absence of Con A.

The drastic effect of Con A on the association rate of α-BGT to one site raises the possibility that in Torpedo AcChR a sugar moiety is in close proximity to such site, and that the second sites either is close to a different carbohydrate chain unable to bind Con A or does not have nearby sugar domains at all. These considerations have interesting ramifications regarding both the sequence segments which form the two sites and their subunit location. If the two α–BGT

sites are on the two α subunits of a Torpedo AcChR oligomer, their differential sensitivity to the presence of bound Con A would be well explain by previous reports that the two Torpedo α subunits are glycosylated to a different extent (Raftery et al. 1983, Lindstrom et al. 1983, Ratnam et al. 1986, Hall et al. 1983 and Conti-Tronconi et al. 1984). On the other hand in another study it was suggested that the carbohydrate parts of the two α subunits in the AcChR monomer are identical (Nomoto et al. 1986). If this is indeed the case, it is more difficult to explain the effect of Con A on one site only if both sites are on α subunits, unless their different microenvironment, i.e. their different flanking domains, allows Con A binding to one α subunit only. Alternatively one could speculate that the lower affinity site is on a different subunit, and because of its intrinsic lower affinity its binding cannot be detected after AcChR denaturation, while α-BGT binding to the α subunit is still demonstrable (Haggerty & Froener 1981; Gershoni et al. 1983; Wilson et al. 1984).

A direct demonstration that the two binding sites for snake α- neurotoxins are structurally different has been attained by studying the binding of derivatives of Naja aigrricollis toxin α, spin labelled on a single amino group, by Electron Spin Resonance (ESR) (Rousselet et al. 1984). It was found that the derivatives labelled at residue Lys^{47}, and Lys^{51} which is known to be directly involved in the interaction with AcChR gave, upon binding to AcChR, complex ESR signals which could be interpreted as indicating the existence of two physically different binding site.

At difference with α-BGT, α-NTX binds in a similar, semi-irreversible fashion to both sites, in both S-AcChR and M-AcChR. The present data confirm previous studies showing that i) α-NTX binds "irreversibly" to Torpedo AcChR with a stoichiometry of two toxin molecules to one AcChR molecule both in M-AcChR (Conti-Tronconi & Raftery 1986) and in S-AcChR (Conti-Tronconi & Raftery, unpublished); ii) when the binding to Torpedo postsynaptic membranes of either α-BGT or α-NTX was assayed by fluorescence titration methods which measure both irreversible and reversible binding, the same number of sites two for each AcChR molecule was demonstrated for both toxins (Neubig & Cohen 1979); iii) in kinetic studies on the interaction of fluorescein isothiocyanate labeled α-NTX to Torpedo membrane bound AcChR (Chang et al.

1984) or to solubilized Electrophorus AcChR (Kang & Maelicke 1980) the association was a single, bimolecular process, with a homogeneous class of binding sites; iv) in the muscle cell line BC3H1, α–NTX binds randomly to either of two sites, in spite of their intrinsic different binding properties for other cholinergic antagonists, like d-tubocurarine (Sine & Taylor 1980,1981, Culver et al. 1984).

The ability of α-NTX to bind in a very slowly reversible fashion to both sites, and to substitute reversibly bound α-BGT, explains the accelerated dissociation of α-BGT from its irreversible site (Figure 6). As a result of α-NTX binding to the second site, a conformational change must occurr, which modifies the binding properties of the other site, and α-BGT is more quickly released. An accelerated dissociation of α-BGT/M-AcChR complexes can be induced by the α-neurotoxin from Dendroaspis viridis venom (α-DTX) , which binds to four sites on the AcChR molecule (Conti-Tronconi & Raftery 1986). The dissociation of complexes between certain brain AcChRs and α-BGT is accelerated by high concentrations of small cholinergic ligands (Wang et al. 1978) presumably by a similar mechanism. Small cholinergic ligands can also accelerate the dissociation of α-NTX from solubilized Electrophorus AcChR (Kang & Maelicke 1980), in a manner dependent from the concentration of cholinergic ligand present (Maelicke et al., 1977, Kang & Maelicke, 1980). The dissociation rate of α-NTX/AcChR complexes can be accelerated several hundred-fold above its intrinsic value by additio nof high concentratiosn of cholinergic ligands (Kang & Maelicke, 1980). This effect may explain the ability of small cholinergic ligands to competitively elute solubilized AcChR from α-NTX affinity resin, within a faster time course than it would be expected from the slow dissociation rate of α-NTX/AcChR complexes. The effect of the cholinergic ligands on the dissociation of α-NTX/AcChR complexes may be due either to association with a subsite partially overlapping the α-NTX site, therefore destabilizing α-NTX binding, or to association with a site(s) not recognized by α-NTX, like the two extra sites of α-DTX.

Cholinergic antagonists exist which bind irreversibly to as many as four sites (α-DTX, Conti-Tronconi & Raftery 1986), or as few as one site (lophotoxin, Culver et al.1984) on the

AcChR molecule. Evidence from spectroscopic studies on cholinergic agonists binding to Torpedo AcChR whose two α subunit sites were blocked by covalently attached bromoacetylcholine suggested multiple sites, possibly four, exist on the AcChR molecule, and that activation and desensitization could be independent, parallel processes, triggered by ligand binding to different sites or subsites (Dunn et al. 1983). All of this suggests that a network of interacting binding sites may exist on the AcChR molecule, possibly as many as five, on the homologous domains of the AcChR, characterized by different binding properties for the different agonists and antagonists. The final functional effects of ligand binding would therefore result from the temporal summation of the single effects caused by the interplay of the differeny activated sites. In this respect irreversible ligands like lophotoxin, α-BGT, α -NTX and α-DTX, which bind to an increasing number of irreversible and reversible sites, elegantly demonstrate the spectrum of possibilities that one needs to confront when studying ligand binding to the nicotinic receptor molecule.

REFERENCES

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H.-P.H. & Raftery, M. A. (1979) Biochemistry 18: 1875-1883.

Bon, F., Lebrun, E., Gomel, J., Van Rapenbusch, R., Cartand, J., Popst, J. L. & Changeux, J. P. (1984) J. Mol. Biol. 176: 205-237

Boyd, N. D. & Cohen, J. B. (1984) Biochemistry 23: 4023-4033.

Cahill, S. & Schmidt, J. (1984) Biochem. Biophys. Res. Commun. 122: 602-608.

Chang, R. S. L., Potter, L. T. & Smith, I. S. (1977) Tissue Cell 9: 623-644.

Chang, H. W., Bock, E. & Neumann, E. (1984) Biochemistry 23: 4546-4556.

Conti-Tronconi, B. M. & Raftery, M. A. (1982) Ann. Rev. Biochem. 51: 491-530.

Conti-Tronconi, B. M., Dunn, S. M. J. & Raftery, M. A. (1982a) Biochem. Biophys. Res. Commun. 107:123-129.

Conti-Tronconi, B. M., Dunn, S. M. J. & Raftery, M. A. (1982b) Biochemistry 21: 893-899.

Conti-Tronconi, B. M. Hunkapiller, M. W. & Raftery, M. A. (1984) Proc. Natl. Acad. Sci. 81: 2631-2634.

Conti-Tronconi, B. M. & Raftery, M. A. (1986) Proc. Natl. Acad. Sci. USA 83: 6646-6650.

Culver, P., Fenical, W. & Taylor, P. (1984) J. Biol. Chem. 259: 3763-3770.

Damle, V. N., McLaughlin, M. & Karlin, A. (1978) Biochem. Biophys. Res. Commun. 84, 845-851.

Dunn, S. M. J. & Raftery, M. A. (1982a) Proc. Natl. Acad. Sci. USA 79: 6757-6761.

Dunn, S. M. J. & Raftery, M. A. (1982b) Biochemistry 21: 6264-6272.

Dunn, S. M. J., Conti-Tronconi, B. M. & Raftery, M. A. (1983) Biochemistry 22: 2512-2518.

Elliot, J. K, Dunn, S. M. J., Blanchard, S. & Raftery, M. A. (1979) Proc. Natl. Acad. Sci. USA 76: 2576-2580.

Elliot, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J. & Raftery, M. A. (1980) Biochem. J. 185: 667-677.

Froehner, S. (1981) Biochemistry 20:4905-4915.

Gershoni, J. M., Hawrot, E. & Lentz, T. L. (1983) Proc. Natl. Acad. Sci. USA 80: 4973-4977.

Haggerty, J. G. & Froehner, S. C. (1981) J. Biol. Chem. 256: 8294-8297.

Hall, J. W., Roisin, M. P. Gy, Y. & Gorin, P. D. (1983) Cold Spring Harb. Symp. Quant. Biol. 48: 101-108.

Hartig, P. & Raftery, M. A. (1979) Biochemistry 18: 1146-1150.

Hazelbauer, R. P. & Changeux, J.-P. (1974) Proc. Natl. Acad. Sci. USA 71: 1479-1483.

Heidmann, T., Sobel, A., Popot, J.-L. & Changeux, J.-P. (1980) Eur. J. Biochem. 110: 35-55.

Heidmann, T., Oswald, R. & Changeux, J.-P. (1983) Biochemistry 22: 3112-3127.

James, R. W., Kato, A. C., Rey, M.-J. & Fulpus, B. W. (1980) North-Holland Biomedical Press 120: 145-148.

Kang, S. & Maelicke, A. (1980) J. Biol. Chem. 255: 7326-7332.

Kao, P. N. & Karlin, A. (1986) J. Biol. Chem. 261: 8085-8088.

Katz, B. & Thesleff, S. (1957) J. Physiol. 138: 63-80.

Klett, R.P., Fulpius, B.W., Cooper, D., Smith, M., Reich, E. and Possani, L.D. (1973) J. Biol. Chem. 248:6841-6853.

Laemmli, U. K. (1970) Nature (London) 227: 680-685.

Lindstrom, J., Gullick, W., Conti-Tronconi, B. M. & Ellismann, M. (1980) Biochemistry 19:4291-4795

Lindstrom, J., Einarson, B and Tzartos, S. (1981) Methods in Enzymology 74: 432-460.

Lindstrom, J., Tzartos, S., Gullick, W., Mochswender, S., Swanson, L., Sargent, P., Jacob, M. & Montal, M. (1983) Cold Spring Harb. Symp. Quant. Biol. 48: 89-99.

Lippiello, P. M. & Fernandes, K.G. (1986) Mol Pharmacol 29: 448-454

Leprince, P., Noble R. & Hesse, G. P. (1981) Biochemistry 20: 5565-5570.

Lukas, R., Morimoto, H., Hanley, M. R. & Bennet, E. L. (1981) Biochemistry 20: 7373-7378.

Lyddiatt, A., Sumikawa, K., Wolosin, J. M., Dolly, J. O. & Barnard, E. A. (1979) FEBS Lett. 108: 20-24.

Maelicke, A. (1987) in: "Handbook of Experimental Pharmacology", G.V.R. Boin, A. Farah, M. Merken, A.D. Welch Eds, vol, "The Cholinergic Synapse", V.P. Whittaker Ed., Springer-Verlag, in press.

Maelicke, A., Fulprius, B. W., Caspar, D., Smith, M., Reich, E. and Possani, L.D. (1973) J. Biol. Chem. 6841-6853.

McCarthy, M. P., Earnest, J. P., Young, F. F., Choes, J. & Stroud, R. (1986) Ann. Rev. Neurosci. 9: 383-413.

Moore, H.-P. H. & Raftery, M. A. (1979) Biochemistry 10: 1862-1867.

Moscovitz, R. & Gershoni, J. M. (1988) J. Biol. Chem. 263:1017-1022.

Neubig, R. R. & Cohen, J. B. (1979) Biochemistry 18:5464-5475.

Neubig, R. R., Krodel, E. K., Boyd, N. D. & Cohen, J. B. (1979) Proc. Natl. Sci. USA 76: 690-694.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M. & Fuchs, S. (1986a) Proc. Natl. Acad. Sci. USA 83:3008-3011.

Nomoto, H., Takahashi, N., Nagaki, Y., Endo, S., Arata, Y. & Hayashi, K. (1986) Eur. J. Biochem. 157: 233-242.

Ong, D. E. & Brady, R. M. (1974) Biochemistry 13: 2822-2827.

Raftery, M. A., Vandler, R. L., Reed, K. L. & Lee, T. (1976) Cold Spring Harbor Symp. Quant. Bio. 40: 193-202.

Raftery, M. A., Dunn, S. M. J., Conti-Tronconi, B. M., Middlemas, D. S. & Crawford, R. D. (1983) Cold Spring Harb. Quant. Biol. 48: 21-33.

Rang, M. P. & Ritter, J. M (1970a) Mol. Pharmacol. 6: 357-383.

Rang, M. P. & Ritter, J. M (1970b) Mol. Pharmacol. 6: 383-390.

Ratnam, M., Gullick, W., Spiess, J., Wan, K., Criado, M. and Lindstrom, J. (1986) Biochemistry 25: 4268-4275.

Rousselet, A., Faure, G., Boulain, J.-C. & Menez, A. (1984) Eur. J. Biochem. 140: 31-37.

Schmidt, J. & Raftery, M. A. (1973) Anal. Biochem. 52: 349-354.

Sine, S. M. & Taylor, P. (1980) J. Biol. Chem. 255: 10144-1056.

Sine, S. M. & Taylor, P. (1981) J. Biol. Chem. 256: 6692-6699.

Sobel, A. Weber, M. & Changeux, J. P. (1977) Eur. J. Biochem 80: 215-224.

St. John, P. A., Frohener, S., Goodenough, D. A. & Cohen, J. B. (1982) J. Cell Biol. 92:333-342.

Strader, C. D. & Raftery, M. A. (1980) Proc. Natl. Acad. Sci. USA 77:5807-5811.

Sugiyama, H. & Changeux, J. P. (1975) Eur. J. Biochem. 55: 505-15.

Wang, G. K., Molinaro, S. & Schmidt, J. (1978) Biol. Chem. 253: 8507-8512

Wang, G. K. & Schmidt, J. (1980) J. Biol. Chem. 255: 11156-11162

Wonnacott, S., Harrison, R. & Lunt, G. G. (1980) Life Sciences 27: 1769-1775

Wilson, P. T., Gershoni, J. M., Hawrot, E. & Lentz, T. L. (1984) Proc. Natl. Acad. Sci. USA 81: 2553-2557.

Wolosin, J. M., Lyddiatt, A., Dolly, J. O. & Barnard, E. A. (1980) Eur. J. Biochem. 109: 495-505.

Young, A. P., Brown, F. F., Halsey, M. J. & Sigman, D. S. (1978) Proc. Natl. Acad. Sci USA 75: 4563-7.

Table 1

Irreversible binding of ^{125}I-α-BGT to AcChR-rich Torpedo membrane fragments before and after solubilization with Triton X-100.

preparation #	before Triton X-100 (nmol/ml)	after Triton X-100 (nmol/ml)
1	7.90	15.71
2	5.40	9.85
3	4.60	9.10
4	4.20	9.30
5	9.5	19.10
6	5.6	11.30
7	8.05	16.15

The binding was measured by DEAE disk assay as described in the "Methods" section.

Table 2

Irreversible binding of ^{125}I-α-NTX to AcChR-rich Torpedo membrane fragments before and after solubilization with Triton X-100.

preparation #	before Triton X-100 (cpm x 10^{-3}/ml)	after Triton X-100 (cpm x 10^{-3}/ml)
1	16,550	17,500
2	21,000	20,900
3	21,210	14,490
4	17,475	16,225
4	15,050	16,750
5	13,900	13,700

The binding was measured by DEAE disk assay as described in the "Methods" section.

Table 3

Effect of ConA on Irreversible Binding of ^{125}I-α-BGT

to Triton Solubilized (S-AcChR) or Membrane Bound (M-AcChR) of AcChR

	S-AcChR (n mole/ml)			M-AcChR (n mole/ml)		
Preparation #	No Con A	2 x ConA	10 x Con A	No Con A	2 x ConA	10 x Con A
1.	8.17	9.06	7.63	ND	ND	ND
2.	9.45	10.20	9.54	ND	ND	ND
3.	9.68	8.64	8.64	ND	ND	ND
4.	8.25	9.87	9.80	4.30	5.43	5.25

The binding was measured by DEAE disk assay as described in the "Methods" section.

Figure 1: SDS gel electrophoresis pattern of a typical Torpedo membrane preparation enriched in AcChR, after treatment at pH 11. The four AcChR subunits (α, β, γ, δ) are the main protein constituent. A contaminant protein of approximate M_r 90,000 is also present. The faint protein bands of low M_r are degradation products of the AcChR subunits.

Figure 2: Increasing amounts of AcChR-rich membrane fragments dissolved in Triton X-100 (S-AcChR, open squares, curve A) or not [M-AcChR, triangles (curve C) and black squares (curve B)] were incubated with excess (approximately 500 nM) ^{125}I-α-BGT. For the samples indicated by open and black squares the amount of bound ^{125}I-α-BGT was measured by DEAE disks, for samples indicated by triangles by centrifugation assay. The values obtained for S-AcChR by disk assay and for M-AcChR by centrifugation assay are identical, while only half as many α-BGT binding sites were revealed for M-AcChR when the disk assay was used.

Figure 3: Measurement of ^{125}I-α-BGT bound to M-AcChR by centrifugation assay. Aliquots (250 μL) containing 10.74 pmol of M-AcChR, measured as α-BGT binding sites in the presence of Triton X-100, were incubated wih increasing concentrations of ^{125}I-α-BGT. The number of sites of this membrane preparation measured by disk assay without Triton X-100 was exactly 50% of those measured in the presence of the detergent (inset, bars A and B). In the centrifugation assay, the amount of bound ^{125}I-α-BGT inreases linearly up to the point where the ^{125}I-α-BGT concentration is approximately equivalent to half of the total concentration of binding sites, yielding a titratio ncurve of the high-affinity sites. The slope then decreases and reaches saturation at approximately the concentration of the sites measured for S-AcChR (inset, bar C).

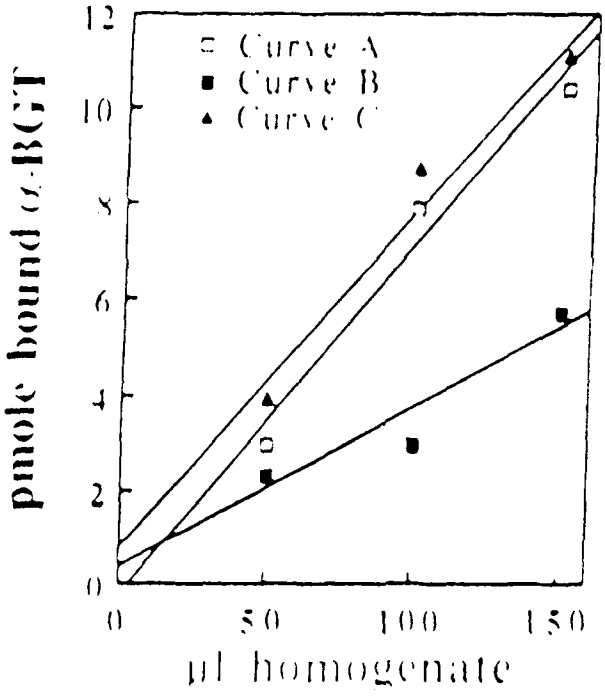
Figure 4: Presence of residual reversible binding sites, measured by centrifugation assay in a preparation of M-AcChR pretreated with unlabeled α-BGT and washed free of the unreacted reversibly bound toxin. The samples thus treated (black squares) still bind approximately 50% of the toxin bound by the control, untreated samples (open squares). Unspecific binding has been subtracted from both curves. See text for experimental details.

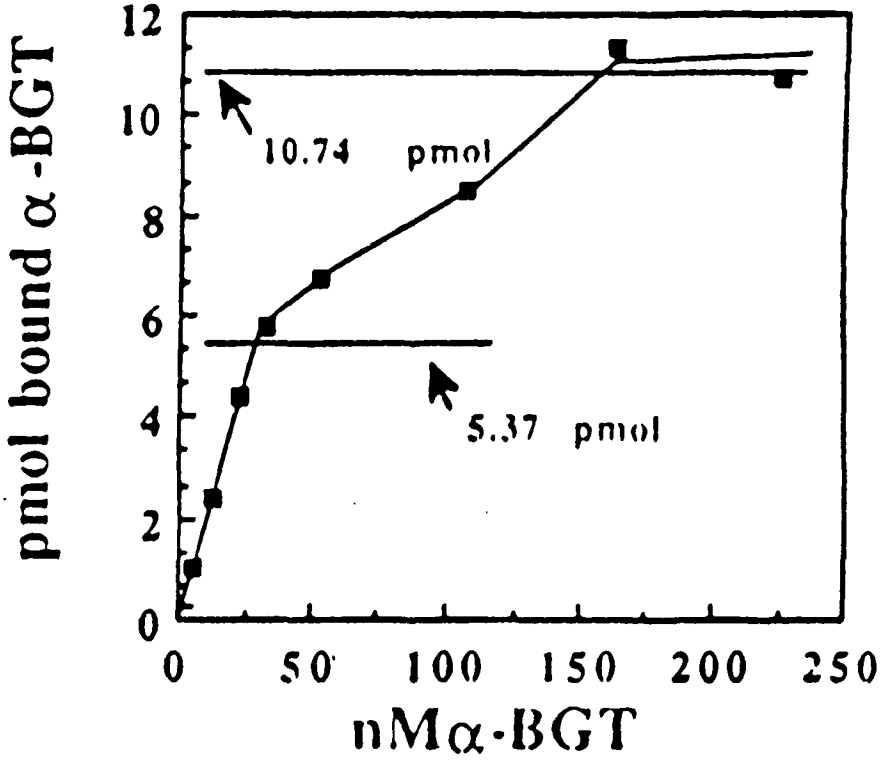
Figure 5: Effect of Con A on the rate of association of ^{125}I-α-BGT to S-AcChR. S-AcChR (1 nM) was incubated with 2 nM α-BGT for 2 h with 10 μM Con A (circles) or without any Con A

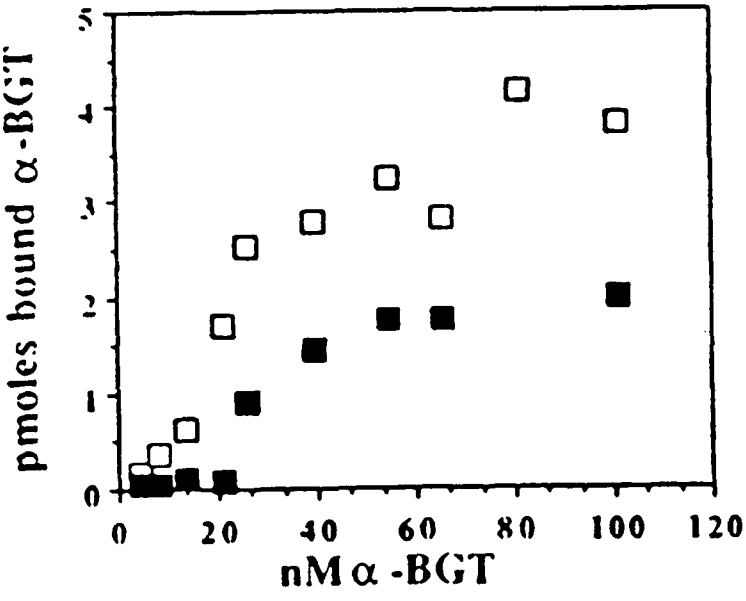
(triangles). At different time intervals, aliquots were taken and the amount of bound ^{125}I-α-BGT measured by DEAE disk assay. The data were fitted by using an exponential fitting program to a single-exponential equation in the case of the curve obtained in the absence of Con A (triangles) and to a double-exponential equation for the data obtained in the presence of 10 μM Con A (circles), as described under Materials and Methods. In the absence of Con A the calculated maximum amount of bound α-BGT was 37.5 ± 0.58 fmol, with a rate of association of 0.114 ± 0.005 min^{-1}. In the presence of Con A the calculated maximum amount of bound α-BGT was 30.3 ± 6.69 fmol, and the association rates were 0.130 ± 0.040 min^{-1} for a slow associating site accounting for the remaining binding.

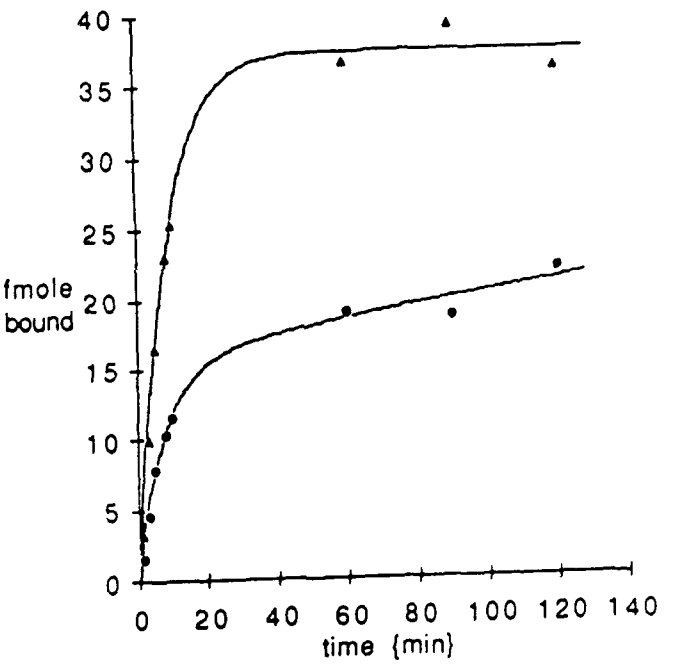
Figure 6: Effect of the presence of an excess of unlabeled α-NTX (black squares) or α-BGT (open triangles) on the dissociation rate of complexes formed between ^{125}I-α-BGT and M-AcChR. Each curve reports the results obtained in three independent experiments, carried out with different preparations of both M-AcChR and toxins. The concentrations used were the following: M-AcChR/^{125}I-α-BGT complexes, 1 μM; α-NTX; 8 μM in two experiments and 10 μM in on eexperiment; α-BGT, 8 μM in two experiments and 7 μM in one experiment. Both the consistency and the magnitude of the effect are evident. The rate of dissociation in the presence of unlabeled α-BGT is very slow ($t_{1/2}$ for this curve equals 290 h) and it is markedly accelerated by the presence of α-NTX ($t_{1/2}$ for this curve equals 8.75 h; correlation coefficient, 0.79). See text for further details.

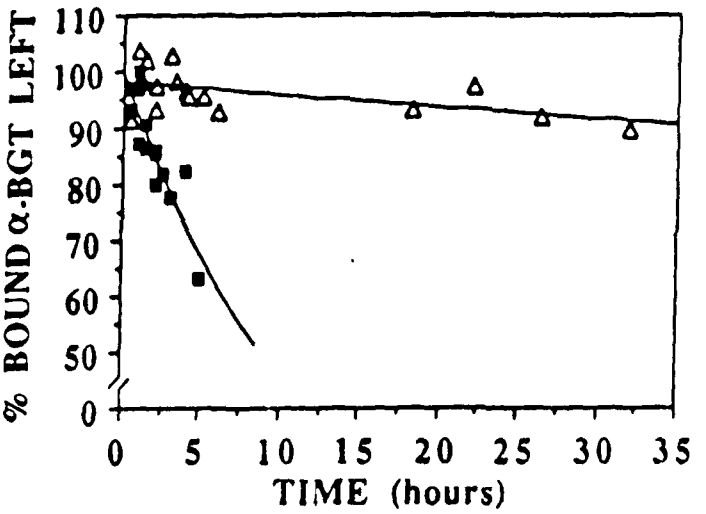

90K
δ
γ
β
α


pmole bound α-BGT
μl homogenate
Curve A
Curve B
Curve C
0
2
4
6
8
10
12
0
50
100
150


pmol bound α-BGT
12
10
8
6
4
2
0
10.74 pmol
5.37 pmol
0
50
100
150
200
250
nMα-BGT


pmoles bound α-BGT
0
1
2
3
4
5
0
20
40
60
80
100
120
nM α -BGT


40
35
30
25
fmole
bound
20
15
10
5
0
0
20
40
60
80
100
120
140
time (min)


% BOUND α-BGT LEFT
110
100
90
80
70
60
50
0
0
5
10
15
20
25
30
35
TIME (hours)

Structural Determinants Within Residues 180-199 of the Rodent α5 Nicotinic Acetylcholine Receptor Subunit Involved in α-Bungarotoxin Binding†

TEXTUAL FOOTNOTES

[1] Abbreviations used include α-BTX (alpha-bungarotoxin), nAChR (nicotinic acetylcholine receptor), IAA (iodoacetamide), DTT (dithiothreitol), IOBA (2-iodosobenzoic acid), CM-Cys (S-carboxymethylcysteine), HPLC (high pressure liquid chromatography).

[2] Data for the carboxymethylation analysis is available upon request.

ABSTRACT

Synthetic peptides corresponding to sequence segments of the nicotinic acetylcholine receptor (nAChR) α subunits have been used to identify regions that contribute to formation of the binding sites for cholinergic ligands. We have previously defined an α-bungarotoxin (α-BTX) binding sequence between residues 180-199 of a putative neuronal nAChR α subunit, designated α5 (McLane, K. E., Wu, X., and Conti-Tronconi, B. M., 1990, J. Biol. Chem. 265, 9816-9824). In the present study, the effects of single amino acid substitutions of Gly or Ala for each residue of the α5(180-199) sequence were tested, using a competition assay, in which peptides compete for ^{125}I-α-BTX binding with native *Torpedo* nAChR. Four residues appeared to be critical for α-BTX binding to the α5 sequence segment -- Lys184, Arg187, Cys191 and Pro195. Peptides containing other amino acid substitutions (Gly185, Asn186, Asp189, Trp193, Tyr194, and Tyr196) retained a measurable level of binding activity, but their affinity for α-BTX was 3 to 10-fold lower compared to the peptide corresponding to the native sequence. The importance of Cys191 and Cys192 for α-BTX binding was difficult to assess, as peptides substituted at either of these positions tended to form dimers. Oxidation of the peptide corresponding to the native sequence resulted in monomers and dimers, which retained ~60% and ~40% binding activity, respectively, and reduction and alkylation with iodoacetamide slightly decreased its ability to bind α-BTX (~70-80% activity). The same treatment of peptides substituted at Cys191 or Cys192 converted them to monomers, and resulted in ~70-80% binding activity for both peptides. These results indicate that a disulfide bridge between the vicinal cysteines at positions 191 and 192 of the α5 sequence is not an absolute requirement for α-BTX binding activity.

INTRODUCTION

Elapid snakes paralyze their prey through postsynaptic curaremimetic antagonists of the muscle nicotinic acetylcholine receptors (nAChR)[1]. α-Neurotoxins from snake venoms, such as α-bungarotoxin (α-BTX) from *Bungarus multicinctus,* bind with subnanomolar affinity to the muscle-type nAChR at sites that reside largely or completely on the α subunits. The use of α-neurotoxins made possible the isolation, purification and determination of the complete amino acid sequence of the four subunits of the *Torpedo* and mammalian muscle nAChR (α1, β, γ, and δ), which share considerable homology and are likely of common ancestry (Raftery et al., 1980; Conti-Tronconi et al., 1982; Noda et al., 1982, 1983a, b; La Polla et al., 1984; Boulter et al., 1985, 1986a; Isenberg et al., 1986; Buonanno et al., 1986; Yu et al., 1986). Further evidence for an extended supergene family has come from the identification of several different, but homologous nAChR subunits from rodent, avian and goldfish neurons, referred to as α2, α3, α4 and α5 (Wada et al., 1988; Boulter et al., 1986b, 1990; Goldman et al., 1987; Nef et al., 1988; Cauley et al., 1990; Hieber et al., 1990a) and β2, β3 and β4 (also designated non-α or structural subunits) (Deneris et al., 1988, 1989; Duvoisin et al, 1989; Nef et al, 1988; Schoepfer et al., 1988; Cauley et al., 1989; Hieber et al., 1990b). The complete sequence of two avian neuronal α-BTX binding proteins, designated α-BGTBP α1 (also referred to as α7), and α-BGTBP α2, have been identified (Schoepfer et al., 1990; Couturier et al., 1990) using oligonucleotides corresponding to the N-terminal sequence of a putative nAChR isolated from the chick brain by α-BTX affinity chromatography (Conti-Tronconi et al., 1985). In addition, subunits from α-BTX binding proteins of the *Drosophila* and locust nervous systems (Schloss et al., 1988, Sawruk et al., 1990; Marshall et al., 1990) have been reported. Although sequence homology indicates that these different neuronal subunits are related to the muscle nAChR subunits, expression of functional receptors and demonstration of nicotinic pharmacology has only been achieved for some of these subunits. Coexpression of the α2, α3, α4 subunits with the β2 or β4 subunits in *Xenopus*

oocytes results in the formation of functional nAChRs, which are insensitive to α-BTX (Goldman et al., 1987; Deneris et al., 1988, 1989; Ballivet et al., 1988; Wada et al., 1988; Duvoisin et al., 1989; Papke et al., 1989; Luetje et al., 1990), whereas expression of the α7 subunit as a homoligomer yields an α-BTX sensitive nAChR (Couturier et al., 1990). Other subunits, such as the α5 subunit, have not been successfully expressed and may require other yet unidentified subunits to form a functional nAChR.

The neuronal α-BTX binding protein subunits from chick and *Drosophila* (Schoepfer et al., 1990; Couturier et al., 1990; Sawruk et al., 1990), and the rat α5 subunit (Boulter et al., 1990), have in common with all nAChR α subunits a pair of adjacent cysteine residues at approximately position 190. Cysteines 192 and 193 of the *Torpedo* nAChR α1 subunit may form a vicinal disulfide bond (Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988; Kellaris et al., 1989), and are labelled by cholinergic affinity reagents that compete for α-BTX binding (Kao et al., 1984; Kao and Karlin, 1986; Wilson et al., 1984). A sequence segment containing cysteines 192 and 193 on the *Torpedo* α1 subunit has also been identified as an important component of the α-BTX binding site by several laboratories, using proteolytic fragments (Wilson et al., 1984,1985; Pederson et al., 1986; Neumann et al., 1986a), synthetic peptides (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson and Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990a), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988; Ohana and Gershoni, 1990). Our laboratory has used synthetic peptides to define components of α-BTX binding sites on the sequence segments 181-200 from the *Torpedo* α1 subunit (Conti-Tronconi et al., 1990), vertebrate muscle α1 subunits McLane et al., 1991a), and the avian brain α-BGTBP α1 and α-BGTBP α2 subunits (McLane et al., 1991b), and the sequence segment 199-200 of the rodent α5 subunit (McLane et al., 1990). We have studied the structural requirements for α-BTX binding by comparing the effects of single amino acid substitutions of the *Torpedo* α-BTX binding peptide (Conti-Tronconi et al., 1991), and species-specific substitutions of muscle α1 subunits (McLane et al., 1991a),

which are highly homologous sequences. In contrast to the *Torpedo* and vertebrate muscle nAChR α1 subunits, the α-BTX binding sequences of neuronal α subunits (rat brain α5, *Drosophila* SAD, chick brain α-BGTBP α1 and α2) are relatively divergent, and offer a unique opportunity to determine what structural features are important to α-BTX binding.

In the present study, we identify the critical amino acids for the binding of α--BTX to the peptide sequence 181-200 of the rodent neuronal α5 subunit. We synthesized twenty single residue substitution peptide analogs of the sequence segment 180-199 of the α5 subunit, in which one of the residues was replaced by a glycine, or, if glycine was the amino acid present at that position in the native sequence, it was replaced by alanine. These single substitution analogs were tested for their ability to bind α-BTX using a competition assay, which measured the ability of a peptide to ^{125}I-α-BTX binding to native *Torpedo* nAChR. Several amino acid substitutions drastically altered the ability of the α5 peptides to inhibit binding of α-BTX to native *Torpedo* nAChR, whereas other substitutions had more subtle effects on the interaction of the α5 peptide with α-BTX, detectable as changes in the IC_{50} values.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Characterization

Peptides, 20-21 amino acids long, were synthesized by manual parallel synthesis (Houghten, 1985). The purity of the peptides was assessed by reverse phase HPLC (high pressure liquid chromatography) using a C18 column (Ultrasphere ODS) and an acetonitrile/water gradient (5-70%) containing 0.1% trifluoroacetic acid. A major peak consistently accounted for 65-85% of the total absorbance at 214 nm. The amino acid composition of the peptides, determined by derivatization of amino acid residues released by acid hydrolysis with phenylisothiocyanate, followed by separation on a reverse phase HPLC column (PICO.TAG) as described by Heinrickson and Meredith (1984), gave satisfactory correspondence between experimental and theoretical values for all peptides.

The sequence and purity of peptides with altered α-BTX binding activity, and other randomly selected peptides, were verified by gas-phase sequencing (Applied Biosystems, Foster City, CA). Only the expected sequences were found. Contaminating sequences, which would be expected from truncated peptides randomly missing amino acids from incomplete coupling, were below the level of detectability (<3%). The sequence and codes of the peptides are reported in **Figure 1**.

Modification of Cysteine/Cystine Residues

Synthetic peptides (0.5 mg/ml) in 100 mM potassium phosphate buffer, pH 8.5, were treated with either 2-iodosobenzoic acid (IOBA) (0.1 or 0.8 mM) or dithiothreitol (DTT) (1.5 mM) for five hours at room temperature. Samples of untreated, oxidized and reduced peptides were alkylated with iodoacetamide (IAA) (6 mM) overnight at 4°C. The reactants and peptides were separated by gel permeation chromatography in 10 mM potassium phosphate buffer, pH 7.0, using a Sephadex G-10 column (0.9 x 4 cm), followed by a P6 column (BioRad) (1.8 x 47 cm), which was calibrated with blue dextran 2000 and the following peptides (obtained from Sigma): bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), and human growth hormone releasing factor (5041 daltons). Carboxymethylation of free sulfhydryl groups was assessed by amino acid composition analysis, as described above. The S-carboxymethylcysteine (CM-Cys) derivative was calibrated with a standard (Pierce) and elutes after the Asp and Glu derivatives, and prior to the Ser derivative using 140 mM sodium acetate buffer (pH 6.4) containing 0.05% triethylamine and 6% acetonitrile (McLane et al., 1991a,b).

Preparation and Calibration of Radiolabelled α-BTX

α-BTX was isolated from *Bungarus multicinctus* venom (Biotoxins Inc.) as described by Clark et al. (1972). The purity of α-BTX, assessed by gas phase sequencing, indicated that contaminating sequences, if present, were below the level of detectability (< 3-5%). α-BTX was radiolabelled with carrier free ^{125}I (Lindstrom et al., 1981) and

calibrated as described by Blanchard et al. (1979) using membrane bound nAChR prepared from *Torpedo californica* electric organ (Neubig et al., 1979; Elliot et al., 1980). The specific activity of the ^{125}I-α-BTX was 52-180 Ci/mmol.

Competitive Inhibition of ^{125}I-α-BTX Binding by Peptides

Peptides (1-250 μg/ml) were preincubated overnight at 4°C with ^{125}I-α-BTX (20 nM) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mg/ml cytochrome c. Membrane bound *Torpedo* nAChR (0.2-1 pmole) (Neubig et al., 1979; Elliot et al., 1980) was added to 100 μl of peptide/toxin solutions. After 3 min. at room temperature, the assay tubes were centrifuged at 14,000 x g for 45 min., washed with 10 mM potassium phosphate buffer containing 100 mM NaCl, and recentrifuged. The pellet was counted in a gamma counter. Nonspecific binding was determined by preincubation of *Torpedo* nAChR with 20 nM unlabelled α-BTX for 10 min. prior to addition to the ^{125}I-α-BTX solutions. Positive controls (100% binding) contained ^{125}I-α-BTX preincubated with buffer in the absence of peptide. The IC_{50} values were determined using the programs EBDA and LIGAND (Munson and Rodbard, 1980; McPherson, 1983). The relative affinities of peptides for α-BTX were also compared by plotting the data in the form of a Hofstee plot (Molinoff et al., 1981), in which the negative slope is equal to the IC_{50}.

RESULTS

Torpedo nAChR Competition Assay: Correlation with the Physiological Sensitivity to α-BTX of the Corresponding nAChRs

In the competition assay used in the present study, peptides were preincubated with ^{125}I-α-BTX, and their ability to inhibit ^{125}I-α-BTX binding to native *Torpedo* nAChR was determined. This assay adequately reflects the ability of the corresponding α subunits to bind α-BTX as demonstrated in previous studies, where we tested the ability of α-BTX to bind to peptides corresponding to the sequence region 180-200 from α subunits of nAChRs known to differ in their ability to bind α-BTX (McLane et al., 1990, 1991a). The

neuronal α2, α3, and α4 rodent subunits, when coexpressed with the β2 subunit in *Xenopus* oocytes, have been shown to form an nAChR insensitive to α-BTX, and peptides corresponding to their sequence segment 181-200 do not compete for α-BTX in the *Torpedo* competition assays, whereas the nAChRs formed by *Torpedo* α1 and rodent α1 coexpressed with β1, γ and δ subunits are sensitive to α-BTX, and peptides corresponding to these α1 sequences are effective competitors for α-BTX (Mishina et al., 1984; Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988; McLane et al., 1990). In addition, the cobra muscle nAChR is unique among the vertebrate muscle nAChRs in that it is insensitive to α-BTX (Burden et al., 1975), and synthetic peptides corresponding to the sequence segment 181-200 of the α1 subunit of different species have been shown to bind α-BTX using the Torpedo competition assay, with the exception of the cobra sequence, which differs by only a few amino acid residues (McLane et al., 1991a).

Single Amino Acid Substitutions of the Sequence Segment α5(180-199) Alter the Binding of α-BTX

The sequence segment α5(180-199) competitively inhibits ^{125}I-α-BTX binding to native membrane-bound *Torpedo* nAChR (McLane et al., 1990). We examined the effects on α-BTX binding of single amino acid substitutions of this sequence. The single amino acid substitution analogs are depicted in **Figure 1**. Each amino acid from positions 181 to 190 was sequentially replaced by Gly, or by Ala when the native sequence had a glycine residue. This panel of twenty peptides was tested using the *Torpedo* nAChR competition assay to determine the effect of each substitution. The results of a typical experiment (n=9), where the same concentration of each peptide was used (100 μg/ml, i.e., 40 μM), is shown in **Figure 2**. At this concentration, peptide α5(180-199)$_{\text{Native}}$ typically inhibits the binding of ^{125}I-α-BTX to *Torpedo* nAChR by >90% (**Figure 2** and McLane et al., 1990). The most profound effect on α-BTX binding was found for substitutions of Lys184 (16% inhibition), Arg187 (20% inhibition), Cys191 (48% inhibition) and Pro195 (61% inhibition). The concentration dependence of the inhibition by peptides with these

substitutions was compared with the native peptide over a concentration range (3-74 μM) at which >50% inhibition is reached when peptide $\alpha5(180\text{-}199)_{Native}$ is used. The approximate IC_{50} values for peptide $\alpha5(180\text{-}199)_{Native}$, and for peptides substituted at Cys191 and Pro195 were 2 μM, 100 μM and 50 μM, respectively (data not shown). A 50% level of inhibition by the peptides substituted at Lys185 and Arg187 could not be reached within the range of solubility of these peptides.

Other substitutions slightly reduced the ability of the peptide to compete for ^{125}I-α-BTX, but these effects were too small to be assessed using the single concentration of peptide as inhibitor of the experiments depicted in **Fig. 2.** To evaluate subtle effects of amino acid substitutions on the ability of the α5 sequence segment to bind α-BTX, the concentration dependence of inhibition by peptides was determined for peptide concentrations from 1 to 100 μM. The results of a typical experiment (n=3) is given in **Figure 3,** where the relative affinities of peptides for α-BTX are compared by plotting the data in the form of a Hofstee plot (Molinoff et al., 1981), in which the negative slope is equal to the IC_{50}. The values obtained in three independent experiments were determined by analysis using the program EBDA (Munson and Rodbard, 1980) and are summarized in **Table I**. The most notable changes in the IC_{50} values were caused by substitutions of Asp189 (~9-fold increase), Asn186 (~5-fold increase), Trp193 (~4-fold increase), and substitutions of Tyr residues at positions 194 and 196 (4 to 5-fold increases). Other substitutions had smaller effects on the affinity of α5(180-199) for α-BTX, as indicated in **Table I**.

Characterization of the Redox State of Cysteine Residues of Peptides $\alpha5(180\text{-}199)_{Native}$, $\alpha5(180\text{-}199)_{C191}$ and $\alpha5(180\text{-}199)_{C192}$

In order to investigate if the oxidation state of the vicinal cysteine residues of the sequence α5(180-199) affects α-BTX binding, peptides $\alpha5(180\text{-}199)_{Native}$, $\alpha5(180\text{-}199)_{C191}$, and $\alpha5(180\text{-}199)_{C192}$ were modified with sulfhydryl reagents. Peptides were either oxidized with iodosobenzoic acid (**IOBA**), or reduced with dithiothreitol (**DTT**).

Free sulfhydryl groups in untreated peptides, and following reducing or oxidizing treatments, were alkylated with iodoacetamide (**IAA**), and quantitated by amino acid composition analysis as phenylisothiocarbamyl derivatives of amino acid residues released upon acid hydrolysis. In this assay, cysteinyl residues with free sulfhydryl groups in the peptide preparation are present as their alkylated derivative S-carboxymethylcysteine (**CM-Cys**) due to IAA (McLane et al., 1991a,b). Comparison of the CM-Cys peak obtained for untreated peptides and after full reduction indicated that in the untreated peptide $\alpha 5(180\text{-}199)_{Native}$ 73% of the Cys residues have free sulfhydryl groups. Surprisingly, no CM-Cys peak was present for the untreated peptides $\alpha 5(180\text{-}199)_{C191}$ and $\alpha 5(180\text{-}199)_{C192}$, while after reduction the expected molar ratio of CM-Cys was obtained (i.e. ~1). Therefore, the peptide analogs carrying substitutions of one of the vicinal cysteine residues is substituted are fully oxidized in solution, probably in the form of dimers[2].

Gel permeation chromatography of these peptides either before or after oxidation, reduction and alkylation reactions confirmed that peptides $\alpha 5(180\text{-}199)_{C191}$ and $\alpha 5(180\text{-}199)_{C192}$ existed in solution predominantly as dimers (4,800 ± 500 Da and 5,000 ± 500 Da, respectively), whereas untreated peptide $\alpha 5(180\text{-}199)_{Native}$ existed predominantly as a monomer (2,400 ± 200 Da) (**Figure 4A**). After reduction and alkylation all peptides eluted as monomers of the expected molecular weight (~2,300 Da), indicating that the higher molecular weight forms of the untreated peptides found with the vicinal cysteine substitutions are not merely aggregates, but are disulfide linked dimers (**Figure 4B**). Oxidation of peptide $\alpha 5(180\text{-}199)_{Native}$ yielded both monomers and dimers, and amino acid composition analysis showed that no detectable free sulfhydryl groups were present, indicating that both Cys191 and Cys192 of the monomer and dimer forms are either disulfide linked or oxidized to cysteic acid.

Effects of Sulfhydryl Modification of Peptides **$\alpha 5(180\text{-}199)_{Native}$, $\alpha 5(180\text{-}199)_{C191}$ and $\alpha 5(180\text{-}199)_{C192}$ on *^{125}I-α-BTX Binding***

The monomeric or dimeric forms of untreated peptides, and those resulting from oxidation and reduction, were tested for their ability to inhibit ^{125}I-α-BTX binding to membrane-bound *Torpedo* nAChR using the competition assay (**Figure 7** and **Table II**). Only the monomeric form of peptide $\alpha 5(180\text{-}199)_{Native}$ could be obtained from untreated peptide, whereas both monomer and dimer forms were obtained in sufficient quantities after oxidation. For peptides $\alpha 5(180\text{-}199)_{C191}$ and $\alpha 5(180\text{-}199)_{C192}$, only dimers could be isolated from untreated peptides, or peptides treated with only IAA or IOBA. For all peptides, only the monomeric form could be isolated and tested after reduction and alkylation. The results of a typical assay using 100 μg/ml peptide solutions (n=6) are shown in **Figure 5**. The ability of treated peptides to inhibit ^{125}I-α-BTX binding relative to peptide $\alpha 5(180\text{-}199)_{Native}$, determined in six independent experiments, are summarized in **Table II**.

Alkylation of peptide $\alpha 5(180\text{-}199)_{Native}$, which contains both Cys 191 and 192, reduced its ability to compete for α-BTX by ~30% with or without prior reduction with DTT. Reduction and/or alkylation of peptide $\alpha 5(180\text{-}199)_{C192}$ did not significantly affect its ability to compete for α-BTX (70-80% inhibition). Peptide $\alpha 5(180\text{-}199)_{C191}$, on the other hand, was relatively ineffective as a competitor for ^{125}I-α-BTX binding (40-50% inhibition), but, upon conversion to the monomer by reduction and alkylation, some activity was restored (~70% inhibition). Although the role of free sulfhydryls in these peptides cannot be tested directly, as conditions necessary to keep the sulfhydryl groups of the peptide in the reduced state would destroy the disulfide loops of α-BTX necessary for activity (Martin et al, 1983), the results of alkylation experiments indicate that a free sulfhydryl is not necessary for α-BTX to bind.

Oxidation of $\alpha 5(180\text{-}199)_{Native}$ with IOBA resulted in the formation of both monomers and dimers. The monomer, which presumably represents the formation of a

vicinal disulfide bond, retained 86% α-BTX binding activity, whereas the oxidized dimer was only 50% active. These results confirm that dimerization of the α5(180-199) peptides reduces, but does not eliminate, α-BTX binding activity. In addition, the lack of enhanced α-BTX binding by the completely oxidized monomer of peptide $\alpha5(180\text{-}199)_{Native}$ indicates that the small proportion of oxidized monomer in untreated preparations of this peptide (~20%) are not responsible for the α-BTX binding observed. Therefore, it appears that α-BTX binding is not affected by the redox state of the adjacent Cys residues at positions 191 and 192.

We are unable at present to explain the significantly reduced ability of peptide $\alpha5(180\text{-}199)_{C192}$ to bind α-BTX after treatment with IOBA, as this peptide exists predominantly as a dimer in untreated solutions. Iodoso compounds, such as IOBA, are mild oxidants, and are known to oxidize cysteine residues to both disulfides and to cysteic acids (Allison, 1976; Torchinsky, 1981; Fontana et al., 1981), and methionine residues to sulfoxides and sulfones (Mahoney and Hermodson, 1979; Fontana et al., 1981). From our amino acid composition analysis (data not shown) it is apparent that the low yield of methionine upon treatment of peptides with IOBA may be due to the formation of the sulfone, which could not be directly quantitated as it coelutes with Asp (Fontana et al., 1981). It is possible that the double "mutation" presented as the Met181 sulfone and the substituted Cys residue of oxidized peptides $\alpha5(180\text{-}199)_{C191}$ and $\alpha5(180\text{-}199)_{C192}$ confers lower α-BTX binding activity than observed for the single amino acid substitutions of either Cys. The results presented for peptides oxidized with IOBA should be interpreted in light of the known side reactions.

DISCUSSION

Recently we have identified a sequence segment between amino acid residues 180-199 of the α5 subunit that contributes to forming the binding site for α-BTX in this putative neuronal nAChR (McLane et al., 1990). In the present study, a panel of peptides

containing single amino acid substitutions of the sequence α5(180-199) were tested for their ability to compete for ^{125}I-α-BTX binding with native membrane bound *Torpedo* nAChR. Substitutions by glycine of several residues within the sequence α5(180-199) sequence markedly reduced the ability the corresponding peptides to inhibit ^{125}I-α-BTX binding to *Torpedo* nAChR, when compared to peptide α5(180-199)$_{Native}$. These residues were: Lys184 (~15% inhibition), Arg187 (~20% inhibition), Cys191 (~50% inhibition), and Pro195 (~60% inhibition). In the sequences of α subunits known to bind α-BTX (such as *Torpedo* and mouse α1 subunits, chick brain α-BGTBP α1 and α2 subunits, and the *Drosophila* SAD subunit), Lys184 is conserved, or conservatively replaced by Arg (See **Figure 6A**). On the other hand, this Lys residue is replaced by Trp in the α-BTX-insensitive cobra a1 sequence (**Figure 1**), indicating that it may be a common, critical residue for α-BTX binding. If an insertion is allowed, Pro195 of the α5 sequence aligns with the highly conserved Pro194 residue of the *Torpedo* α1 sequence (**Figure 6A**), which we previously showed to be critical for α-BTX binding (Conti-Tronconi et al., 1991). Arginine187, which is unique to the α5 sequence, may replace the positive charge of the Arg181 or His186 residues of the *Torpedo* and muscle α1 subunits. The positively charged Lys residue at position 187 of the *Drosophila* SAD subunit may play a similar role. Alternatively, realignment of Arg187 with the α-BGTBP α1 sequence, as shown in **Figure 6B** suggests the conservation of the sequence **RT(E/D)S**. Our results also indicate that Cys191 is important for α-BTX binding, but the extent of its contribution is difficult to assess due to the tendency of peptide α5(180-199)$_{C191}$, as well as α5(180-199)$_{C192}$ to form dimers, as discussed below.

Subtle changes in the affinity for α-BTX occurred when other amino acid residues were substituted, most notably for peptides with substitutions of Asp189 (~10-fold increase in IC_{50}s relative to the native sequence), Tyr194, Tyr196, Asn186 and Trp193 (4 to 5-fold increase in IC_{50}s). Aromatic amino acid residues have been implicated in the high affinity α-BTX binding of the *Torpedo* sequence by comparison with the α1 subunit

sequences of other species that bind α-BTX with lower affinity (Wilson and Lentz, 1988; Ohana and Gershoni, 1990; McLane et al., 1991a), and Trp 187 of the *Torpedo* α1 sequence has been demonstrated to be important to α-BTX binding by the chemical modification in the synthetic peptide α1(185-196) (Neumann et al., 1986b). On the basis of the structural analysis of α-BTX, the toxin/nAChR interface is thought to involve primarily hydrophobic and hydrogen bonding interactions, and only a few charged amino acids (Love and Stroud, 1986). Further support for the role of aromatic residues in formation of the cholinergic site has been indicated by the binding of acetylcholine to a completely synthetic receptor comprised primarily of aromatic rings, which accommodate the quaternary ammonium group through a stabilizing cation-π electron interaction (Doughtery and Stauffer, 1990). Thus, despite the apparent divergence of the α5 sequence from other α-BTX binding α subunits, certain common features, such as an abundance of aromatic residues and amino acids able to participate in electrostatic and/or hydrogen bonding interactions, may indicate conserved structural elements that are required for α-BTX binding. The failure to find a common sequence motif by comparison of the sequence segments of α subunits known to bind α-BTX is similar to the search for the specific peptide sequences that bind to receptors involved in sorting proteins into cellular organelles and membrane compartments (reviewed in Verner and Schatz, 1988; Hartl et al., 1989; Keegstra, 1989; Pfanner and Neupert, 1990; Dice, 1990). In these cases it has been difficult to identify common recognition sequences, and instead compositional motifs define the targeting sequences of proteins that share a cellular compartment, in which certain amino acids or residues with similar physical characteristics are common, but the exact sequence is unimportant. For example, retention of certain proteins in the endoplasmic reticulum can be conferred by two appropriately placed C-terminal Lys residues (Jackson et al., 1990), and the signal motif for proteins targeted for lysosomal proteolysis involves amino acid residues of the recognition sequence KFERQ, in which the amino acid residues may occur in any order and can be reversed (Dice et al., 1990). In this

respect, it has been found using site-directed mutagenesis that although a single amino acid substitution can obliterate activity of an enzyme, secondary nonconservative substitutions can restore function (Blacklow and Knowles, 1990), also indicating that the order of presentation of critical structural residues is not always important. It is therefore conceivable that the important structural features of the divergent α-BTX binding proteins of the nAChR superfamily can be presented in a different sequence order.

Several disulfide bonds in the intact *Torpedo* α1 subunit may exist, including a vicinal disulfide between Cys at positions 192 and 193, which appears to be uniquely sensitive to reducing agents (Kao et al., 1984; Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988; Kellaris et al., 1989). A disulfide bridge between flanking cysteine residues requires a *cis,* nonplanar peptide bond, which is an unusual configuration likely to alter the reactivity of the sensitive cystine (Dayhoff, 1976; Schultz and Schirmer, 1979; Thorton, 1981; Ovchinnikov et al., 1985,1988). On the other hand, we found here, in agreement with previous determinations of the homologous *Torpedo* and vertebrate muscle peptides (Conti-Tronconi et al., 1991, McLane et al., 1991a), that ~30% of the Cys residues of the untreated peptide $\alpha5(180\text{-}199)_{\text{Native}}$ are oxidized as disulfide bridges, and as the amount of dimer present in these preparations is insufficient to account for the total peptide present in oxidized form, we must conclude that a disulfide bridge forms spontaneously between the vicinal Cys of the peptide. The role of vicinal disulfide in α-BTX binding to the *Torpedo* α1 sequence, however, remains equivocal. In the intact *Torpedo* nAChR, reduction, or reduction and alkylation, do not change the number of α-BTX binding sites (Moore and Raftery, 1979; Walker et al., 1981). Similarly, Mosckovitz and Gershoni (1988) have shown that alkylation of the vicinal cysteine residues with a large adduct, such as 3-(N-maleimidopropionyl)biocytin, does not interfere with α-BTX binding to either the isolated *Torpedo* α1 subunit, or to an 18 kDa proteolytic fragment containing the cholinergic site. The role of the adjacent cysteines in α-BTX binding to the intact *Torpedo* receptor has also been studied by mutagenesis of the cloned α1 subunit and

expression in *Xenopus* oocytes with the other unmodified subunits (Mishina et al., 1985). In these studies, replacement of either Cys192 or Cys193 reduced, but did not eliminate α-BTX binding (39% and 28% activity, respectively.)

The role of the vicinal cysteine residues at positions 191 and 192 of the α5 sequence can only partially be evaluated due to the tendency for peptides $\alpha5(180\text{-}199)_{C191}$ and $\alpha5(180\text{-}199)_{C192}$ to form dimers. Interestingly, the dimer of peptide $\alpha5(180\text{-}199)_{C192}$ retained some activity (~70-80% inhibition), whereas the dimer of peptide $\alpha5(180\text{-}199)_{C191}$ was only 40-50% as active as the native peptide, and its reduction and alkylation restored ~70% activity. It is possible that the $\alpha5(180\text{-}199)_{C192}$ peptide dimer formed through Cys191 mimics a disulfide bridge found in the native α5 receptor subunit. In the untreated peptide $\alpha5(180\text{-}199)_{Native}$, on the other hand, approximately 70-80% of the Cys residues have free sulfhydryl groups. Reduction and alkylation decreased the ability of this peptide to compete for ^{125}I-α-BTX binding by 20-30%, whereas oxidation with IOBA to monomer and dimer reduced to ~60% and ~40%, respectively, the α-BTX binding activity relative to untreated peptide. In studies of synthetic peptides corresponding to the *Torpedo* sequence segments α1(172-205) (Ralston et al, 1987), α1(185-196) (Neumann et al., 1986b, and α1(181-200) (Conti-Tronconi et al., 1991), reduction and alkylation has been reported to decrease α-BTX binding, and these findings have been interpreted as indicating that a disulfide bond between adjacent cysteine residues is important for α-BTX binding. In the case of the α5(180-199) sequence, however, our results demonstrate that both reduced and oxidized monomer forms of the peptide are active, and, in addition, ~70% of the α-BTX binding activity is retained after alkylation of the cysteine residues. Thus, although the cysteine residues may assist in the formation of a functional binding surface for α-BTX in the intact receptor, it does not appear that a vicinal disulfide bond is necessary for inducing an active conformation of the α5 subunit.

In conclusion, we have demonstrated that synthetic peptides with single amino acid substitutions can be used to identify critical residues involved in formation of the functional

binding surface of a α-BTX binding site on the α5 subunit. The use of synthetic peptides to study the effects of single amino acid changes has several advantages over mutagenesis and expression of cloned cDNA sequences, such as i) problems due to difference in the level of expression as a result of mutagenesis do not arise; ii) the resultant secondary structural changes in peptides is more amenable to analysis than are the intact subunits; and 3) more selective chemical modification is possible with peptides than is with the membrane bound or solubilized nAChR. Using synthetic peptides instead of the intact receptor to define the binding sites of ligands, however, requires that enough structural information be contained in a continuous segment of the protein sequence, that the sequence segment is able to fold into the active conformation in the absence of neighboring sequences, and that the affinity of that sequence in the absence of other segments, which may assist in ligand binding in the intact receptor, be high enough to be detectable by the assay method. In the case of the nAChR and α-BTX, we and other investigators have been fortunate.

REFERENCES

Allison, W.S. (1976) *Acc. Chem. Res. 9*, 293-299.

Aronheim, A., Eshel, Y., Mosckovitz, R., & Gershoni, J.M. (1988) *J. Biol. Chem. 263*, 9933-9937.

Ballivet, M., Nef, P., Couturier, S., Rungger, D., Bader, C. R., Bertrand, D., & Cooper, E. (1988) *Neuron 1*, 847-852.

Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S.J., & Ballivet, M. (1987) *Science 235*, 77-80.

Blacklow, S. C., & Knowles, J. R. (1990) *Biochemistry 29*, 4099-4108.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H.-P. H., & Raftery, M.A. (1979) *Biochemistry 18*, 1875-1883.

Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heinemann, S., & Patrick, J. (1985) *J. Neurosci. 5*, 2545-2552.

Boulter, J., Evans, K., Martin, G., Mason, P., Stengelin, S., Goldman, D., Heinemann, S., & Patrick, J. (1986a) *J. Neurosci. Res. 16*, 37-49.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., & Patrick, J. (1986b) *Nature **319***, 368-374.

Boulter, J., Connolly, J., Deneris, E., Goldman, D., Heinemann, S., & Patrick, J. (1987) *Proc. Natl. Acad. Sci. U. S. A. 84*, 7763-7767.

Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D.,Heinemann, S., Patrick, J. (1990) *J.. Biol. Chem. 265*, 4472-4482.

Buonanno, A., Mudd, J., Shah, V., & Merlie, J. P. (1986) *J. Biol. Chem. 261*, 16451-16458.

Burden, S. Y., Hartzell, M. C. & Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. U. S. A. 72*, 3245-3249

Cauley, K., Agranoff, B. W., & Goldman, D. (1989) *J. Cell Biol. 108*, 637-645.

Cauley, K., Agranoff, B. W., & Goldman, D. (1990) *J. Neurosci. 10*, 670-683.

Clark, D.G., Macmurchie, D.D., Elliot, E., Wolcott, R.G., Landel, A.M., & Raftery, M.A. (1972) *Biochemistry 11*, 1663-1668.

Conti-Tronconi, B. M., Gotti, C. M., Hunkapillar, M. W., & Raftery, M. A. (1982) *Science 218*, 1227-1229.

Conti-Tronconi, B.M., Dunn, S.M.J., Barnard, E.A., Dolly, J.O., Lai, F.A., Ray, N., & Raftery, M.A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5208-5212.

Conti-Tronconi, B.M., Tzartos, S., Spencer, S.R., Kokla, A., Tang, F., & Maelicke, A. (1988) in *Nicotinic Acetylcholine Receptors in the Nervous System*, (Clementi, F., Gotti, C., & Sher, E., eds.) NATO-ASI, series H, Vol. 25, pp. 119-136, Springer-Verlag, Berlin.

Conti-Tronconi, B.M., Fels, G., McLane, K., Tang, F., Bellone, M., Kokla, A., Tzartos, S., Milius, R. & Maelicke, A. (1989) in *Molecular Neurobiology of Neuroreceptors & Ion Channels*, (Maelicke, A., ed.) NATO-ASI, series H, Vol. 32, pp. 291-310, Springer-Verlag, Berlin.

Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., & Maelicke, A. (1990) *Biochemistry 29*, 6221-6230.

Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, T., Schroder, B., Reinhardt-Maelicke, S., & Maelicke, A. (1991) *Biochemistry 30*, 2575-2584

Couturier, S., Bertrand, D., Matter, J.-M., Herandez, M.-C., Bertrand, S., Millar, N., Valera, S., Barkas, T., & Ballivet, M. (1990) *Neuron 5*, 847-856.

Dayhoff, M. D. (1976) in *Atlas of Protein Sequence & Structure*, Vol. 5, pp. 77-268, National Biomedical Research Foundation, Washington, D. C.

Deneris, E. S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L. W., Patrick, J., & Heinemann, S. (1988) *Neuron 1*, 45-54.

Deneris, E. S., Boulter, J., Swanson, L. W., Patrick, J., & Heinemann, S. (1989) *J. Biol. Chem. 264*, 6268-6272.

Dice, J. F. (1990) *Trends Biochem. Sci. 15*, 305-309.

Dougherty, D. A., & Stauffer, D. A. (1990) *Science 250*, 1558-1560.

Duvoisin, R. M., Deneris, E. S., Patrick, J., & Heinemann, S. (1989) *Neuron 3*, 487-496.

Elliot, J., Blanchard, S.G., Wu, W., Miller, J., Strader, C.D., Hartig, P., Moore, H.-P., Racs, J., & Raftery, M. (1980) *Biochem. J. 185*, 667-677.

Fontana, A., Dalzoppo, D., Grandi, C., & Zambonin, M. (1981) *Biochemistry 20*, 6997-7004.

Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., & Heinemann, S. (1987) *Cell 48*, 965-973.

Hartl, F. U., Pfanner, N., Nicholson, D. W., & Neupert, W. (1989) *Biochim. Biophys. Acta 988*, 1-45.

Heinrickson, S., & Meredith, B. (1984) *Anal. Biochem. 136*, 65-74.

Hieber, V., Bouchey, J., Agranoff, B. W., & Goldman, D. (1990a) *Nucleic Acids Res. 18*, 5293.

Hieber, V., Bouchey, J., Agranoff, B. W., & Goldman, D. (1990b) *Nucleic Acids Res. 18*, 5307.

Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5131-5135.

Isenberg, K. E., Mudd, J., Shah, V., & Merlie, J. P. (1986) *Nucleic Acids Res. 14*, 5111-5118.

Jackson, M. R., Nilsson, T., & Peterson, P. A. (1990) *EMBO J. 9*, 3153-3162.

Kao, P. N., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., & Karlin, A. (1984) *J Biol. Chem. 259*, 11662-11665.

Kao, P. N., & Karlin, A. (1986) *J. Biol. Chem. 261*, 8085-8088.

Keegstra, K. (1989) *Cell 56*, 247-253.

Kellaris, K. V., Ware, D. K., Smith, S., & Kyte, J. (1989) *Biochemistry 28*, 3469-3482.

La Polla, R. J., Mixter-Mayne, K., & Davidson, N. (1984) *Proc. Natl. Acad Sci. U. S. A. 81*, 7970-7974.

Lindstrom, J., Einarson, E. & Tzartos, S. (1981) in *Methods in Enzymology* Vol. 74, (Langone, J.J., & van Vunakis, H. eds.) pp. 432-460, Academic Press, New York.

Loring, R. H., & Zigmond, R. E. (1988) *Trends Neurosci. 11*, 73-78.

Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37-46.

Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., & Patrick, J. (1990) *J. Neurochem. 55*, 632-640.

Mahoney, W. C., & Hermodson, M. A. (1979) *Biochemistry 18*, 3810-3814.

Marshall, J., Buckingham, S. D., Shingai, R., Lunt, G. G., Goosey, M. W., Darlison, M. G., Sattelle, D. B., & Barnard, E. A. (1990) *EMBO J. 9*, 4391-4398.

Martin, B. M., Chibber, B. A., & Maelicke, A. (1983) *J. Biol. Chem. 258*, 8714-8722.

McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1990) *J. Biol. Chem. 265*, 9816-9824.

McLane, K. E., Wu, X., & Conti-Tronconi, B. M. (1991a) *Biochemistry* 30,

McLane, K. E., Wu, X., Schoepfer, R., Lindstrom, J. M., & Conti-Tronconi, B. M. (1991b) *J. Biol. Chem., in press.*

McPherson, G. A. (1983) *Computer Prog. Biomed. 17,* 107-111.

Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J.,Takahashi, T., Kuno, M., & Numa, S. (1984) *Nature 307*, 604-608.

Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M., & Numa, S. (1985) *Nature 313*, 364-369.

Molinoff, P. B., Wolfe, B. B., & Weiland, G. A. (1981) *Life Sci. 29*, 427-438.

Moore, H.-P., & Raftery, M. A. (1979) *Biochemistry 18*, 1907-1919.

Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 1017-1022.

Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220-239.

Nef, P., Oneyser, C., Alliod, C., Couturier, S., & Ballivet, M. (1988) *EMBO J. 7*, 595-601.

Neubig, R. R., Krodel, E. K., Boyd, N. D. & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690-694.

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., & Fuchs, S. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3008-3011.

Neumann, D., Barchan, D., Fridkin, M., & Fuchs, S. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9250-9253.

Neumann, D., Barchan, D., Horowitz, M., Kochva, E., & Fuchs, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7255-7259.

Noda, M., Takahashi, H., Tanabe, T , Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., & Numa, S. (1982) *Nature 299*, 793-797.

Noda, M. , Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., & Numa, S. (1983a) *Nature 302*, 528-532.

Noda, M., Furutani, Y.,Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T.,Hirose, T., Inayama, S., & Numa, S. (1983b) *Nature 305*, 818-823.

Ohana, B., & Gershoni, J. M. (1990) *Biochemistry* 29, 6409-6415.

Ovchinnikov, Y. A., Lipkin, V. M., Shuvaeva, T. M., Bogachuk, A. P., & Shemyakin, V. V. (1985) *FEBS Lett 179*, 107-110.

Ovchinnikov, Y. A., Abdulaev, N. G., & Bogachuk, A. P. (1988) *FEBS Lett 230*, 1-5.

Papke, R. L., Boulter, J., Patrick, J., & Heinemann, S. (1989) *Neuron 3*, 589-596.

Pedersen, S. E., Dreyer, E. B., & Cohen, J. B. (1986) *J. Biol. Chem. 261*, 13735-13742.

Pfanner, N., & Neupert, W. (1990) *Ann. Rev. Biochem. 59*, 331-353.

Raftery, M. A., Hunkapillar, M. W., Strader, C. D., & Hood, L. E. (1980) *Science 208*, 1454-1457.

Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, L., & Lindstrom, J. (1987) *Biochemistry 26*, 3261-3266.

Rodbard, D., & Frazier, G. R. (1975) in *Methods in Enzymology*, Vol. 37 (Part B) (O'Malley, B. W., & Hardman, J. G. eds.) pp. 3-22, , Academic Press, New York.

Sawruk, E., Schloss, P., Betz, H., & Schmitt, B. (1990) *EMBO J. 9*, 2671-2677.

Scloss, P., Hermans-Borgmeyer, I., Betz, H., Gundelfinger, E. D. (1988) *EMBO J. 7*, 2889-2894.

Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S., & Lindstrom, J. (1988) *Neuron* 1, 241-248.

Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M., & Lindstrom, J. (1990) *Neuron 4*, 35-48.

Schultz, G. E., & Schirmer, R. H. (1979) in *Principles of Protein Structure* (Cantor, C. R., ed.), p. 55, Springer-Verlag, New York.

Thorton, J. M. (1981) *J. Mol. Biol. 151*, 261-287.

Torchinsky, Y. M. (1981) in *Sulfur in Proteins*, p.48, Pergamon Press, New York.

Verner, K., & Schatz, G. (1988) *Ann. Rev. Biochem. 59,* 331-353.

Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E. S., Swanson, L. W., Heinemann, S., & Patrick, J. (1988) *Science 240*, 330-334.

Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191-2199.

Whiting, P. J., Scoepfer, R., Swanson, L. W., Simmons, D. M., Lindstrom, J. M. (1987) *Nature 327*, 515-518.

Wilson, P. T., Gershoni, J. M., Hawrot, E., & Lentz, T. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 2553-2557.

Wilson, P. T., Lentz, T. L., & Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8790-8794.

Wilson, P. T., Hawrot, E., & Lentz, T. L. (1988) *Mol. Pharmacol. 34*, 643-651.

Wilson, P. T., & Lentz, T. L. (1988) *Biochemistry 27*, 6667-6674.

Yu, L., La Polla, R. J., & Davidson, N. (1986) *Nucleic Acids Res. 14*, 2539-2555.

Table I: IC50 Values of Single Amino Acid Substitution Peptide Analogs of the Sequence α5(180-199).

Amino Acid Substituted	IC_{50} (μM)[a]	Fold Increase Relative to Native
Native	3.0 (0.9)	1.0
M181	5.3 (2.5)	1.8
G182	6.4 (2.6)	2.1
S183	7.0 (1.6)	2.3
K184	N.D.	----
G185	8.9 (1.8)	3.0
N186	14.0 (0.8)	4.7
R187	N.D.	----
T188	4.5 (3.8)	1.5
D189	26.3 (7.8)	8.8
S190	5.5 (0.7)	1.8
C191	[~100]	> 30
C192	4.7 (3.1)	1.6
W193	11.7 (1.1)	3.9
Y194	13.9 (4.3)	4.6
P195	[~50]	> 15
Y196	11.3 (3.7)	3.8
I197	5.7 (3.1)	1.9
T198	6.0 (2.8)	2.0
Y199	7.3 (3.0)	2.4

[a]The IC_{50} values are the means of three experiments and standard deviations are given in in parentheses. Values that could not be determined by linear regression and were estimated by inspection are indicated by brackets. N.D. indicates that IC_{50} values were not determinable.

Table II. Inhibition of ^{125}I-α-BTX Binding to *Torpedo* nAChR By Peptides Treated with Sulfhydryl Reagents

	Percent Inhibition Relative to Native[a]			
Peptide	Untreated	IAA Only	DTT/IAA	IOBA/IAA
α5(180-199)$_{Native}$				
Monomer	100%	76%	69%	56%
Dimer	-----	-----	-----	35%
α5(180-199)$_{C1}$				
Monomer	-----	-----	7[illegible]%	-----
Dimer	44%	38%	-----	18%
α5(180-199)$_{C192}$				
Monomer	-----	-----	76%	-----
Dimer	80%	74%	-----	32%

[a]The relative level of inhibition of binding of ^{125}I-α-BTX to *Torpedo* nAChR by different peptides (~40 μM) was averaged for different experiments (n=6) and divided by the mean level of inhibition observed for untreated peptide α5(180-199)$_{Native}$ (77%).

FIGURE LEGENDS

Figure 1. Peptide Sequences and Codes

The rat α5 sequence segment corresponding to residues 180-199 (Boulter et al., 1990) was previously shown to bind ^{125}I-α-BTX (McLane et al., 1990). Each residue of this sequence was sequentially replaced in the synthesis of a panel of peptides corresponding to single amino acid substitutions of Gly for the native residue, or Ala in the case that the native residue was Gly. The sequences and codes of the substituted peptides are shown. For each α5(180-199) amino acid substituted, the final letter corresponds to the amino acid substituted, followed by the residue position in the intact α5 subunit. In the other figures the peptides are designated by abbreviations including only the amino acid substituted and its position number.

Figure 2. *Torpedo* Competition Assay Using Peptides Corresponding to Single Amino Acid Substitutions of the α5(180-199) Sequence.

Peptides (100 μg/ml) were preincubated with ^{125}I-α-BTX (20 nM, 110 cpm/fmole) prior to addition of 0.2 pmoles of membrane bound *Torpedo* nAChR. Assays were performed as described in "Experimental Procedures". "-BTX" refers to a negative control with *Torpedo* nAChR that has been preincubated with 20 μM unlabeled α-BTX for 10 min. prior to addition to assays, and "No Peptide" corresponds to assays conducted in the absence of peptide. The values given are the mean of triplicate determinations and the error bars are standard deviations.

Figure 3. Concentration Effects on Inhibition of ^{125}I-α-BTX Binding to *Torpedo* nAChR by Peptides Corresponding to Single Amino Acid Substitutions of the α5(180-199) Sequence.

Assays were performed as described in Figure 2 with the exception that the concentration of peptide was varied from 3-100 μM. The data points are the average of triplicate determinations. The graphical representation is a Hofstee plot (Molinoff et al., 1981), in which the IC_{50} values can be directly compared as the negative slopes of the linear regression lines. The IC_{50} values were determined using the analysis program EBDA (Munson and Rodbard, 1980).

Figure 4. P6 Chromatography of Untreated and Reduced and Alkylated Peptides α5(180-199)$_{Native}$, α5(180-199)$_{C191}$, and α5(180-199)$_{C192}$.

Untreated peptides (**top**) and peptides which had been reduced with DTT and alkylated with IAA (**bottom**) as described in "Experimental Procedures" were chromatographed on a Biogel P6 column (1.8x47 cm) in 10 mM potassium phosphate buffer, pH 7.0. The molecular weight markers are bradykinin (1060 daltons), renin substrate tetradecapeptide (1759 daltons), β-endorphin (3465 daltons), and growth hormone releasing factor (5041 daltons).

Figure 5. *Torpedo* nAChR Competition Assay Using Peptides α5(180-199)$_{Native}$, α5(180-199)$_{C191}$, and α5(180-199)$_{C192}$ Before and After Treatment with Sulfhydryl Reagents.

Peptides were modified as described in "Experimental Procedures" and purified as described in Figure 4. *Torpedo* nAChR competition assays were performed using 20 nM ^{125}I-α-BTX (380 cpm/fmole) and 0.3 pmoles of *Torpedo* nAChR per assay, and ~40 μM peptides. The results are expressed as the mean of triplicate determinations and the error bars are standard deviations.

Figure 6. Comparison of the α5(180-199) Sequence with Different α-BTX Binding α Subunits.

(A) Alignment of the Vicinal Cysteines.

The sequences are aligned with respect to the vicinal cysteine pair at positions 192/193, relative to the *Torpedo* α1 sequence (Noda et al., 1982). The sequences of the avian brain α-BGTBP α1 and α2 subunits (Schoepfer et al., 1990), and *Drosophila* SAD (Sawruk et al., 1990) are included for comparison. Conserved residues, or conservative substitutions relative to the Torpedo α1 sequence are indicated by a black background.

(B) Alignment of Arg187 of the α5 Subunit with Arg183 of the Avian Brain α-BGTBP α1 Subunit.

Conserved residues relavant to the "Discussion" are indicated by a black background.

$\alpha5(180\text{-}199)_{Native}$	AMGSKGNRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{M181}$	A**G**GSKGNRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{G182}$	AM**A**SKGNRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{S183}$	AMG**G**KGNRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{K184}$	AMGS**G**GNRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{G185}$	AMGSK**A**NRTDSCCWYPYITY
$\alpha5(180\text{-}199)_{N186}$	AMGSKG**G**RTDSCCWYPYITY
$\alpha5(180\text{-}199)_{R187}$	AMGSKGN**G**TDSCCWYPYITY
$\alpha5(180\text{-}199)_{T188}$	AMGSKGNR**G**DSCCWYPYITY
$\alpha5(180\text{-}199)_{D189}$	AMGSKGNRT**G**SCCWYPYITY
$\alpha5(180\text{-}199)_{S190}$	AMGSKGNRTD**G**CCWYPYITY
$\alpha5(180\text{-}199)_{C191}$	AMGSKGNRTDS**G**CWYPYITY
$\alpha5(180\text{-}199)_{C192}$	AMGSKGNRTDSC**G**WYPYITY
$\alpha5(180\text{-}199)_{W193}$	AMGSKGNRTDSCC**G**YPYITY
$\alpha5(180\text{-}199)_{Y194}$	AMGSKGNRTDSCCW**G**PYITY
$\alpha5(180\text{-}199)_{P195}$	AMGSKGNRTDSCCWY**G**YITY
$\alpha5(180\text{-}199)_{Y196}$	AMGSKGNRTDSCCWYP**G**ITY
$\alpha5(180\text{-}199)_{I197}$	AMGSKGNRTDSCCWYPY**G**TY
$\alpha5(180\text{-}199)_{T198}$	AMGSKGNRTDSCCWYPYI**G**Y
$\alpha5(180\text{-}199)_{Y199}$	AMGSKGNRTDSCCWYPYIT**G**

Figure 2

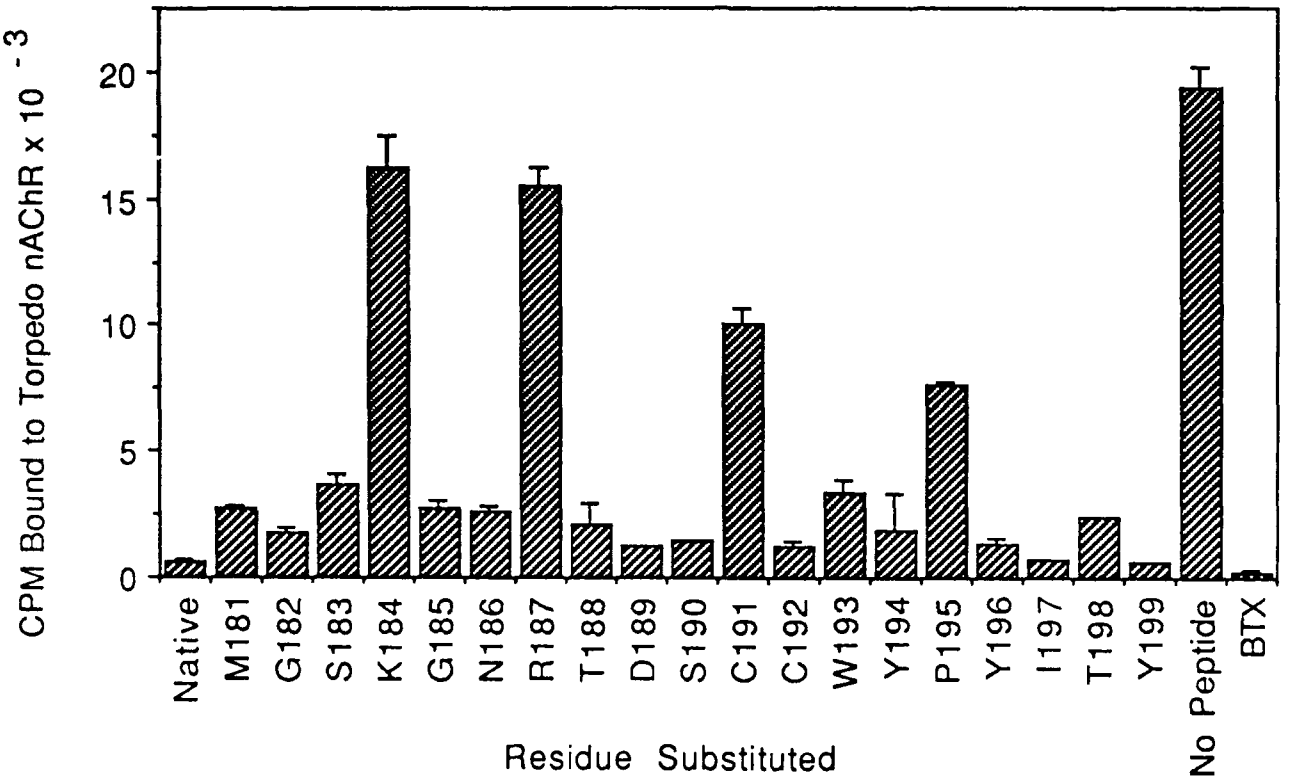
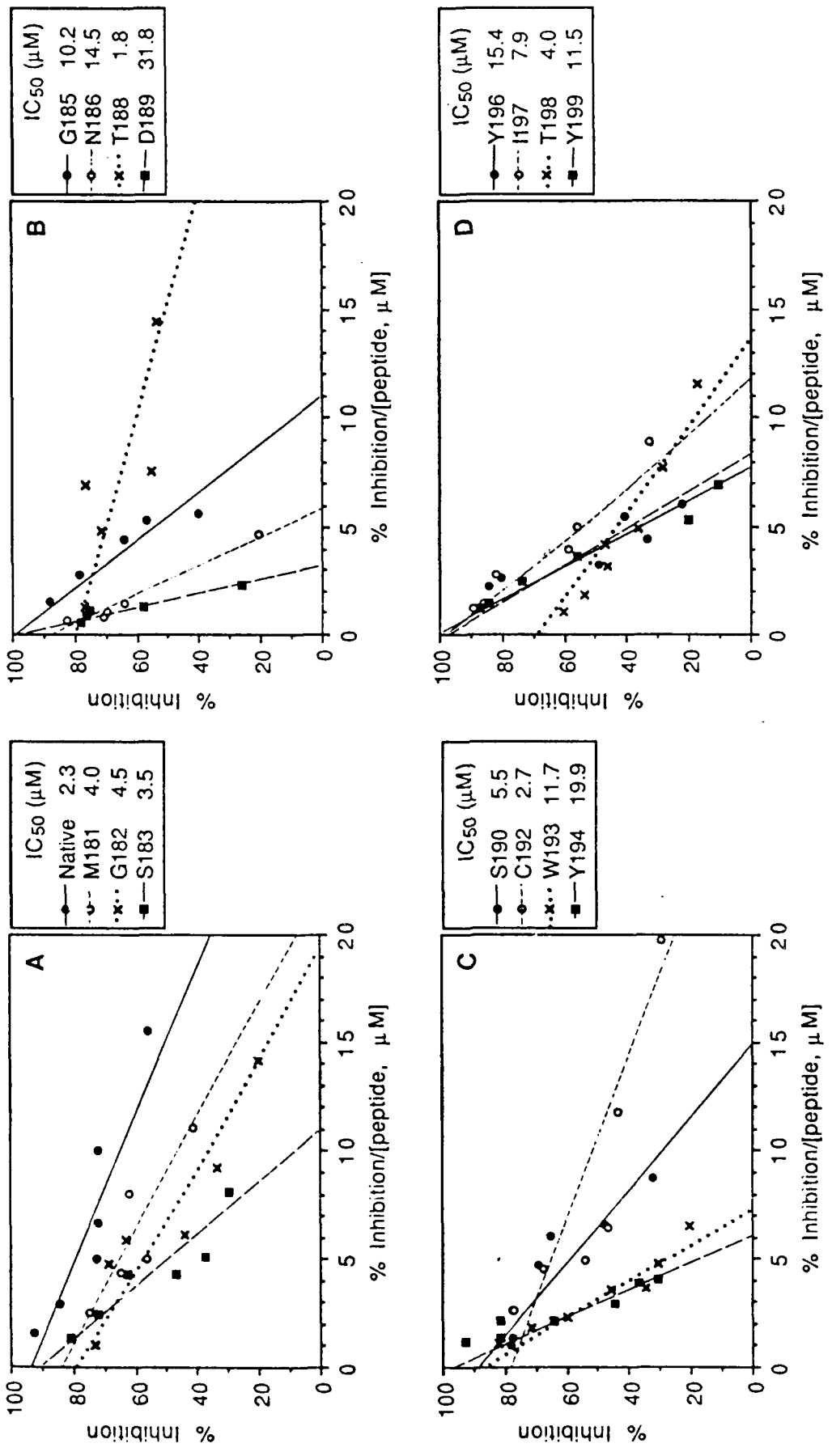
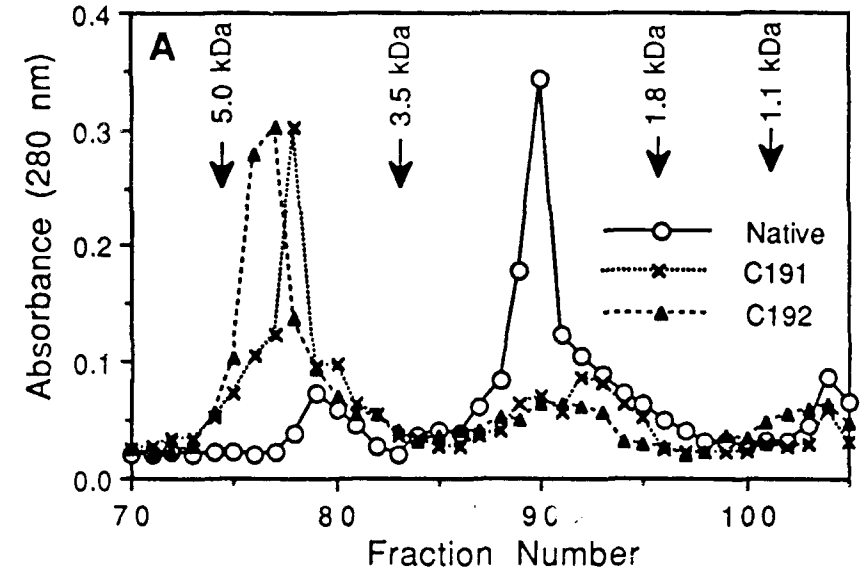
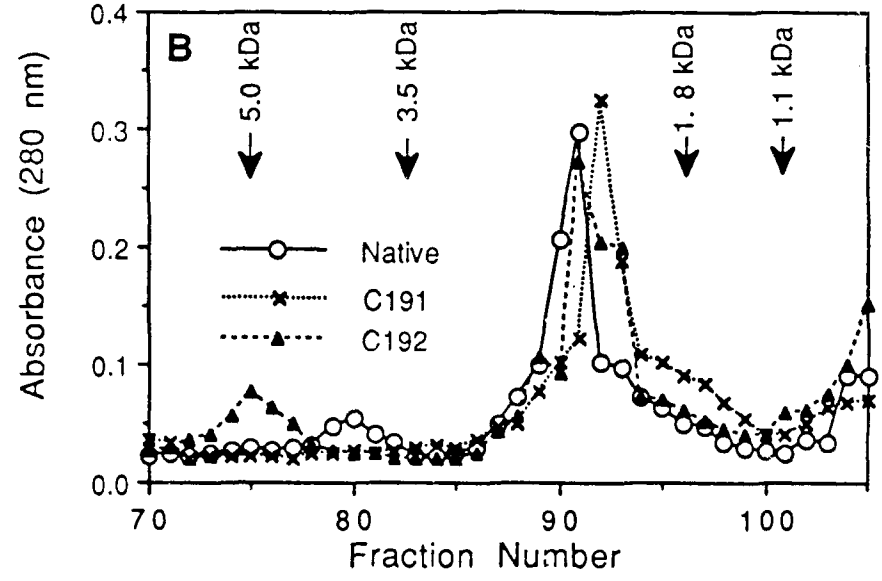
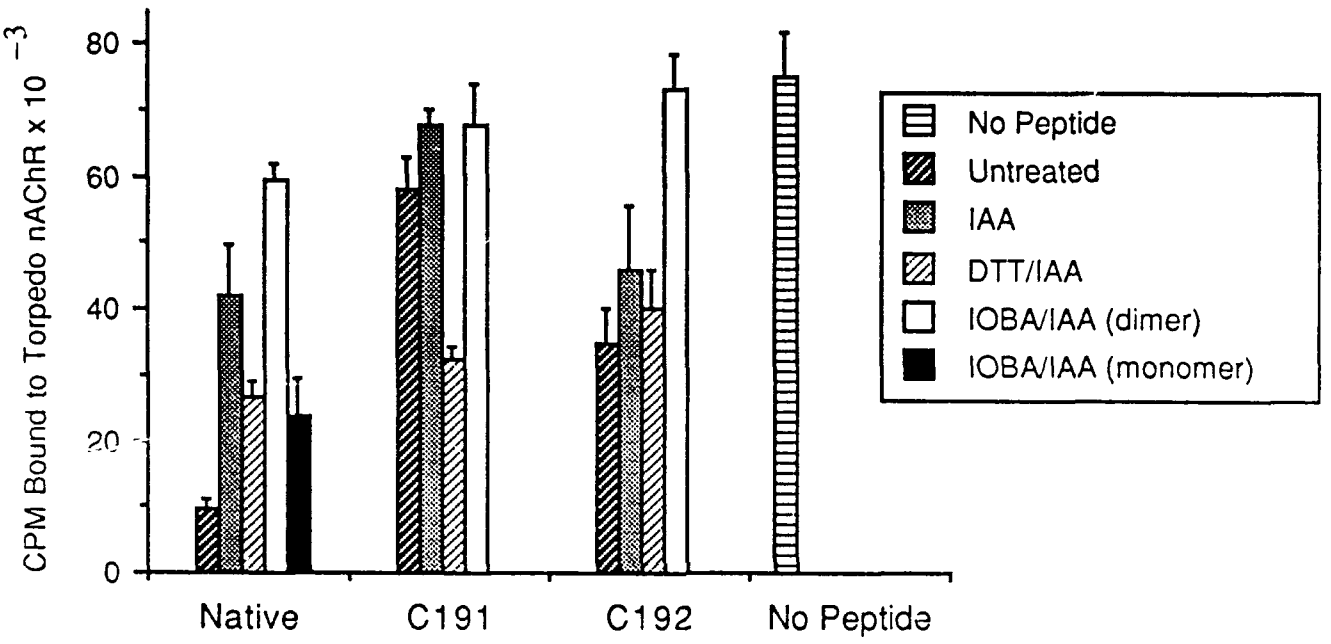

CPM Bound to Torpedo nAChR x 10 - 3
20
15
10
5
0
Native
M181
G182
S183
K184
G185
N186
R187
T188
D189
S190
C191
C192
W193
Y194
P195
Y196
I197
T198
Y199
No Peptide
BTX
Residue Substituted

Figure 3

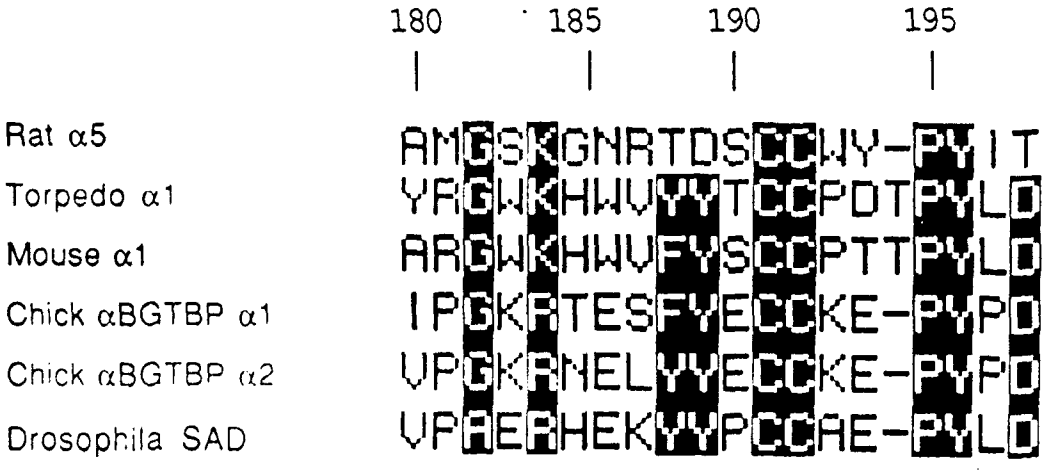

A
B
C
D
% Inhibition
% Inhibition/[peptide, μM]
IC50 (μM)
Native 2.3
M181 4.0
G182 4.5
S183 3.5
G185 10.2
N186 14.5
T188 1.8
D189 31.8
S190 5.5
C192 2.7
W193 11.7
Y194 19.9
Y196 15.4
I197 7.9
T198 4.0
Y199 11.5


A
5.0 kDa
3.5 kDa
1.8 kDa
1.1 kDa
Native
C191
C192
Absorbance (280 nm)
0.4
0.3
0.2
0.1
0.0
70
80
90
100
Fraction Number

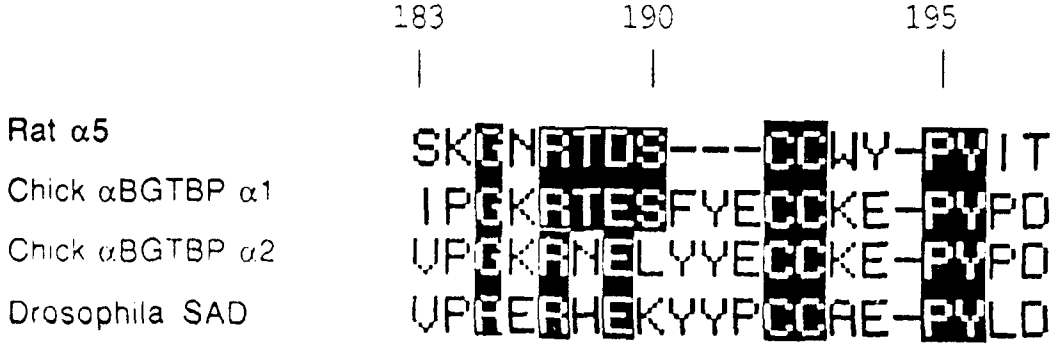

B
5.0 kDa
3.5 kDa
1. 8 kDa
1.1 kDa
Native
C191
C192
Absorbance (280 nm)
0.4
0.3
0.2
0.1
0.0
70
80
90
100
Fraction Number


CPM Bound to Torpedo nAChR x 10 $^{-3}$
80
60
40
20
0
Native
C191
C192
No Peptide
No Peptide
Untreated
IAA
DTT/IAA
IOBA/IAA (dimer)
IOBA/IAA (monomer)

A


180
185
190
195
Rat α5
Torpedo α1
Mouse α1
Chick αBGTBP α1
Chick αBGTBP α2
Drosophila SAD

B


183
190
195
Rat α5
Chick αBGTBP α1
Chick αBGTBP α2
Drosophila SAD

α-BUNGAROTOXIN AND THE COMPETING ANTIBODY WF6 INTERACT WITH DIFFERENT AMINO ACIDS WITHIN THE SAME CHOLINERGIC SUBSITE

ABSTRACT

In the nicotinic acetylcholine receptors (AChR), the sequence segment surrounding two invariant vicinal cysteinyl residues at positions 192 and 193 of the α subunit contains important structural component(s) of the binding site for acetylcholine and high molecular weight cholinergic antagonists, like snake α-neurotoxins. At least a second sequence region contributes to the formation of the cholinergic site. Studying the binding of α-bungarotoxin and three different monoclonal antibodies able to compete with α-neurotoxins and cholinergic ligands, to a panel of synthetic peptides as representative structural elements of the AChR from Torpedo, we recently identified the sequence segments α181-200 and α55-74 as contributing to form the cholinergic site (Conti-Tronconi et al., 1990). As a first attempt to elucidate the structural requirements for ligand binding to the subsite formed by the sequence α181-200, we have now studied the binding of α-bungarotoxin and of antibody WF6 to the synthetic peptide α181-200, and to a panel of peptide analogues differing from the parental sequence α181-200 by substitution of a single amino acid residue. CD-spectral analysis of the synthetic peptide analogues indicated that they all have comparable structures in solution, and they can therefore be used to analyze the influence of single amino acid residues on ligand binding.

Distinct clusters of amino acid residues, discontinuously positioned along the sequence 181-200, seem to serve as attachment points for the two ligands studied, and the residues necessary for binding of α-bungarotoxin are different from those crucial for binding of antibody WF6. In particular, residues at positions 188-190 (VYY) and 192-194 (CCP) were necessary for binding of α-bungarotoxin, while residues W_{187}, T_{191}, Y_{198} and the three residues at positions 193-195 (CPD) were necessary for binding of WF6.

Comparison of the CD-spectra of the toxin/peptide complexes, and those obtained for the same peptides and α-bungarotoxin in solution, indicates that structural changes of the ligand(s) occur upon binding, with a net increase of the β-structure component.

The cholinergic binding site is therefore a complex surface area, formed by discontinuous clusters of amino acid residues from different sequence regions. Such complex structural

arrangement is similar to the "discontinuous epitopes" observed by X-ray diffraction studies of antibody-antigen complexes [reviewed in Davies et al. (1988)]. Within this relatively large structure, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of the attachment points exist. This may be the molecular basis of the wide spectra of binding affinities, kinetic parameters and pharmacologic properties observed for the different cholinergic ligands.

INTRODUCTION

The nicotinic acetylcholine receptors (AChRs) are complex transmembrane proteins formed by homologous subunits [reviewed in McCarthy et al. (1986), Lindstrom et al. (1987) and Maelicke (1988)] which in peripheral tissues, such as fish electroplax and skeletal muscle, are assembled in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Conti-Tronconi et al., 1982). The AChRs are among the best characterized ligand-gated ion channels. Binding to the AChR of acetylcholine or of other cholinergic agonists induces transient openings of the channel (reviewed in Sine & Taylor, 1980; Conti-Tronconi & Raftery, 1982; Maelicke, 1988). Antagonists compete with agonists for AChR binding, and block the agonist-induced activation of the channel.

The α subunits contain two cysteinyl (Cys) residues at positions 192 and 193 [reviewed in McCarthy et al. (1987) and Maelicke (1988)] which are labeled by the cholinergic affinity labels (Kao et al., 1984; Dennis et al., 1988). α-Neurotoxins from elapid snakes like α-bungarotoxin (αBTX) are high affinity, slowly dissociating ligands of the AChR from peripheral tissues [reviewed in Klett et al., 1973; Blanchard et al., 1979; Sine and Taylor, 1980; Wang and Schmidt, 1980; Chang et al., 1984; Maelicke, 1988]. Several studies of the binding of ^{125}I-α-BTX and other similar neurotoxins to proteolytic, synthetic, or biosynthetic peptides suggested that a sequence segment of the AChR α subunit flanking and including Cys_{192} and Cys_{192}, contains important constituent elements of the cholinergic site (Wilson et al., 1984; Oblas et al., 1986; Neumann et al., 1986a,b; Gershoni, 1987; Ralston et al., 1987; Gotti et al., 1988; Aronheim et al., 1988; Conti-Tronconi et al., 1988, 1990; Wilson & Lentz, 1988; McLane et al., 1990a,b; Guy et al., 1990; Ohana & Gershoni, 1990).

Anti-AChR monoclonal antibodies (mAbs) can be produced, which bind with high affinity to the cholinergic binding site, and compete with α-neurotoxins and small cholinergic ligands (Watters & Maelicke, 1983; Fels et al., 1986). These mAbs and snake α-neurotoxins are high affinity, non-irreversible proteinaceous ligands which offer a good alternative to irreversible affinity labels (Kao et al., 1984; Dennis et al., 1988) for mapping of cholinergic binding sites. An advantage of their use is that they may identify the amino acid residues on the AChR surface with

which they interact, and answer the question as to whether the cholinergic binding site is formed by a very small area, i.e. by a single amino acid residue or a pair of residues, or whether it comprises a larger surface, formed by the interaction of clusters of several amino acid residues.

We have recently shown, studying the binding of αBTX and of three different monoclonal antibodies (mAbs) able to compete with αBTX and with other cholinergic ligands, to a panel of overlapping synthetic peptides screening the complete sequence of Torpedo α subunit, that the latter models applies to the cholinergic binding site. Both α-BTX and the mAbs bound to the same two discontinuous sequence regions of the AChR α-subunit, and the relative contributions to ligand binding of each of these subsites varied in a ligand-specific fashion (Conti-Tronconi et al., 1990). Specifically αBTX and mAb WF6 preferentially but not exclusively interacted with elements within the sequence region α181-200, while mAbs W2 and WF5 preferentially interacted with elements within the segment α55-74. Furthermore, we observed that binding of αBTX is facilitated by the existence of a disulfide bridge between cysteines α192 and α193, while WF6 binds with even higher affinity to the AChR (or to the synthetic peptide α181-200) when this disulfide bridge is reduced and the cysteines alkylated. These results, which are consistent with ligand-specific multipoint interactions between AChR and cholinergic ligands, prompted us to further investigate the interaction of proteinic ligands with their subsites on the Torpedo AChR molecule.

As a first step into this direction, we have synthesized a set of peptides homologous to α181-200. Each of these peptides differs by just one amino acid exchange from the parent sequence, resulting in the set of twenty peptides shown in Fig. 1. We have studied the binding of α-BTX and mAb WF6 to these peptide analogues, both using solid phase assays of the direct binding of α-BTX to the peptides, and competition assays of the ability of the peptides in solution to compete with native Torpedo AChR for binding to α-BTX and WF6. The contribution to αBTX and WF6 binding of each individual amino acid residue within the sequence α181-200 was determined. We found striking differences in the patterns of amino acids which are crucial for interaction with αBTX and with WF6. Therefore these ligands have distinct multipoint

attachments even at the level of this subsite. This mechanism of binding may account for the species specificity and tissue specificity of the binding of αBTX and WF6.

MATERIALS AND METHODS

Peptide synthesis and characterization. Peptides were synthesized according to Houghten (1985). Their purity was analyzed by high pressure liquid chromatography (HPLC), amino acid analysis and gas-phase sequencing as described before (Conti-Tronconi et al., 1990). The codes and the amino acid sequence of the peptides are reported in fig. 1.

Purification and radiolabeling of α-bungarotoxin (αBTX). αBTX was purified from Bungarus multicinctus venom (Biotoxins Inc., Fla.) according to Clark et al. (1972). The toxin was characterized and radiolabeled with ^{125}iodine as described before (Conti-Tronconi et al., 1990). It was separated from unreacted iodine and other low molecular weight contaminants by gel chromatography. The specific activity of ^{125}I-α-BTX was determined according to Blanchard et al. (1979).

Purification of monoclonal antibody WF6. The production and properties of antibody WF6 have been described previously (Watters & Maelicke, 1983; Fels et al., 1986; Schröder et al., 1989). It was purified by chromatography on protein A Sepharose 4B (Pharmacia) from supernatants of hybridoma cells raised in IgG-free medium. WF6 binds mutually exclusively with αBTX to membrane-bound or solubilized AChR from Torpedo electric tissue (Conti-Tronconi et al., 1990) but it blocks only one of the two acetylcholine binding sites per AChR monomer (Fels et al., 1986).

Preparation of AChR-rich Torpedo membrane fragments. AChR-rich membrane fragments were prepared from Torpedo californica or from Torpedo marmorata electric tissue according to established procedures (Elliot et al., 1980; Neubig et al., 1979; Fels et al., 1986). The source of starting material and the level of enrichment did not affect the results reported here.

Reduction/alkylation or oxidation of Cys192-193 disulfide bridge. Peptides (100-400 μM) in 10 mM sodium phosphate buffer, pH 8.0, were treated with dithiothreitol (final concentration 3.5 mM) for 1 hr at room temperature under nitrogen. Iodoacetamide was added to a final concentration of 10 mM and incubated for a few minutes at room temperature. Finally, the

mixture was dialyzed against 10 mM sodium phosphate buffer, pH 7.0 using dialysis tubing with a cut-off of 1000 Da (Spectraphor 1000).

For oxidation, the peptide solution (100-400 μM in 10 mM sodium phosphate buffer, pH 8.0) was made 1.75 mM in iodosobenzoic acid and was incubated for 1 hr at room temperature under nitrogen, followed by dialysis as described above. The concentration of sulfhydryl groups in the native, reduced/alkylated or oxidized peptide was determined by reaction with 5,5-dithio-bis-2-nitrobenzoic acid (Ellman et al., 1961) employing a molar extinction coefficient of the colored product of 13.600 at 420 nm.

Circular dichroism. The elipticity of the peptides was followed with a Jasco J 41 C spectropolarimeter (Jasco Inc., Easton, MD) interfaced by means of an Adalab A/D converter (Adalab-PC Interactive Microwave Inc., State College, PA) to an IBM-XT compatible personal computer. Water-jacketed, thermostated quartz cuvettes with path lengths of 0.5 cm, 1 mm and 0.1 mm were obtained from Hellma (Forest Hills, N.Y.). Calibration of the instrument was done with (±)-10-camphorsulphonic acid. Peptides were solubilized at pH 11 and spectra of the random coil structure were taken. The pH was then lowered to 7.4 at which some peptides were partially insoluble. In these cases, sample was centrifuged to eliminate undissolved material, and the supernatant was used for the recording of spectra. Protein concentation was assayed in the sample used to obtain the CD spectra. Absorption was recorded from 500 nm to 190 nm with a Perkin Elmer Lambda-5 Spectrophotometer, with the following absorption coefficients used: 2.88 at 1 mM concentration for peptides containing two tryptophanyl and four tyrosyl residues, and 2.03 for peptides containing two tryptophanyl and three tyrosinyl residues.

The values of the CD-spectra are expressed as mean elipticity per residue, θ, which has the dimensions of degrees * cm^{2-1} * $decimole^{-1}$. The mean residue weight (MRW) used was 128.2 for α181-200, 126.1 for $\alpha 181\text{-}200_{G188}$, 122.9 for $\alpha 181\text{-}200_{G189}$, 126 for $\alpha 181\text{-}200_{G191}$, and 125.9 for $\alpha 181\text{-}200_{G192}$ and $\alpha 181\text{-}200_{G193}$. The spectra of αBTX were taken in 5 mM Tris HCl, pH 7.4. Solubilized toxin (2 mg/ml) was used to solubilize an equimolar amount of peptide,

and the sample was then diluted to 1 mg/ml of toxin concentration. The differential spectra of peptides in the bound form were obtained by subtracting the spectrum of the free toxin.

Digitized data were analyzed with a fitting program based on the reference spectra of Chang et al. (1978), and the approximate relative contents of types of secondary structure were normalized to 100.

Binding of α-BTX to synthetic peptides .

(a) ***Dot blot assay .*** The peptide was spotted onto nitrocellulose strips, and the assay was performed with four or five different concentrations of ^{125}I-αBTX, as indicated in the legends to fig. 4. All other conditions as described in Conti-Tronconi et al. (1990). The dried strips were autoradiographed, and/or the dots were cut out and counted in a Beckman Gamma 5500 counter. Unspecific binding was determined in the presence of 80-100 μM unlabeled αBTX and was subtracted.

(b) ***Competition binding assay .*** ^{125}I-αBTX (2 pmoles) was incubated with given concentrations of peptide in PBS (see fig. 5) in a final volume of 100 μl for at least 4 h at 4°C. One pmol of membrane bound AChR in 5 μl PBS was added and, after incubation for 5 min at room temperature, the mixture was centrifuged at 13,000 rpm in a Sorvall SH-MT rotor. The pellet was resuspended in PBS and washed twice by centrifugation as described above. The supernatant was carefully sucked off, and the amount of ^{125}I-αBTX bound to the membrane was determined by counting in a Beckman Gamma 5500 counter.

Binding of mAb WF6 to the synthetic peptides .

(a) ***Dot blot assay .*** The assay was performed as described before (Conti-Tronconi et al. (1990) using ^{125}I-protein A [1.2 x 10^6 cpm/ml, labelled by the chloramin T method of Greenwood et al. (1963)] to monitor the concentration of bound WF6.

(b) ***Solid phase radioimmuno assay (SPRIA) .*** The assay protocol of Wilson and Lentz (1988) with the modifications of Conti-Tronconi et al. (1990) was employed.

(c) ***Enzyme linked immunosorbent assay (ELISA) .*** 50 μl of peptide solution (0.05 mg/ml in PBS) was added to each well of flexible microtiter plates (Falcon) followed by one

hour of incubation at 37°C. After washing and incubation with a solution of 3% bovine serum albumin (BSA) in PBS (150 µl/well, 1 h at 37°C), 50 µl/well of WF6 in PBS was added and the plates were incubated overnight at 4°C. After three washes with PBS-Tween (PBS containing 0.05% Tween 20), the wells were treated with 50 µl of a solution of peroxidase-linked rabbit anti-mouse IgG (Dakopatts), diluted 750 fold with 1% BSA-PBS. After two washes, 100 µl of substrate solution (0.15 M 1,2-phenylenediamin, 0.05% H_2O_2, 0.1 M sodium citrate, pH 5.0) was added The plates were incubated for constant periods of time, and the reaction was stopped by the addition of 25 µl 4.5 M sulfonic acid. Absorption at 490 nm was read in a ImmunoReader NG-2000 (InterMed).

To quantify this assay, the peptide was applied at a concentration at which it is completely bound to the wells. The binding capacity of the wells was determined by titration, determining the amount of unbound peptide by ELISA after absorption to a second well. Complete immobilization of peptides up to 0.2 µg/well was observed.

(d) ***Competition binding assay.*** 50 µl of Torpedo membrane fragments (1:1000 in 0.1 M $NaHCO_3$, pH 9.0) was added to each well and after incubation and several washes, the wells were further coated with BSA as described above. Aliquots of a solution of WF6 in PBS (2 nM) were incubated with various concentrations of synthetic peptides (0.16-5,200 nM) for 2 h at 37°C, after which time 50 µl of this mixture was added to the AChR-coated wells (triplicates). After 1 h of incubation at room temperature, the plates were washed as described above, followed by incubation with the peroxidase-linked second antibody. The enzyme reaction was performed at room temperature for 600 ± 5 sec following the procedure described above.

To quantify this assay, the concentration of immobilized AChR was determined by binding of ^{3}H-αBTX (spec. radioactivity 4.48 TBq/mmol, Amersham). AChR-coated microtiter wells were incubated with various concentrations of ^{3}H-αBTX overnight at 37°C, after which time the concentration of unbound αBTX was determined by counting in a scintillation counter the radioactivity of a 40 µl supernatant aliquot mixed with 5 ml of Quickzint 212 scintillation solution (Zinsser). After washing the plates three times with PBS-Tween, the wells were cut off, and the

concentration of bound ^{3}H-αBTX was determined as described above. Good agreement was observed between calculated and measured values for bound ^{3}H-αBTX.

Quantification of antibody binding data. We assumed that the concentration of immobilized peptide is identical to that in the incubation mixture (see ELISA assay for details), while the concentration of purified antibody was determined by UV-absorption at 280 nm (1.4 OD_{280} = 1 mg/ml). The relative binding can be assayed by ELISAs applying identical conditions for each antibody concentration studied. This is not a true equilibrium binding assay, and thus the binding affinities determined in this way may be considerably lower than the real binding affinity.

The experimental conditions of the competition ELISA were too complicated to allow sophisticated data analysis. To obtain a rough estimate for the binding affinity of the mutated peptide analogues, we determined "apparent K_I"-values, i.e. the concentrations of peptide at which the binding of WF6 to immobilized AChR was reduced by 50% under the experimental conditions used.

RESULTS

Structural properties of synthetic peptides . The amino acid sequences and the codes of the synthetic peptides used in this study are shown in Fig. 1. The parent peptide $T\alpha 181\text{-}200_{unmod}$ corresponds to the sequence segment 181-200 of the α-subunit of the AChR from Torpedo californica and Torpedo marmorata. The other peptides are analogues which differ from the parent peptide by single amino acid substitutions. The individual residues of the sequence Tα181-200 were changed to a glycin residue. The residue Gly_{183} or the parent sequence was changed to alanin. The purity of the peptides, assessed as described before (Conti-Tronconi et al., 1990), was 85% or higher.

In order to be suitable structural representations of the main cholinergic subsite of the AChR, the structure in solution of the parent peptide should not be random, and the peptide structure should not change substantially as a consequence of these single residue substitutions . These requirements were tested by CD spectral analysis. Fig. 2 shows the far UV CD-spectra of equimolar amounts of α181-200 and of three peptides containing single residue substitutions ($\alpha 181\text{-}200_{G188}$, $\alpha 181\text{-}200_{G189}$, $\alpha 181\text{-}200_{G191}$). Analysis according to Chang et al. (1978) yielded for $\alpha 181\text{-}200_{unmod}$ approx. 50% of β-pleated sheet structure and only 30% of random coil structure. The three modified peptides, which were selected because of their different ability to bind αBTX (see below), had similar structural contents as the parent peptide. These findings suggest that any differences found in the binding properties of the substituted peptides as compared to peptide $T\alpha 181\text{-}200_{unmod}$, are not due to gross changes in the peptide structure, but rather reflect the specific contribution to ligand binding of the substituted residue.

The above conclusion does not exclude the possibility of conformational adjustments upon ligand binding Indeed, as shown in Fig. 3, the CD-spectra of a mixture of peptide $\alpha 181\text{-}200_{unmod}$ and of αBTX is not simply the sum of the two independent spectra but rather shows an increase of 20-25% in β-structure at the expense of random coil structure. Similar effects were observed for peptides $\alpha 181\text{-}200_{G186}$ (Fig. 3C), $\alpha 181\text{-}200_{G191}$ (not shown) and $\alpha 181\text{-}200_{G199}$ (not shown), and were absent in mixtures of αBTX with peptides which do not mimic cholinergic

subsites (Fig. 3D). Thus the observed increase in β-pleated sheet structure was the result of peptide/αBTX interactions, and it may have been caused either by limited conformational adjustments of the peptide (or αBTX, or both) in the course of complex formation, or by the very nature of the interaction between two polypeptides of mainly β-structure (see Discussion). From these results, irrespective of their alternative interpretations, we concludes that peptide $\alpha 181\text{-}200_{unmod}$ and the set of single-residue substitued analogue peptides used here are suitable models for the main cholinergic subsite and its single residue mutations.

Binding of αBTX to the synthetic peptides. To study the binding of αBTX to the panel of homologous synthetic peptides, we employed the same two assays previously developed for mapping of the sequence regions of Torpedo AChR α-subunit which form the cholinergic binding site (Conti-Tronconi et al., 1990). In the "direct binding" assay, the peptides were immobilized on nitrocellulose strips and, after incubation with ^{125}I-αBTX and washing, the strips were autoradiographed and/or cut and counted in a Gamma counter. Fig. 4 shows the results of a representative experiment using four different concentrations of ^{125}I-αBTX. Consistently, the apparent K_D values for αBTX binding to the peptides either remained unchanged (i.e. approx. 1 μM), or went up by a factor of 4-6, or there was no measurable binding under the experimental conditions used. From the B_{max} values (not shown), it appeared that at least peptide $\alpha 181\text{-}200_{G196}$ did not bind to nitrocellulose as well as the other peptides, and this may cause an overestimate by this assay of the influence of residue T_{196} on αBTX binding. The same may apply to some of the modified peptides that do not show any measurable binding of αBTX. The opposite (i.e. somewhat larger Bmax values for the modified peptides) was observed for peptides in which any of the residues Y_{181}, G_{183}, W_{184}, H_{186} and W_{187} had been substituted.

In the "competition binding" assay, binding of ^{125}I-αBTX to the peptides occurred in solution, and the concentration of unbound ^{125}I-αBTX was determined by binding to an excess of Torpedo AChR (see Methods section). Although it is less sensitive than the direct binding assay described above, the competition assay avoided ambiguities resulting from variations in the peptides attachment to solid supports. Fig. 5 depicts the results of one such experiment, at three

different concentrations of inhibiting synthetic peptides. Because of the very large differences in the binding affinity of αBTX to native AChR, as compared to its binding affinity to the peptides, rather large concentrations of peptides must be used to observe measurable effects. A clear pattern of more or less essential residues for αBTX binding emerged from these experiments, which was largely consistent with the results of the dot blot assays (Fig. 4). Therefore the interaction of αBTX with the main cholinergic subsite formed by the sequence segment α181-200 appears to involve at least six amino acid residues (V_{188}, Y_{189}, Y_{190}, C_{192}, C_{193} and P_{194}). A few other residues seem to have an accessory role, because the effect of their substitution, although significant, were of lesser importance.

Binding of antibody WF6 to the synthetic peptides . Four assays were developed (see Method section) and were performed independently in both laboratories. Fig. 6 depicts the results of a dot blot experiment using four different dilutions of antibody. Again, as shown in Fig. 4 for αBTX binding, substitution of some amino acids profoundly affected the affinity of the peptides for the antibody, while other substitutions did not significantly affect WF6 binding. Similar results were obtained with the ELISA and SPRIA assays described in the Methods section (Fig. 9). To quantify the variations in binding affinity for the antibody induced by single amino acid substitutions within the sequence α181-200, the ELISA assay was performed under conditions of known concentrations of immobilized peptide and antibody (see Methods section). Because of the solid-phase nature of this assay, the "K_D"-values thus obtained (table II) should be considered upper limits of the true equilibrium dissociation constants of the antibody-peptide interaction. Fig. 7 summarizes one set of such data in terms of relative affinities of binding. Three ranges of affinities were apparent, as follows. Changes of less than a factor of two were considered insignificant, i.e. within the experimental range of error. Amino acid substitutions which caused affinity changes of up to a factor of 5 were considered "influential", those causing even larger changes in affinity (including "no measurable binding") were considered "essential". Following these criteria, we identified residues T_{191}, P_{194}, D_{195} and Y_{198} as essential, and W_{187}, C_{193}, T_{196}, P_{197} and L_{199} as influential for the interaction with antibody

WF6. Because, as discussed before for αBTX binding, the peptides vary considerably in their ability to adhere to the plastic surface (and possibly also in their conformation and accessibility when attached), and may compromise the conclusions drawn from direct binding studies.

To avoid such ambiguity, we investigated the ability of the peptides to bind mAb WF6 by the competition binding assay. After the test peptide and antibody WF6 were incubated in solution, the mixture was distributed into the wells of a microtiter plate, which had been coated with a fixed concentration of Torpedo AChR. The relative concentration of WF6 available for AChR binding was then assayed by ELISA (for details see Methods section). From the resulting plots, the concentration of peptide which reduced by 50% the binding of WF6 to immobilized AChR was deduced. This apparent "K_i"-value is related to the binding affinity of peptide and antibody in solution (table II).

Fig. 8 reports the related data in terms of relative affinities of binding. Consistent with the direct binding data, T_{191}, P_{194}, D_{195} and Y_{198} were identified as "essential" amino acids. In addition, substitution of residues W_{187} and C_{193} strongly affected the binding affinity for WF6, while substitution of residues Y_{190}, T_{196} and P_{197} had much smaller effects. The differences between the results of the dot blot assay and the competition ELISA assay may be due both to the higher sensitivity of the former assay, and to its potential pitfalls, as discussed above.

Fig. 9 depicts schematic representation of the effect of single residue substitution on WF6 binding, measured with the different techniques reported.

In summary, binding of the antibody WF6 to the main cholinergic subsite appears to involve at least six amino acid residues discontinuously distributed within the sequence α181-200. These residues only partially overlap those crucial for αBTX binding.

DISCUSSION

We previously showed that the cholinergic binding site on the α subunit of Torpedo AChR is not a single narrow sequence region, but rather it comprises a rather large area, formed by at least two sequence segments, folded together in the native structure of the AChR (Conti-Tronconi et al., 1990). As a conclusion of that study, we suggested that the interaction between AChR and α-BTX is not mediated by single residue or pair of them, as it may occur for the active site of enzymes, but that it involves instead a complex interaction of several amino acid residues on both the AChR and the α-BTX molecules, thus explaining the very high affinity with which this interaction occurs ($k_D < 10^{-2}$ M, reviewed in Lee, 1979). In the present paper we demonstrated that this is indeed the case, because even within the "subsite" formed by the residues within the sequence segment α181-200 the interaction of AChR with α-BTX is mediated by at least six amino acid residues, discontinuously distributed along the sequence region which forms this subsite. The conclusion of our results are in excellent agreement with earlier suggestions, based on the structure of α-neurotoxins (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988) and the properties of their binding to AChR (Martin et al., 1983), that these ligands recognize a large area on the extracellular AChR surface. Furthermore, the residues essential for ligand binding are different for the two proteinic ligands investigated (Fig. 9). Ligand-specific attachment points may therefore provide the molecular basis for the well established specificity in ligand recognition and primary response of nicotinic receptors (reviewed in Maelicke, 1984).

The experimental approach used for this study, which utilizes synthetic peptides as structural models for selected regions of receptors (Conti-Tronconi et al., 1989) deserves some comments. Successful application of this approach requires a sufficiently high affinity of the model peptides for the ligands. Albeit several orders of magnitude lower than the affinity of native AChR for αBTX (reviewed in Lee, 1979) and WF6 (Fels et al., 1986), peptide α181-200 still binds these ligands with affinities comparable to ACh binding to native AChR (Conti-Tronconi, 1990 and Fig. 4). This is probably due to the relatively high content of secondary structure of α181-200 in solution (Fig. 2). The binding affinity of peptide Tα181-200 for αBTX is within the

range found by other groups for αBTX binding to denatured α subunit or proteolytic/synthetic α subunit peptides (Haggerty & Frohener, 1981; Wilson et al., 1984; Neumann et al., 1986a,b; Gershoni, 1987). CD spectral analysis indicates that the content of secondary structure is not severely compromised by the single residue substitutions (Fig. 2), so that our studies may indeed expose the individual contributions to ligand binding of each amino acid within the sequence α181-200. This may be a lucky situation which may not apply to other sequence regions of the AChR or other receptors.

The sequence region α181-200 may form a part of the AChR molecule endowed with unusually high structural stability and resistance to denaturation also in the native form of this receptor. This is suggested by i) the ability of α-neurotoxins and WF6 to bind to the denatured α subunit (Haggerty & Frohener, 1981; Fels et al., 1986; Conti-Tronconi et al., 1990; Neumann et al., 1986a,b); ii) the finding that newly synthesized α subunit can bind αBTX at a much earlier stage of structural maturation than small cholinergic ligands (Merlie, 1984; Carlin et al., 1986) and iii) partially digested AChR preparations retain the ability to bind cholinergic ligands (Lindstrom et al., 1980; Fels et al., 1982; Boheim et al., 1987; Conti-Tronconi et al., 1982) while the ion channel contained within the AChR molecule not always can be activated under these conditions (Fels et al., 1982; Boheim et al., 1982). It seems possible that the structural stability of the sequence region α181-200 may be responsible for the residual binding activity of denatured or partially digested AChR preparations, while channel activation requires concerted binding to all subsites of the cholinergic binding region, including less stable ones, and is therefore lost upon denaturation/digestion of the AChR molecule.

The observed interaction of antibody WF6 with several residues even within this particular cholinergic subsite is in excellent agreement with the described interaction between antibodies and their protein antigen, as deduced by X-ray diffraction studies of antigen/antibody complexes. Antibodies bind to large areas (690-750 Å), and 14-16 residues on the protein antigen are directly involved in antibody binding [reviewed in Davies et al. (1988)]. Although in all cases studied so far antibody epitopes are formed by juxtaposition of several discrete segments of the antigen

sequence, brought in contact as a result of the tertiary folding of the protein, long segments may occur, as in the case of a lysozyme epitope which is formed by 16 residues arranged in two 9-residue loops. A similar situation may occur for the AChR area to which α-BTX and the three mAbs studied here bind, where several residues are in contact with the WF6 or α-BTX, six of which (Val_{188}, Tyr_{189}, Tyr_{190}, Cys_{192}, Cys_{193} and Pro_{194} for α-BTX, Trp_{187}, Tre_{191}, Cys_{193}, Pro_{194}, Asp_{195} and Tyr_{198} for WF6) are within the segment α181-200 and they have been identified here. Other not yet identified residues should reside within the segment α55-74, identified by our previous study (Conti-Tronconi et al., 1990) as contributing to the binding site of αBTX and WF6.

α-BTX binds to all known AChRs from vertebrate muscle but ot to cobra muscle AChR, nor to several rat neuronal AChRs, whose constituent α subunits have been sequences (rat α_2, α_3 and α_5 subunits). WF6 cross-reacts with the AChRs from both the tissues tested (Electrphorus electroplax and rat muscle, see Watters & Maelicke, 1983). Comparison of the sequence segments α181-200 from the different AChR, in the light of the results reported here, should explain the spectrum of binding of α-BTX and WF6 at the level of thd single amino acid residues. Table III shows an alignment of this sequence segment from Torpedo AChR, from different vertebrate muscle AChR, including cobra, and from the three rat neuronal α subunits known not to bind α-BTX. Conserved residues and conservative substitutions are indicated as boldface characteris. It clearly appears that the six residues crucial for α-BTX binding (indicated in Table III with an asterisk*) are all conserved or conservatively substituted in all vertebrate muscle AChR, with the only exception of cobra muscle AChR, where both Tyr_{189} and Pro_{194} are non-conservatively changed (Tyr -> Asn, Pro -> Leu). Also in the neuronal subunits which do not bind α-BTX three of these crucial residues (Val_{188}, Tyr_{189} and Pro_{194}) are consistently substituted non-conservatively. Tyr_{189} is changed to threonine in the human α subunit. This change, although conservative, may explain the described lesser ability to bind α-BTX of synthetic and biosynthetic peptides corresponding to the human sequence (Lentz et al., 1987; Wilson & Lentz, 1988; Ohana & Gershoni, 1990; Griesmann et al., 1990). The good affinity of α-BTX for native human

muscle AChR is well explained by the multipoint attachment reported here, so that substitution of one or few of the many residues involved in α-BTX may be functionally silent. Of the six residues crucial for WF6 binding (indicated in Table III with an arrow), four are conserved in all muscle AChR, but not in cobra AChR where both Trp_{187} and Pro_{194} are nonconservatively changed. The other two residues (Thr_{191} and Asp_{195}) are also non-conservatively changed in some muscle AChR and one may predict that WF6 may not be a "universal" probe for all peripheral nicotinic receptors.

The observed multipoint attachment of antibody WF6 to the main cholinergic subsite well explains why anti-AChR antibodies are rather species-specific and subunit specific, although extensive sequence homology exists among AChR from different species, and amongst the subunits of an individual AChR (Maelicke, 1988). As shown here, the exchange of a single residue in the epitope sequence may already reduce the binding affinity by a factor of 20-100 (table II, Figs. 7,8). Similarly, variations in distance between essential residues may strongly influence antibody binding. Thus, antibodies can discriminate between homologous sequences much more efficiently than may be expected on the basis of linear sequence differences. This is evident when considering the species-specificity of antibody WF6, in view of the minute sequence variations existing between AChRs from different species in the highly conserved region of the cholinergic subsite investigated here. A similar explanation may apply to the specificity of different α-neurotoxins for different AChR subtypes (Loring et al., 1984; Grant & Chiapinelli, 1985; McLane et al., 1990).

The observed multipoint attachment of αBTX to the main cholinergic subsite well explains the inability of previous studies to identifying within the α-neurotoxin molecule single amino acid residues (or pairs of them as essential for neurotoxicity (reviewed in Low, 1980). Studies on the binding properties of chemically modified α-cobratoxin have shown that none of its positively charged residues, nor tyrosine or tryptopahn, nor the integrity of its disulfide bridges is essential for binding to the AChR (Martin et al., 1983). Because the chemical modification of single amino acid residues or groups of residues induced only graded changes in binding affinity, it was

concluded that several structural elements of the toxin must be involved in binding. Tfese studies (Martin et al., 1983) and the detailed structural information obtained from X-ray and NMR studies (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988) suggested that a large area of the α-neurotoxin molecules formed by three sequence segments, i.e., both sides of the central loop and the lower tip of loop III, is involved in AChR binding. A corresponding large area on the AChR surface, formed by several residues within the sequence segments α181-200 and α55-74 (Conti-Tronconi et al., 1990) should make contact with the α-neurotoxins. The high affinity [K_D = 10^{-12}-10^{-10} M reviewed in Lee (1979)] and very slow reversibility [K_{off} ~ 2 x 10^{-4}-5 x 10^{-5} and 4 x 10^{-3} x 10^{-4} min^{-1} for α-BTX and α-CTX, respectively; e.g., see Blanchard et al. (1979) and Kang and Maelicke (1980)] of the binding of these toxins is consistent with interaction with the AChR via large surfaces, with formation of a large number of attachment points. In view of the results presented here, a testable model of the interaction between the sequence regions (and their essential residues) of neurotoxins and AChR within their complexes may soon be feasible.

A result of the present study which deserve further comment is the increase in β-pleated sheet structure determined for complexes of αBTX with the main cholinegic subsite. As shown by X-ray crystallographic analysis and NMR studies (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988), the "long" α-neurotoxins have a similar structure, consisting of a globular head the folding of which is determined by four disulfide bridges, and three loops hanging down from the head arranged side-by-side in a β-sheet and configuration. These loops are rather flexible and their regular β-configuration can, be disrupted like in crystals of α-BTX, where the regular β-sheet configuration of the region proposed with the AChR is disrupted by an irregular orientation of residue Lys_{27} (Love & Stroud, 1986). It was therefore proposed that α-neurotoxins may change their conformation upon binding to the AChR (Kistler & Stroud, 1987). Indeed spectral changes of fluorescen tissue upon binding to AChR strongly support the possibility of conformational adjustments of the α-neurotoxins induced by the binding (Kang and Maelicke, 1980; Johnson et al., 1984; Cheung et al., 1984). The results reported here further support the formation of extended β-structures between the sequence loops forming the binding area on the α-

neurotoxin, and sequence regions of the AChR binding the cholinergic site and AChR involving some of the amino acids identified here as attachment points between those molecules. Structural rearrangement of protein ligands upon binding have been described as for the interaction between a monoclonal antibody and lysozyme (Amit et al., 1986).

Table I: Structural contents of synthetic peptides obtained by CD-spectral analysis:

Amino acid exchange	α-helix	β-sheet	β-turn	random coil
$(V = G)_{188}$	0.09	0.67	0.00	0.24
$(Y = G)_{189}$	0.06	0.74	0.00	0.20
$(C = G)_{192}$	0.05	0.35	0.26	0.34
$(C = G)_{193}$	0.03	0.64	0.07	0.27

CD-spectra were taken as described in Methods and in the legend of Fig. 2. They were analyzed according to Chang et al. (1978).

Table II: Apparent equilibrium dissociation constants of the binding of antibody WF6 to the panel of synthetic peptides

Peptide	Direct ELISA		Competition ELISA	
	K_D (μM)	rel. affinity	K_I (μM)	rel. affinity
unmod	0.17	1.0	0.35	1.0
Y181	0.13	1.3	0.44	0.8
R182	0.20	0.9	0.42	0.8
G183	0.06	2.8	0.36	1.0
W184	0.11	1.5	0.5	0.7
K185	0.10	1.7	0.39	1.0
H186	0.18	0.9	0.76	0.5
W187	0.38	0.4	>100	<0.01
V188	0.17	1.0	0.63	0.6
Y189	0.10	1.7	0.70	0.5
Y190	0.12	1.4	2.1	0.2
T191	>1.5	<0.1	>100	<0.01
C192	0.04	4.2	0.89	0.4
C193	0.6	0.3	27	0.01
P194	>1.5	<0.1	38	0.01
D195	>1.5	<0.1	>100	<0.01
T196	0.48	0.4	0.95	0.4
P197	0.42	0.4	1.5	0.2
Y198	0.90	0.2	>100	<0.01
L199	0.50	0.3	0.49	0.7
D200	0.22	0.8	0.37	1.0

FIGURE LEGENDS:

Fig. 1: Internal code and amino acid sequence of peptide α181-200 and the panel of homologous peptides employed in this study. The single letter notation for amino acids was used.

Fig. 2: Far UV circular dichroism spectra of $\alpha 181\text{-}200_{unmod}$ and three homologous peptide of our panel.

$\alpha 181\text{-}200_{G188}$ (panel C) and $\alpha 181\text{-}200_{G189}$ (panel D). Solid lines refer to the respective peptide in 5 mM TrisHCl, pH 7.4; dotted lines refer to the unfolded peptide in Tris-HCl, pH 11. Digitized data were analysed by a fitting program based on the reference spectra of Chang et al. (1978) yielding for all four peptides similar contents of β-pleated sheet structure (50-60%) and of random coil structure (25-30%).

Fig. 3: Effect of complex formation with α-bungarotoxin on the far UV circular dichroism spectra of $\alpha 181\text{-}200_{unmod}$ and $\alpha 181\text{-}200_{G186}$.

αBTX was dissolved at a concentration of 1 mg/ml in 5 mM Tris/HCl, pH 7.4 and the spectrum was taken (panel A). To obtain the CD-spectrum of $\alpha 181\text{-}200_{unmod}$ bound to αBTX, the toxin was dissolved at a concentration of 2 mg/ml, an equimolar amount of the peptide was added, and the sample was diluted to 1 mg/ml toxin concentration. By subtracting the spectrum of free αBTX from that of the reaction mixture, the differential spectrum of bound $\alpha 181\text{-}200_{unmod}$ was obtained (dotted line in panel B). For comparison, also the spectrum of the peptide in the absence of αBTX is shown (solid line in panel B). Panel C shows the CD spectra of free and bound $\alpha 181\text{-}200_{G186}$ obtained in the same way as described above. The CD spectra of a control peptide (α91-110) in the absence and presence of αBTX is shown in panel D. The subtracted spectra for $\alpha 181\text{-}200_{unmod}$ and $\alpha 181\text{-}200_{G186}$ show red shifts of their negative maxima which calculate to an increase of 20-30% in β-structure at the expense of random coil and β-turn structure.

Fig. 4: Dose dependency of the binding of ^{125}I-αBTX to the panel of synthetic peptides, as investigated by the dot blot assay.

(A): Four concentrations of ^{125}I-αBTX (0.1, 0.5, 1.0 and 2 μM) were employed. Unspecific binding was determined for each point in the presence of an excess of unlabelled αBTX and was subtracted. Control refers to ^{125}I-αBTX binding to a non-related peptide, blank refers to ^{125}I-αBTX binding to nitrocellulose without any peptide attached.

We also determined αBTX binding to the overlapping peptide $\alpha 184\text{-}203_{unmod}$. Consistent with the finding that substitution of one each of the first three amino acids of $\alpha 181\text{-}200_{unmod}$ does not significantly alter αBTX binding, $\alpha 184\text{-}200_{unmod}$ binds the toxin with similar affinity as $\alpha 181\text{-}200_{unmod}$.

(B): K_D-values obtained from Scatchard analysis of the dot blot assays depicted in (A).

Fig. 5: Inhibition by synthetic peptides of ^{125}I-αBTX binding to native AChR.

Three concentrations of added peptides were employed. All other experimental conditiosn as described in the METHODS section. "None" refers to ^{125}I-αBTX binding to AChR-rich Torpedo membrane fragments in the absence of any peptide. "Unlab. αBTX" refers to unspecific binding, i.e. in the presence of an excess of unlabelled αBTX. Unspecific binding was not subtracted.

Fig. 6: Dose dependency of the binding of antibody WF6 to the panel of synthetic peptides, as determined by the dot blot assay.

Four different dilutions of the WF6 stock solution (1 mg/ml) were employed; , 1:500; , 1:1000; , 1:2000; , 1:4000. Unspecific binding of ^{125}I-labelled protein A was determined for each point in the absence of the mAb, and was subtracted. "Blank" refers to protein A binding (to WF6) in the absence of immobilized peptide; "control" refers to protein A binding (to WF6) in the presence of an unrelated peptide.

Fig. 7: Semi-quantitative ELISA of the binding of antibody WF6 to the panel of synthetic peptides.

ELISAs (triplicates) were performed for several different concentrations of antibody, and titration curves and Scatchard plots were constructed. The related "K_D"-values are listed in table

II. The figure depicts "relative affinities", i.e. reciprocal K_D-values divided by that for the parent peptide. Arrows denote off range values.

Fig. 8: Inhibition by synthetic peptides of the binding of WF6 to immobilized AChR, as determined by competition ELISA.

Antibody WF6 preincubated in the absence and presence of various concentrations of synthetic peptides was applied onto microtiter wells coated with a fixed concentration of Torpedo AChR. The relative concentration of AChR-bound antibody was assayed by ELISA as described in the METHODS section. Titration curves were constructed, and the concentration of the respective peptide inhibiting by 50% the binding of WF6 to AChR was deduced ("K_I"-value). The related data are listed in table II. The figure depicts "relative affinities" of WF6 binding.

Fig. 9: Schematic representation of the amino acid residues within peptide $\alpha 181\text{-}200_{unmod}$ being "essential" or "influential" for the binding of αBTX and WF6.

The scheme summarizes the results of this study including those not shown here. Black squares refer to essential residues, dotted squares refer to influential residues.

REFERENCES

Amit, A.G., Mariuzza, R.A., Philips, S.E.V. & Poljak, R.J. (1986) Science 233, 747-753.

Aronheim, A., Eshel, Y., Mosckovitz, R. & Gershoni, J.M. (1988) J. Biol. Chem. 263, 9933-9937.

Basus, V.J., Billeter, M., Love, R.A., Stroud, R.M. & Kunitz, I.D. (1988) Biochemistry 27, 2763-2771.

Blanchard, S.G., Quast, U., Reed,K., Lee, T., Schimerlik, M.I., Vandlen, R., Claudio, T., Strader, C.D., Moore, H.-P.H. & Raftery, M.A. (1979) Biochemistry 18, 1875-1883.

Boheim, G., Hanke, W., Barrantes, F.J., Eibl, H., Sakmann, B., Fels, G. & Maelicke, A. (1981) Proc. Natl. Acad. Sci. USA 78, 3586-3590.

Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stenegelin, S., Martin, G., Heinemann, S. & Patrick, J. (1985) J. Neurosci. 5, 2545-2552.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. & Patrick, J. (1986) Nature 319, 368-374.

Carlin, B.E., Lawrence, J.C. Jr., Lindstrom, J.M. & Merlie, J.P. (1986) Proc. Natl. Acad. Sci. USA 83, 498-502.

Chang, C.T., Wu, C.-S.C. & Yang, Y.T. (1978) Analyt. Biochem. 91, 13-31.

Chang, R.S.L., Potter, L.T. & Smith, I.S. (1977) Tissue & Cell 9, 623-644.

Cheung, A.T., Johnson, D.A. & Taylor, P. (1984) Biophys. J. 45, 447-454.

Clark, D.G., Macmurchie, D.D., Elliott, E., Wolcott, R.G., Landel, A.M. & Raftery, M.A. (1972) Biochemistry 11, 1663-1668.

Conti-Tronconi, B.M., Kokla, A., Maelicke, A., Milius, R., Raftery, M.A., Spencer, S.R., Tang, F. & Tzartos, S. (1988) NATO ASI Ser., Ser. Vol. H 25, 119-136.

Conti-Tronconi, B.M., Gotti, C.M., Hunkapiller, M.W. & Raftery, M.A. (1982) Science 218, 1227-1229.

Conti-Tronconi, B.M. & Raftery, M.A. (1982) Ann. Rev. Biochem. 51, 491-530.

Conti-Tronconi, B.M., Tang, F., Diethelm, B.M., Spencer, S.R., Reinhardt-Maelicke, S. & Maelicke, A. (1990) Biochemistry 29, 6221-6230.

Conti-Tronconi, B.M., Dunn, S.M.J. & Raftery, M.A. (1982) Biochemistry 21, 893-899.

Davies, D.R., Sheriff, S. & Padlan, E.A. (1988) J. Biol. Chem. 263, 10541-10544.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chretien, M. & Changeux, J.-P. (1988) Biochemistry 27, 2346-2357.

Ellmann, G.L., Courtney, K.S., Andres, V. & Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88-95.

Elliot, J., Blanchard, S.G., Wu, W., Miller, J., Strader, C.D., Martig, P., Morre, H.-P.H., Racs, J. & Raftery, M.A. (1980) Biochem. J. 185, 667-677.

Fels, G., Wolff, E.K. & Maelicke, A. (1982) Eur. J. Biochem. 127, 31-38.

Fels, G., Plümer-Wilk, R., Schreiber, M. & Maelicke, A. (1986) J. Biol. Chem. 261, 15746-15754.

Gershoni, J.M. (1987) Proc. Natl. Acad. Sci. USA 84, 4318-4321.

Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J. & Heinemann, S. 91987) Cell 48, 965-973.

Gotti, C., Frigerio, F., Bolognesi, M., Longhi, R., Racchetti, G. & Clementi, F. (1988) FEBS Lett. 228, 118-122.

Grant, G.A. & Chiapinelli, V.A. (1985) Biochemistry 24, 1532-1537.

Greenwood, F.C. & Hunter, W.M. (1963) Biochem. J. 89, 114-123.

Griesmann, G.E., McCormick, D.J., De Aizpurua, H.J. & Lennon, V.A. (1990) J. Neurochem. 54, 1541-1547.

Haggerty, J.G. & Froehner, S.C. (1981) J. Biol. Chem. 256, 8294-8297.

Houghten, R.A. (1985) Proc. Natl. Acad. Sci. USA 82, 5131-5135.

Isenberg, K.E., Mudd, K., Shak, V. & Merlie, J.B. (1986) Nucleic Acids Res. 14, 5111-5117.

Johnson, D.A., Voet, J.G. & Taylor, P. (1984) J. Biol. Chem. 259, 5717-5725.

Kang, S. & Maelicke, A. (1980) J. Biol. Chem. 255, 7326-7332.

Kao, P.N., Dwork, A.J., Koldary, R.R.J., Silver, M.L., Wideman, J., Stein, S. & Karlin, A. (1984) J. Biol. Chem. 259, 11662-11665.

Kistler, J., Stroud, R.M., Klymkowsky, M.W., Lalancette, R.A. & Fairclough, R.H. (1982) Biophys. J. 37, 371-383.

Klett, R.P., Fulpuius, B.W., Cooper, D., Smith, M., Reich, E. & Possani, L.D. (1973) J. Biol. Chem. 248, 6841-6853.

Lee, C.Y. (1979) Adv. Cytopharmacol. 3, 1-16.

Lentz, T.L., Hawrot, E. & Wilson, P.T. (1987) Proteins: Struct., Funct., Genet. 2, 298-307.

Lindstrom, J., Schoepfer, R. & Whiting, P. (1987) Mol. Neurobiol. 1, 281-337.

Lindstrom, J., Gullick, W., Conti-Tronconi, B. & Ellisman, M. (1980) Biochemistry 19, 4791-4795.

Loring, R.H., Chiapinelli, V.A., Zigmond, R.E. & Cohen, J.B. (1984) Neuroscience 11, 989-999.

Love, R.A. & Stroud, M. (1986) Protein Eng. 1, 37.

Low, B.W. (1980) in: Handbook of Experiment. Pharmacol. Vol. 52 (Lee, C.Y., ed.), pp. 213-257, Springer, Berlin.

McCarthy, M.P., Earnest, J.P., Young, E.F., Choe, S. & Stroud, R.M. (1986) Ann. Rev. Neurosci. 9, 383-413.

McLane, K.E., Wu, X. & Conti-Tronconi, B.M. (1990a) J. Biol. Chem. 265, 9816-9824.

McLane, K.E., Tang, F. and Conti-Tronconi, B.M. (1990b) J. Biol. Chem. 265, 1537-1544.

Maelicke, A. (1984) Angew. Chem. Int., Ed. Engl. 23, 195-21.

Maelicke, A. (1988) In: Handbook of Experimental Pharmacology (eds. G.V.R. Born, A. Farah, H. Herken, A.D. Welch) Vol.: "The Cholinergic Synapse" (Whittaker, V.P., Ed.) Vol. 86, pp. 267-313, Springer-Verlag Berlin-Heidelberg-New York-Tokyo.

Martin, B.M., Chibber, B.A. & Maelicke, A. (1983) J. Biol. Chem. 258, 8714-8721.

Martin, B.M., Chibbler, B.A. & Maelicke, A. (1983) J. Biol. Chem. 258, 8714-8722.

Merlie, J.P. (1984) Cell 36, 573-575.

Neubig, R.R., Krodel, E.K., Boyd, N.D. & Cohen, J.B. (1979) Proc. Natl. Acad. Sci. USA 76, 690-694.

Neumann, D., Barchan, D., Fridkin, M. & Fuchs, S. (1986a) Proc. Natl. Acad. Sci. USA 83, 9250-9253.

Neumann, D., Barchan, D., Safran, A., Gershoni, J.M. & Fuchs, S. (1986b) Proc.Natl. Acad. Sci. USA 83, 3008-3011.

Neumann, D., Barchan, D., Horowitz, M., Kochva, E. & Fuchs, S. (1989) Proc. Natl. Acad. Sci. USA 86, 7255-7259.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. & Numa, S.(1982) Nature 299, 793-797.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Yanano, T., Hirose, T., Inayama, S. & Numa, S. (1983) Nature 302, 818-823.

Oblas, B., Singer, R.H. & Boyd, N.D. (1986) Mol. Pharmacol. 29, 649-656.

Ohana, B. & Gershoni, J.M. (1990) Biochemistry 29, 6409-6415.

Ralston, S., Sarin, V., Thanh, H.L., Rivier, J., Fox, J.L. & Lindstrom, J. (1987) Biochemistry 26, 3261-3266.

Schoepfer, R., Luther, M. & Lindstrom, J. (1988) FEBS Letters 226, 235-240.

Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S. & Lindstrom, J. (1988) Neuron 1, 241-248.

Schröder, H., Zilles, K., Maelicke, A. & Hajos, F. (1989) Brain Research 502, 287-295.

Sine, S.M. & Taylor, P. (1980) J. Biol. Chem. 255, 10144-10156.

Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E.S., Swanson, L.W., Heinemann, S. & Patrick, J. (1988) Science 240, 330-334.

Walkinshaw, M.D., Saenger, W. & Maelicke, A. (1980) Proc. Natl. Acad. Sci. USA 85, 2899-2903.

Walkinshaw, M.D., Saenger, W. & Maelicke, A. (1980) Proc. Natl. Acad. Sci. USA 77, 2400-2404.

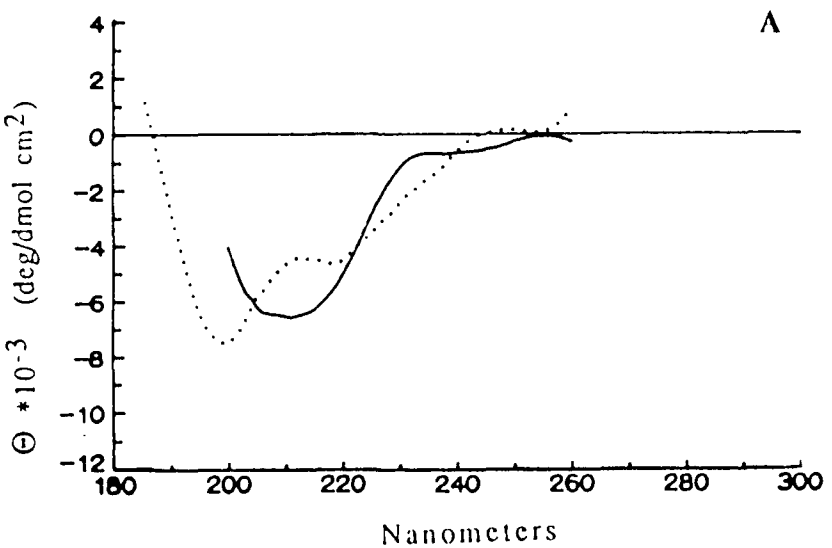
Wang, G.K. & Schmidt, J. (1980) J. Biol. Chem. 255, 11156-11162.

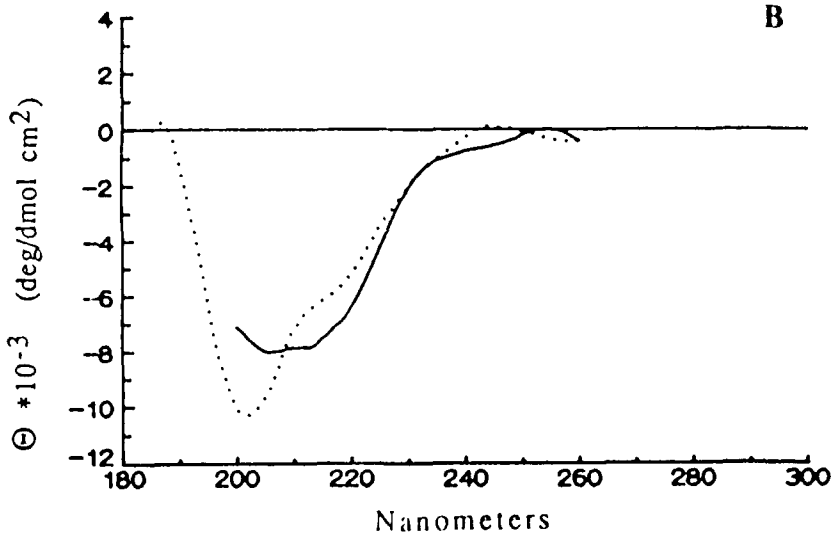
Watters, D. & Maelicke, A. (1983) Biochemistry 22, 1811-1819.

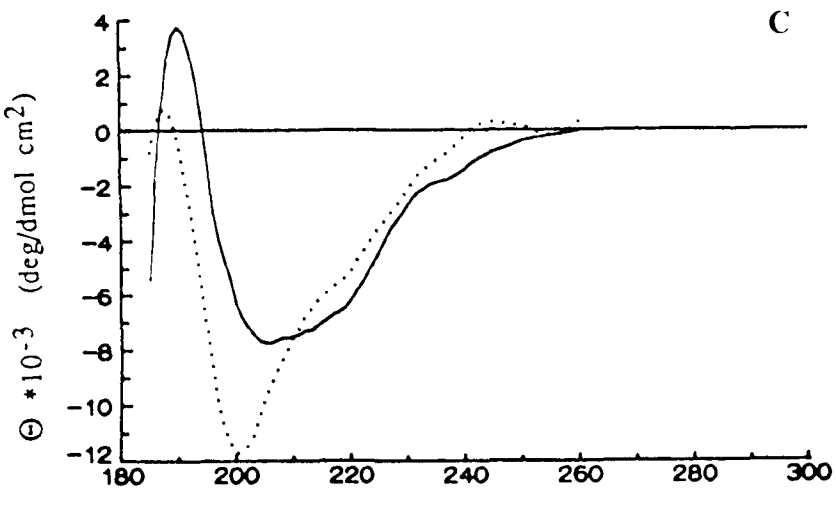
Wilson, P.T., Gershoni, J.M., Haawrot, E. & Lentz, T.L. (1984) Proc. Natl. Acad. Sci. USA 81, 2553-2557.

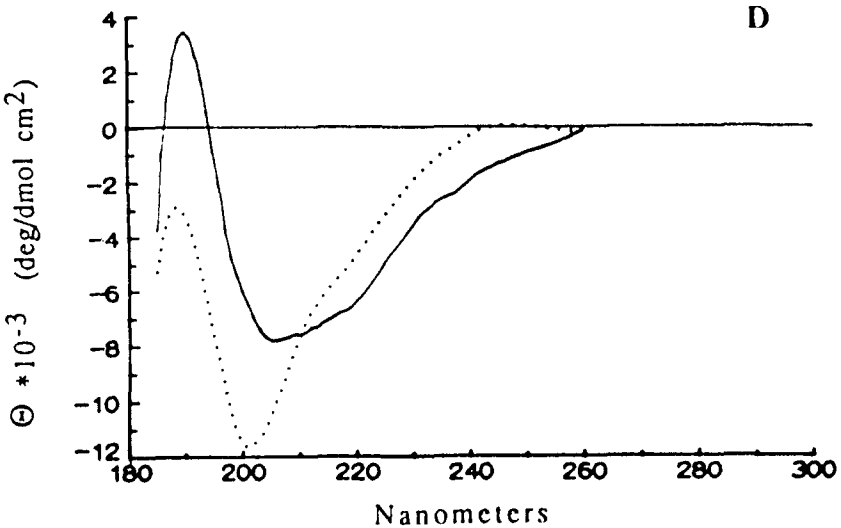
Wilson, P.T. & Lentz, T.L. (1988) Biochemistry 27, 6667-6674.

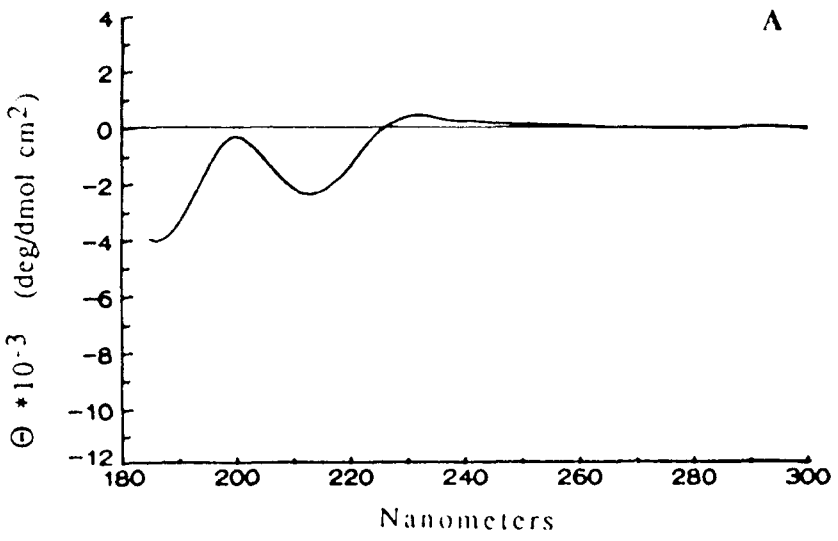
Tα181-200$_{unmod}$	YRGWKHWVYYTCCPDTPYLD
Tα181-200$_{G181}$	**G**RGWKHWVYYTCCPDTPYLD
Tα181-200$_{G182}$	Y**G**GWKHWVYYTCCPDTPYLD
Tα181-200$_{A183}$	YR**A**WKHWVYYTCCPDTPYLD
Tα181-200$_{G184}$	YRG**G**KHWVYYTCCPDTPYLD
Tα181-200$_{G185}$	YRGW**G**HWVYYTCCPDTPYLD
Tα181-200$_{G186}$	YRGWK**G**WVYYTCCPDTPYLD
Tα181-200$_{G187}$	YRGWKH**G**VYYTCCPDTPYLD
Tα181-200$_{G188}$	YRGWKHW**G**YYTCCPDTPYLD
Tα181-200$_{G189}$	YRGWKHWV**G**YTCCPDTPYLD
Tα181-200$_{G190}$	YRGWKHWVY**G**TCCPDTPYLD
Tα181-200$_{G191}$	YRGWKHWVYY**G**CCPDTPYLD
Tα181-200$_{G192}$	YRGWKHWVYYT**G**CPDTPYLD
Tα181-200$_{G193}$	YRGWKHWVYYTC**G**PDTPYLD
Tα181-200$_{G194}$	YRGWKHWVYYTCC**G**DTPYLD
Tα181-200$_{G195}$	YRGWKHWVYYTCCP**G**TPYLD
Tα181-200$_{G196}$	YRGWKHWVYYTCCPD**G**PYLD
Tα181-200$_{G197}$	YRGWKHWVYYTCCPDT**G**YLD
Tα181-200$_{G198}$	YRGWKHWVYYTCCPDTP**G**LD
Tα181-200$_{G199}$	YRGWKHWVYYTCCPDTPY**G**D
Tα181-200$_{G200}$	YRGWKHWVYYTCCPDTPYL**G**

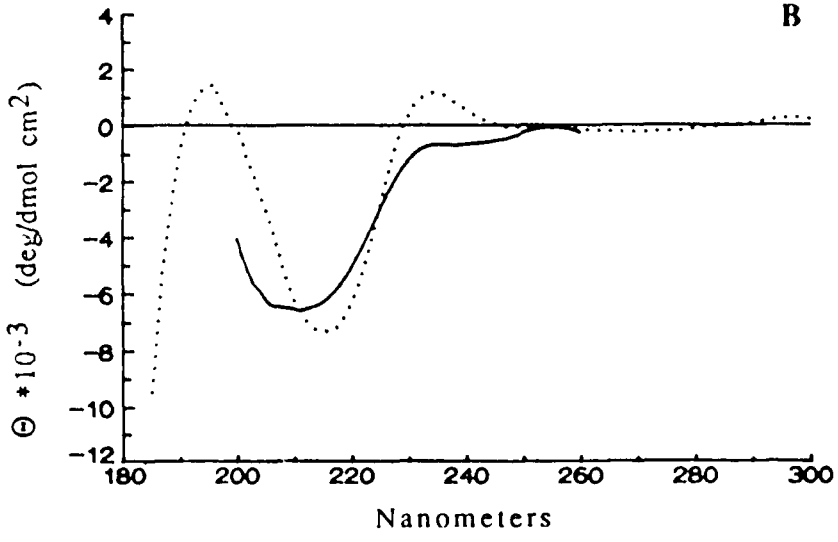

A
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

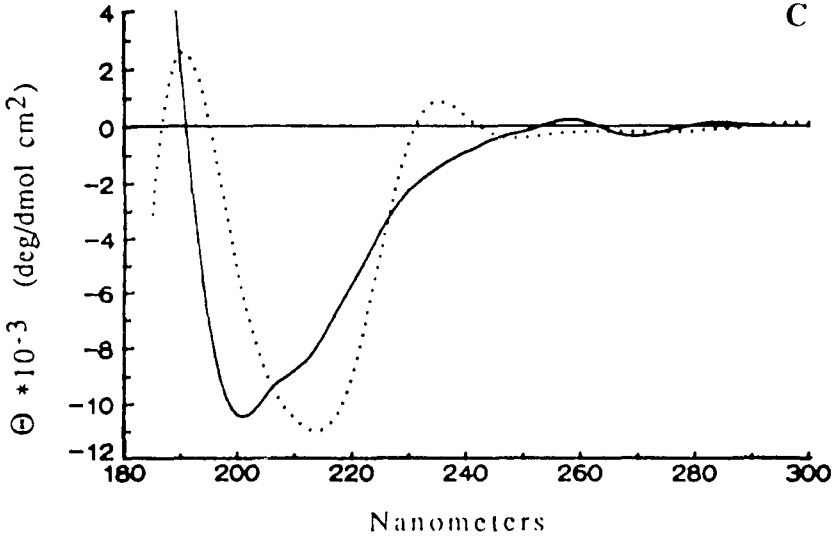

B
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

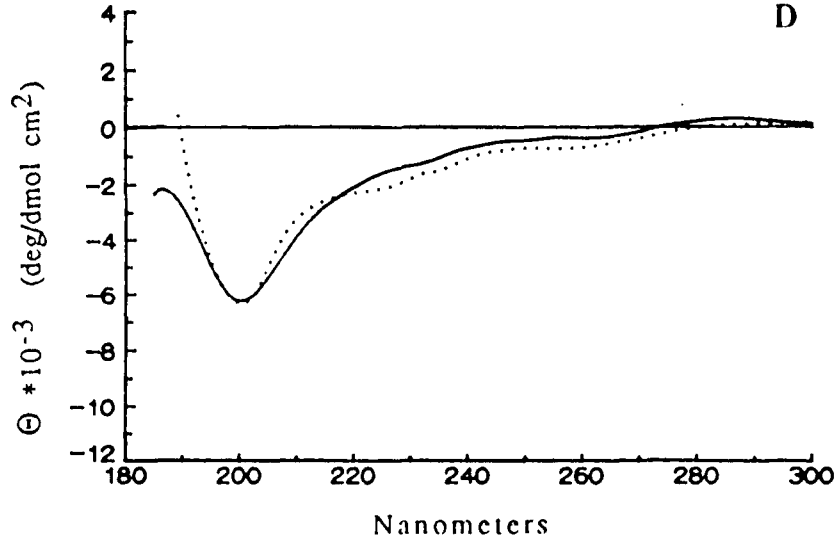

C
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers


D
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

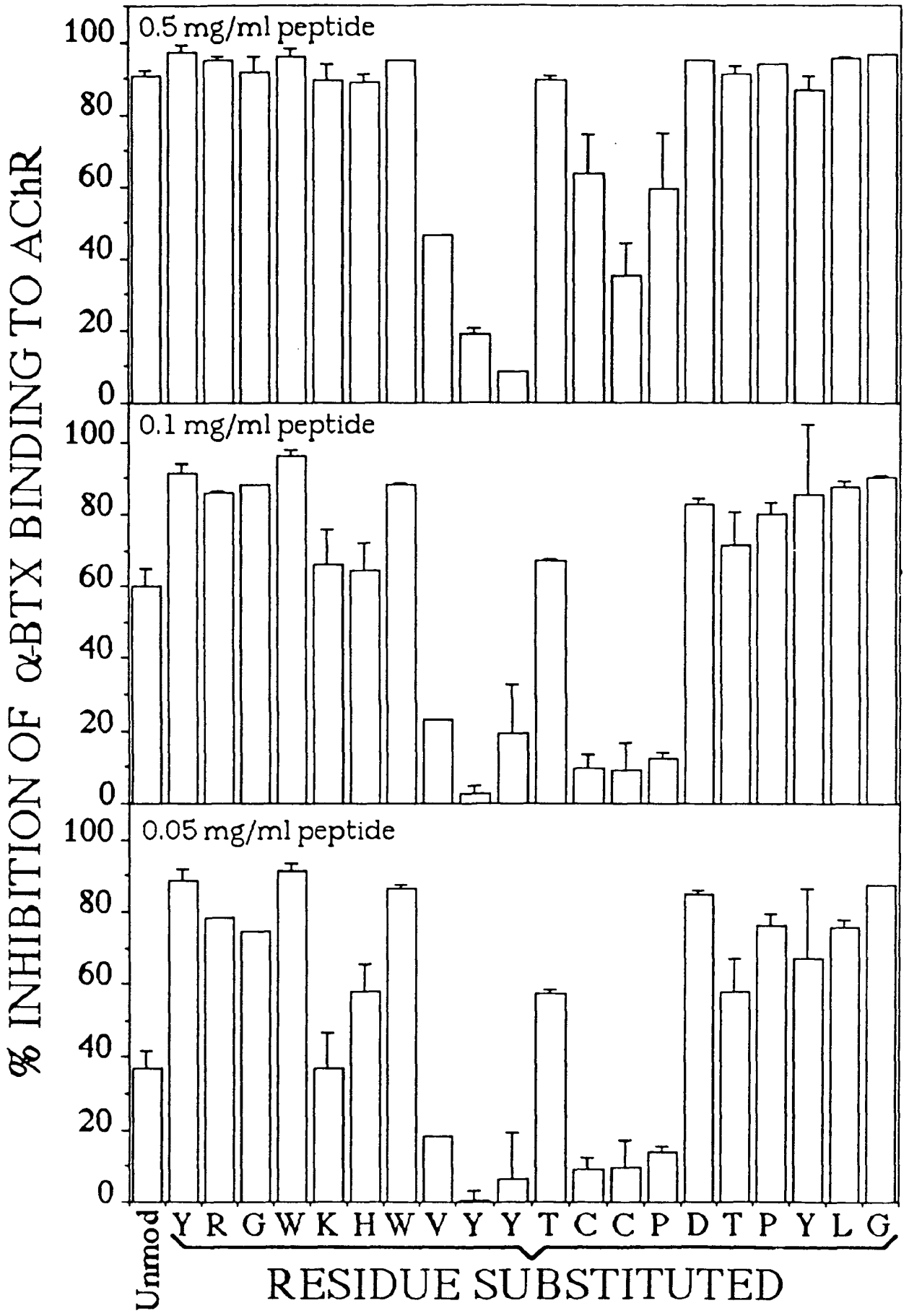

A
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

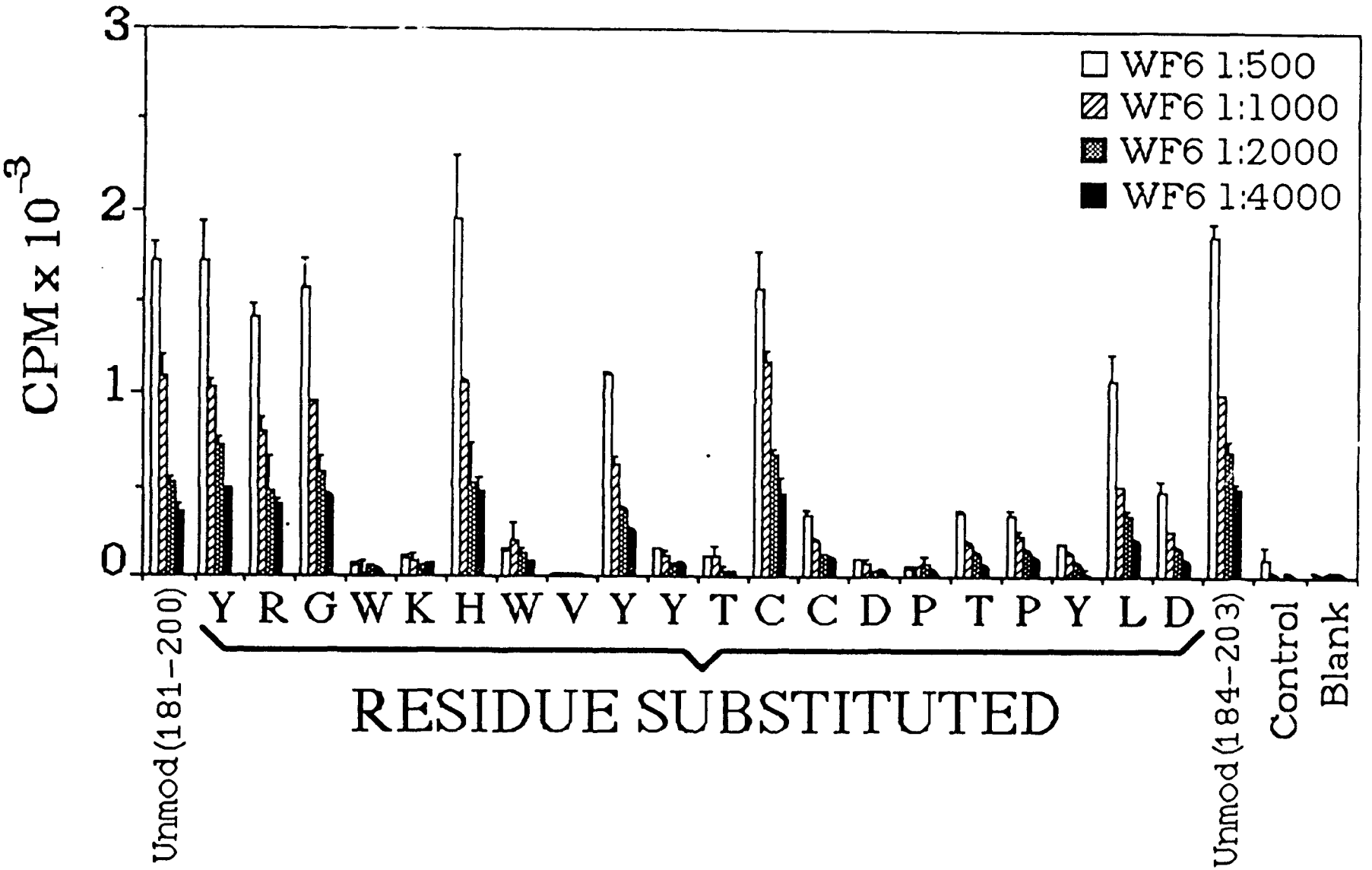

B
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

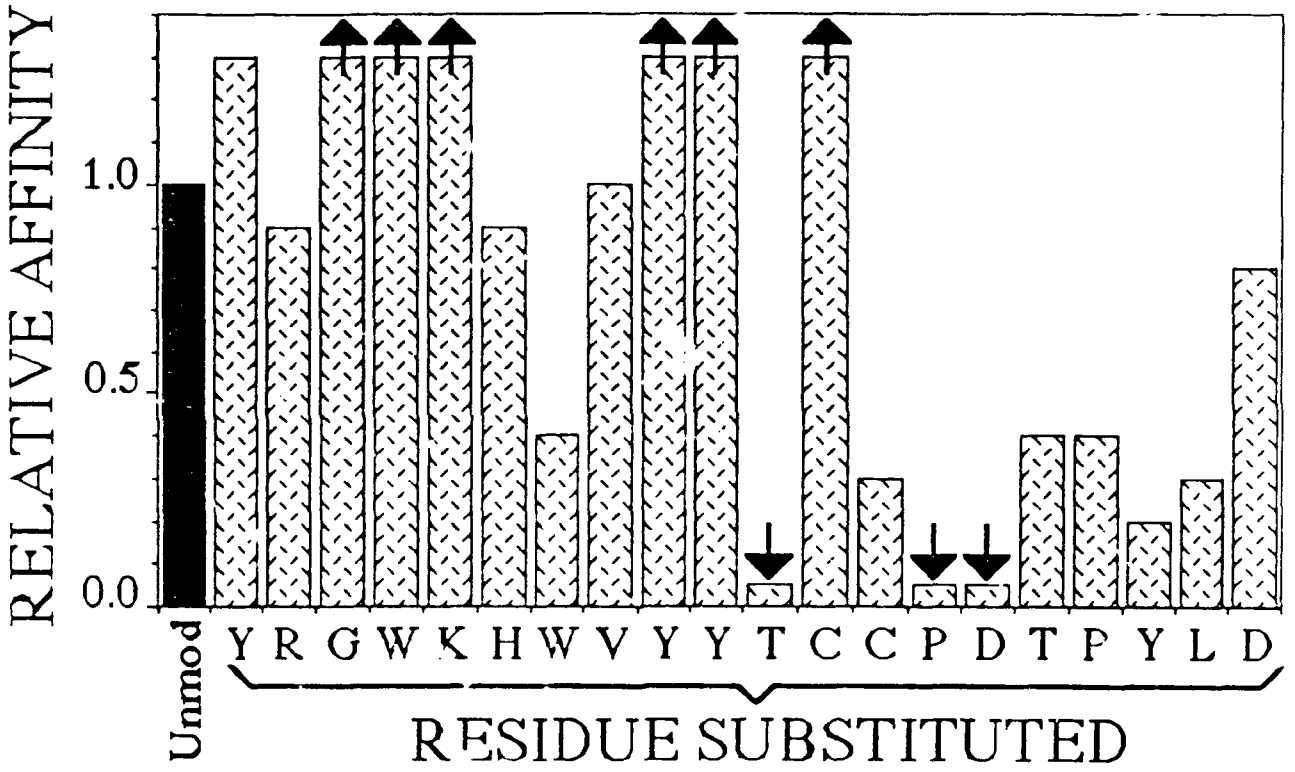

C
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

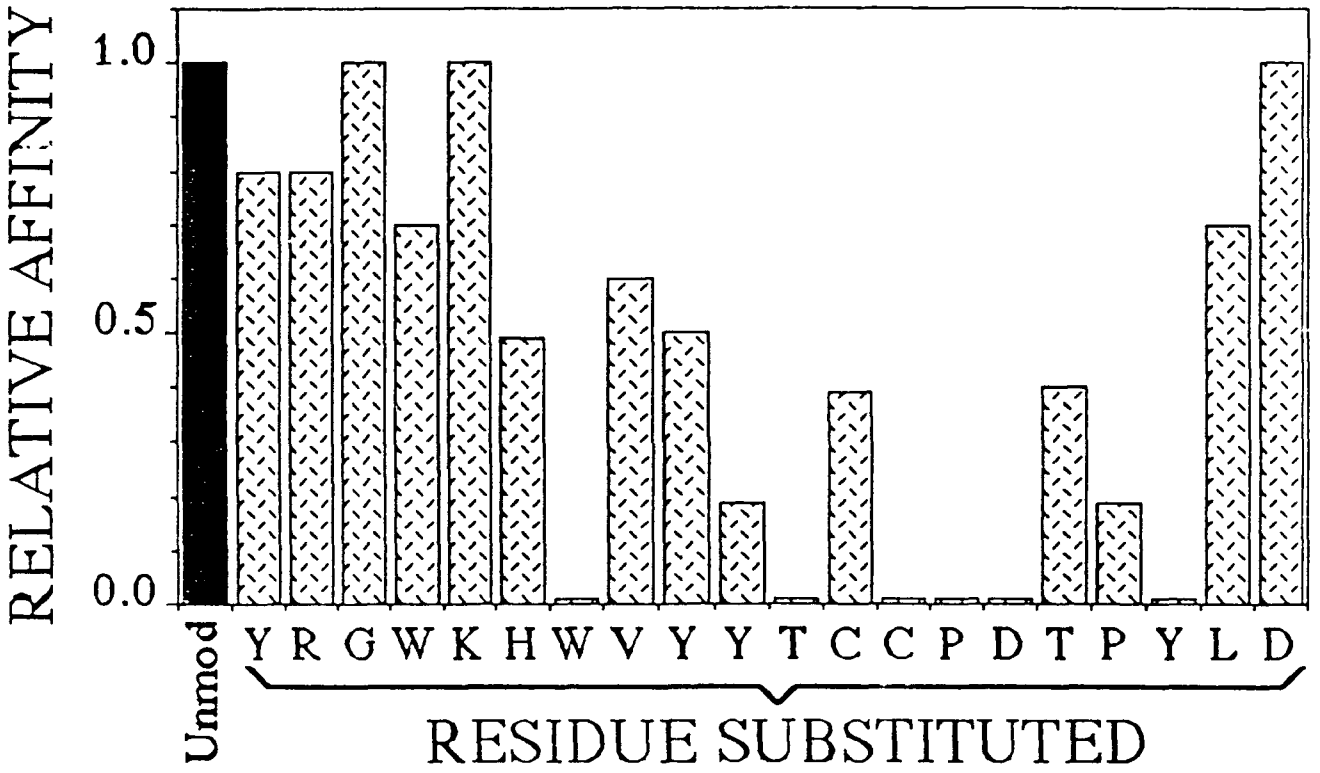

D
4
2
0
-2
-4
-6
-8
-10
-12
180
200
220
240
260
280
300
Θ *10-3 (deg/dmol cm2)
Nanometers

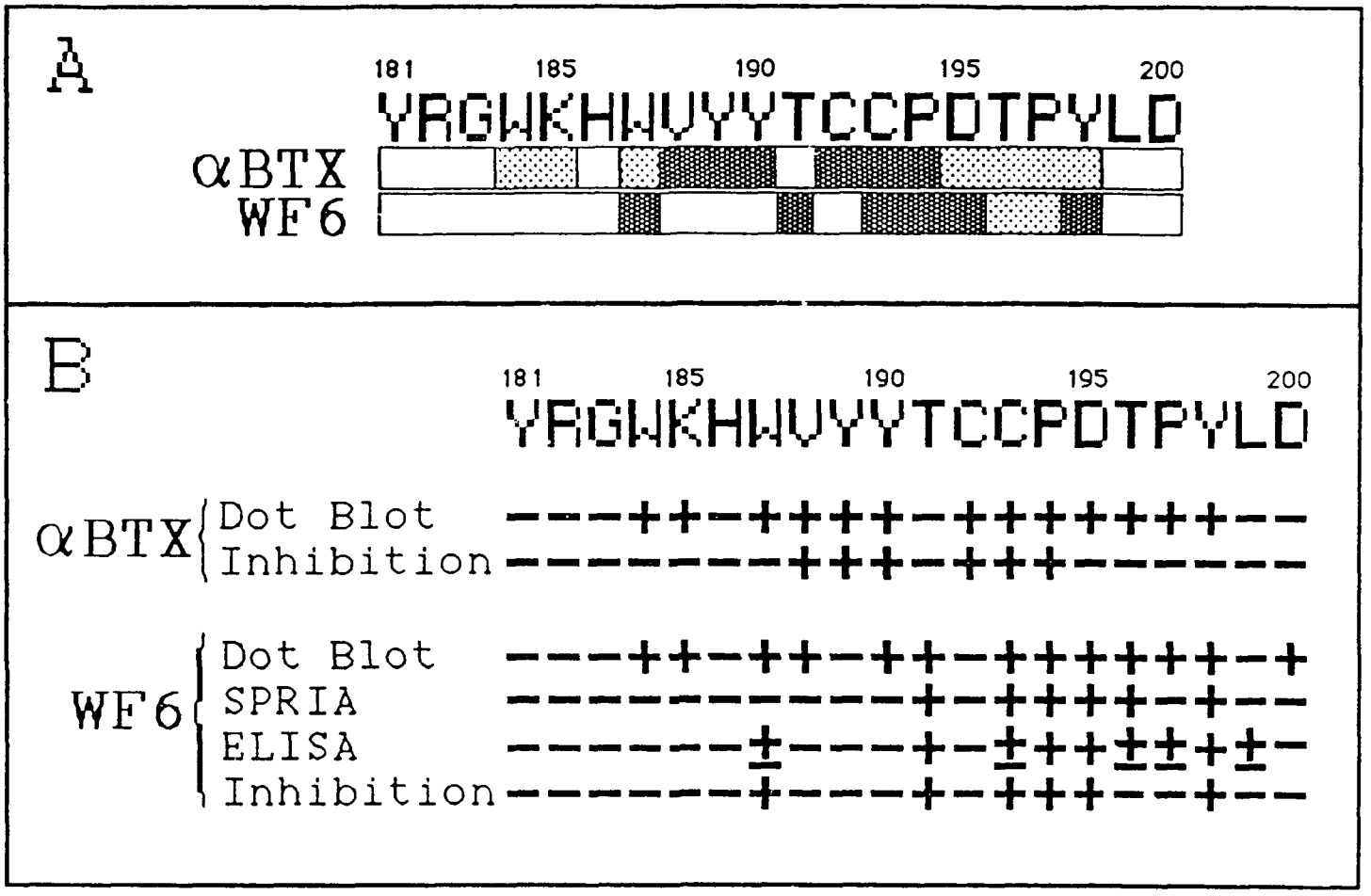
B
K_D, μM
8
6
4
2
0
No measurable binding
Y R G W K H W V Y Y T C C P D T P Y L D
Unmod(181-200)
Unmod(184-203)
RESIDUE SUBSTITUTED
B
A
fmole bound α-BTX
600
500
400
300
200
100
0
0.1 μM α-BTX
0.5 μM α-BTX
1 μM α-BTX
2 μM α-BTX
Control
Blank
Blank
RESIDUE SUBSTITUTED
A


0.5 mg/ml peptide
0.1 mg/ml peptide
0.05 mg/ml peptide
% INHIBITION OF α-BTX BINDING TO AChR
100
80
60
40
20
0
Unmod
Y R G W K H W V Y Y T C C P D T P Y L G
RESIDUE SUBSTITUTED


CPM x 10^-3
3
2
1
0
WF6 1:500
WF6 1:1000
WF6 1:2000
WF6 1:4000
Unmod(181-200)
Y R G W K H W V Y Y T C C D P T P Y L D
Unmod(184-203)
Control
Blank
RESIDUE SUBSTITUTED


RELATIVE AFFINITY
1.0
0.5
0.0
Unmod
Y R G W K H W V Y Y T C C P D T P Y L D
RESIDUE SUBSTITUTED


RELATIVE AFFINITY
1.0
0.5
0.0
Unmod
Y R G W K H W V Y Y T C C P D T P Y L D
RESIDUE SUBSTITUTED


A
181
185
190
195
200
YRGWKHWVYYTCCPDTPYLD
αBTX
WF6
B
181
185
190
195
200
YRGWKHWVYYTCCPDTPYLD
αBTX
Dot Blot
Inhibition
WF6
Dot Blot
SPRIA
ELISA
Inhibition

AMINO ACID RESIDUES FORMING THE INTERFACE OF A NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR WITH κ-BUNGAROTOXIN: A STUDY USING SINGLE RESIDUE SUBSTITUTED PEPTIDE ANALOGS

FOOTNOTES

Abbreviations: αBGTBP (α-bungarotoxin binding protein), and HPLC (high pressure liquid chromatography).

SUMMARY

κ-Bungarotoxin is a high affinity antagonist of neuronal nicotinic acetylcholine receptors of the α3 subtype. Three sequence segments of the α3 subunit that contribute to forming the binding site for κ-bungarotoxin were previously located using synthetic peptides corresponding to the complete α3 subunit, i.e., α3(1-18), α3(50-71) and α3(180-201). Here we use single residue substituted peptide analogs of the α3(50-71) sequence, in which amino acids are sequentially replaced by Gly, to determine which residues are important for κ-bungarotoxin binding activity. Although no single substitution obliterated κ-bungarotoxin binding, several amino acid substitutions lowered the affinity for κ-bungarotoxin -- i.e., two negatively charged residues (Glu51 and Asp62), and several aliphatic and aromatic residues (Leu54, Leu56, and Tyr63). These results indicate that the interface of the α3 subunit with κ-bungarotoxin involves primarily hydrophobic interactions, and a few negatively charged residues.

INTRODUCTION

The venom of the Formosan banded krait, *Bungarus multicinctus*, contains two closely related polypeptide toxins that bind with high affinity to nicotinic acetylcholine receptors -- α-bungarotoxin and κ-bungarotoxin. The latter toxin is also referred to as Toxin F, bungarotoxin 3.1, and neuronal bungarotoxin (1-5). α-Bungarotoxin blocks nicotinic transmission at the neuromuscular junction and a few cholinergic responses in the mammalian brain (6-8), whereas κ-bungarotoxin inhibits nicotinic acetylcholine receptors of the autonomic ganglia and both excitatory and inhibitory nicotinic transmission in various regions of the brain (9-15). Molecular genetic approaches have revealed extensive heterogeneity of the neuronal nicotinic acetylcholine receptors, as indicated by the cloning of multiple cDNAs for α (α2, α3, α4, α5) and β (β2, β3, β4) subunits (16-24), and expression of their transcripts in *Xenopus* oocytes (18, 20, 22, 25, 26). The nicotinic acetylcholine receptors formed by the α3 and α4 subunits are sensitive to κ-bungarotoxin, when coexpressed with the β2 subunit, whereas neither the α2, α3 or α4 nicotinic acetylcholine receptor subtypes are blocked by α-bungarotoxin (18, 20, 22). Interestingly, the α3β4 complex is insensitive to κ-bungarotoxin (22), indicating that the β subunit is also able to affect the ligand binding properties of neuronal nicotinic acetylcholine receptors. The sequences for two neuronal subunits corresponding to chick brain α-bungarotoxin binding proteins have recently been reported, designated αBGTBP α1 (or α7) and αBGTBP α2 (27, 28), and a homomeric receptor complex formed by expression of the αBGTBP α1 subunit in *Xenopus* oocytes has been shown to be sensitive to α-bungarotoxin (28).

Our laboratory has used synthetic peptides, corresponding to the deduced amino acid sequences of the cloned cDNAs, to map the α-bungarotoxin and κ-bungarotoxin binding sites on different nicotinic acetylcholine receptor α subunits (29-33). An α-bungarotoxin binding sequence on the *Torpedo* electric organ and vertebrate muscle α1 subunits has been shown to be between residues 173-204 by several laboratories, using synthetic and biosynthetic peptides (34-40). We have identified two sequence segments that contribute to forming the α-bungarotoxin binding site on the *Torpedo* α1 subunit at positions 55-74 and 181-200 (29, 30). The amino acid residues within the sequence *Torpedo* α1(181-200) that are crucial for α-bungarotoxin binding have been identified by using single residue substitution analogs of the α1(181-200) peptide (31). We have found that the κ-bungarotoxin binding site on the α3 subunit also involves several peptide loops, i.e., sequence segments α3(1-18), α3(50-71) and α3(180-201) (33). In the present study, we determine what residues within this sequence α3(50-71) are important for κ-bungarotoxin binding by sequentially substituting each amino acid of the sequence with Gly, and assessing the change in

affinity of the substituted peptide for [^{125}I]κ-bungarotoxin. Although no single substitution obliterates κ-bungarotoxin binding activity, several amino acid substitutions effectively reduced the apparent affinity of the α3(50-71) peptide sequence for κ-bungarotoxin.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by manual parallel synthesis (41). The purity was assessed by HPLC analysis on a C18 column and amino acid composition analysis of all peptides, and gas-phase sequencing of randomly selected peptides as previously described (30-33). The sequences and codes are shown in Figure 1.

[^{125}I]κ-bungarotoxin. κ-Bungarotoxin was purified from *Bungarus multicinctus* venom, radiolabelled with carrier-free [^{125}I], and calibrated as previously described (33). For some experiments commercial κ-bungarotoxin was used (Biotoxins, St. Cloud, FL). The specific activity of [^{125}I]κ-bungarotoxin was 40-50 Ci/mmol.

Binding Assays. Peptides (25 μg in 10 mM potassium phosphate pH 7.4) were added in triplicate to wells of 96-well Nunc Immunolon plates and incubated overnight at 4°C. The plates were washed twice with 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl and 0.1% Tween-20. The plates were blocked with 10 mg/ml cytochrome c in 10 mM potassium phosphate pH 7.4 for 2 hours at room temperature prior to addition of [^{125}I]κ-bungarotoxin solutions. Nonspecific binding, which accounted for less than 25% of the total binding, was determined by preincubating replicate wells with 10 μM unlabelled κ-bungarotoxin for 2 hours at room temperature prior to addition of [^{125}I]κ-bungarotoxin (0.01-1 μM). After 4 hours at room temperature, the wells were washed 5 times with 10 mM sodium phosphate (pH 7.4) containing 100 mM NaCl and 0.1% Tween-20 and bound radioactivity was removed by addition of 2% sodium dodecyl sulfate (0.2 ml/well) and counted in a gamma counter. Scatchard analysis was performed using the programs EBDA and LIGAND (42, 43). Experiments were repeated three times to insure an accurate determination of the Kd.

RESULTS AND DISCUSSION

Synthetic peptides corresponding to the sequence segment 51-70 of the rat nicotinic acetylcholine receptor α3 subunit, which contains an important structural element of the κ-bungarotoxin binding site (32), and single residue substitution analogs of this sequence segment were used in this study (Figure 1). The peptides were tested for their ability to bind [^{125}I]κ-bungarotoxin using solid phase assay described above, that we have previously used to map the α-bungarotoxin and κ-bungarotoxin binding sequences on neuronal nicotinic acetylcholine receptor α subunits (32, 33). The role of each amino acid residue within of the sequence α3(51-70) for κ-bungarotoxin binding activity was assessed by determining the affinity of each single residue

substituted peptide analog relative to the native sequence. The concentration of [^{125}I]κ-bungarotoxin was varied between 0.01-1 μM, and Scatchard analysis was performed after correction for nonspecific binding. The results of a typical experiment (n=3) are shown in Fig. 2. The values obtained for the Kds in different experiments are summarized in Table I.

Although no single substitution obliterated κ-bungarotoxin binding activity, several substitutions lowered the affinity for κ-bungarotoxin relative to the peptide corresponding to the native sequence, suggesting that these residues are involved in κ-bungarotoxin binding. Amino acid substitutions that resulted in a 3 to 5-fold increase in the apparent Kd included Glu51, Leu54, Leu56, Asp62, and Tyr63. A slight decrease (2 to 3-fold) in the affinity for κ-bungarotoxin was observed for amino acid substitutions of Trp60, Trp67 and Ser70. The Bmax values, which merely reflect the ability of peptides to bind to the plastic matrix of the solid support in an active conformation, varied substantially between different peptides, but seemed to increase when charged amino acids were substituted (See Fig. 2).

Our results indicate that the amino acid residues, which are crucial for interaction of the α3 subunit with κ-bungarotoxin, include i) negatively charged residues (Glu51 and Asp62), which may reflect the requirement for electrostatic interactions with several positively charged amino acid side chains on the binding surface of κ-bungarotoxin, and ii) aliphatic and aromatic residues (Leu54, Leu56, and Tyr63, and perhaps, Trp60 and Trp67). Although the structure of κ-bungarotoxin has not been reported, its sequence and charge properties are homologous to α-bungarotoxin, for which structural data are available (44-46). A model for the binding interface of α-bungarotoxin has been proposed that involves primarily hydrophobic and hydrogen bonding interactions, with the participation of only a few charged groups (44). Further support for the role of aromatic residues in formation of the cholinergic site has been indicated by the binding of acetylcholine to a completely synthetic receptor comprised primarily of aromatic rings, which accommodate the quaternary ammonium group of acetylcholine through cation-π electron interactions (47). The importance of complementary aromatic side chains on the *Torpedo* α1 subunit sequence segment 181-200 in conferring high affinity binding of α-bungarotoxin has been suggested by the lower affinities of peptides corresponding to the homologous sequences of vertebrate muscle α1 subunits from different species for which one or more of the aromatic residues is nonconservatively replaced (37, 40, McLane et al., unpublished). The results reported here indicate that κ-bungarotoxin binding to the α3 subunit has a similar requirement for aromatic amino acids.

In conclusion, we demonstrate that synthetic peptides corresponding to single amino acid substitution analogs can be used to identify the important amino acid side chains that are involved

in the binding of ligands. The success in using this approach to define the α-bungarotoxin and κ-bungarotoxin binding sites on different nicotinic acetylcholine receptor α subunits has relied on the very high affinity of these toxins for the native receptor, and the ability of continuous sequence segments of the α subunits to form independent toxin binding sites in the absence of other surrounding peptide loops of the native α subunit and from other subunits of the nicotinic acetylcholine receptor complex. The ability to rapidly synthesize single site mutants of these toxin binding sequences has provided a powerful method to determine the important structural features of these binding sites.

Acknowledgments -- This work was supported by research grants U. S. NSF BNS-8607289, U. S. NIDA program project grant 5P01-DA05695, and ARO contract DAMD 17-88-C-8120 (to B.M.C.-T.). K.E.M. is a recipient of a predoctoral fellowship as part of the USPHS Training grant 5-T32-GM07323.

REFERENCES

1. Chiappinelli, V.A. (1983) Brain Res. 277, 9-21.
2. Chiappinelli, V.A. (1985) Pharmac. Ther. 31, 1-32.
3. Loring, R. H., Chiappinelli, V. A., Zigmond, R. E., and Cohen, J. B. (1984) Neuroscience 11, 989-999.
4. Halvorsen, S., and Berg, D. (1986) J. Neurosci. 6, 3405-3412.
5. Lindstrom, J., Schoepfer, R., and Whiting, P. (1987) Mol. Neurobiol. 1, 281-337.
6. Dinger, B., Gonzalez, C., Yoshizaki, K., and Fidone, S. (1985) Brain Res. 339, 295-304.
7. de la Garza, R., McGuire, T.J., Freedman, R. and Hoffer, B.J. (1987) Neuroscience 23, 887-891.
8. Zatz, M., and Brownstein, M.J. (1981) Brain Res. 213, 438-442.
9. Lipton, S. A., Aizenmann, E., and Loring, R. H. (1987) Pflugers Archiv. 410, 37-43.
10. Loring, R.H., and Zigmond, R.E. (1988) Trends Neurosci. 11, 73-78.
11. Vidal, C., and Changeux, J.-P. (1989) Neuroscience 29, 261-270.
12. Calabresi, P., Lacey, M. G., and North, R. A. (1989) Br. J. Pharmacol. 98, 135-140.
13. Schultz, D. W., and Zigmond, R. E. (1989) Neurosci. Lett. 98, 310-316.
14. de la Garza, R., Freedman, R., and Hoffer, B. J. (1989) Neurosci. Lett. 99, 95-100.
15. Wong, L. A., and Gallagher, J. P. (1989) Nature 341, 439-442.
16. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., and Patrick, J. (1986) Nature 319, 368-374.
17. Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., and Heinemann, S. (1987) Cell 48, 965-973.
18. Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E.S., Swanson, L.W., Heinemann, S., and Patrick, J. (1988) Science 240, 330-334.
19. Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D.,Heinemann, S., Patrick, J. (1990) J. Biol. Chem. 265, 4472-4482.
20. Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L.W., Patrick, J., and Heinemann, S. (1988) Neuron 1, 45-54.
21. Deneris, E.S., Boulter, J., Swanson, L.W., Patrick, J., and Heinemann, S. (1989) J. Biol. Chem. 264, 6268-6272.

22. Duvoisin, R. M., Deneris, E. S., Patrick, J., and Heinemann, S. (1989) Neuron 3, 487-496.
23. Nef, P., Oneyser, C., Alliod, C., Couturier, S., and Ballivet, M. (1988) EMBO J. 7, 595-601.
24. Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S., and Lindstrom, J. (1988) Neuron 1, 241-248.
25. Papke, R. L., Boulter, J., Patrick, J., and Heinemann, S. (1989) Neuron 3, 589-596.
26. Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., and Patrick, J. (1990a) J. Neurochem. 55, 632-640.
27. Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M., and Lindstrom, J. (1990) Neuron 4, 35-48.
28. Couturier, S., Bertrand, D., Matter, J.-M., Hernandez, M.-C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Ballivet, M. (1990) Neuron (in press).
29. Conti-Tronconi, B.M., Fels, G., McLane, K., Tang, F., Bellone, M., Kokla, A., Tzartos, S., Milius, R. and Maelicke, A. (1989) In Molecular Neurobiology of Neuroreceptors and Ion Channels, (Maelicke, A., ed.) NATO-ASI, series H, Vol. 32, pp. 291-310, Springer-Verlag, Berlin.
30. Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., and Maelicke, A. (1990) Biochemistry 29, 6221-6230.
31. Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, T., Schroder, B., Reinhardt-Maelicke, S., and Maelicke, A. (1990) Biochemistry in press.
32. McLane, K. E., Tang, F., and Conti-Tronconi, B. M. (1990) J. Biol. Chem., 265, 1537-1544.
33. McLane, K. E., Wu, X., and Conti-Tronconi, B. M. (1990) J. Biol. Chem. 265, 9816-9824.
34. Neumann, D., Barchan, D., Fridkin, M., and Fuchs, S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 250-9253.
35. Ohana, B., and Gershoni, J. M. (1990) Biochemistry 29, 6409-6415.
36. Ralston, S., Sarin, V., Thanh, H.L., Rivier, J., Fox, L., and Lindstrom, J. (1987) Biochemistry 26, 3261-3266.
37. Aronheim, A., Eshel, Y., Mosckovitz, R., and Gershoni, J.M. (1988) J. Biol. Chem. 263, 9933-9937.
38. Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S.J., and Ballivet, M. (1987) Science 235, 77-80.
39. Wilson, P.T., Hawrot, E., and Lentz, T.L. (1988) Mol. Pharmacol. 34, 643-651.
40. Wilson, P.T., and Lentz, T.L. (1988) Biochemistry 27, 6667-6674.
41. Houghten, R.A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 5131-5135.
42. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
43. McPherson, G. A. (1983) Computer Prog. Biomed. 17, 107-111.
44. Love, R. A., and Stroud, R. M. (1986) Protein Eng. 1, 37-46.
45. Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., and Kuntz, I. D. (1988) Biochemistry 27, 2772-2775.
46. Kosen, P. A., Finer-Moore, J., McCarthy, M. P., and Basus, V. J. (1988) Biochemistry 27, 2775-2781.
47. Dougherty, D. A., and Stauffer, D. A. (1990) Science 250, 1558-1560.

FIGURE LEGENDS

Figure 1. Single Amino Acid Substitution Analogs of the Sequence Segment α3(50-71).

The sequence and code for each peptide is indicated, and the position of amino acid substituted by Gly is indicated in **bold**.

Figure 2. Scatchard Analysis of [^{125}I]κ-Bungarotoxin Binding to Peptides.

For each peptide, the total radioactivity bound and the nonspecific radioactivity bound (after blocking with 10 μM unlabelled κ-bungarotoxin), were determined at each concentration of κ-bungarotoxin. Specific binding, the total minus the nonspecific bound was used to calculate an apparent Kd using the ligand-binding analysis programs EBDA and LIGAND (42, 43).

PEPTIDE	SEQUENCE
Native	ETNLWLKQIWNDYKLKWKPS
E51	**G**TNLWLKQIWNDYKLKWKPS
T52	E**G**NLWLKQIWNDYKLKWKPS
N53	ET**G**LWLKQIWNDYKLKWKPS
L54	ETN**G**WLKQIWNDYKLKWKPS
W55	ETNL**G**LKQIWNDYKLKWKPS
L56	ETNLW**G**KQIWNDYKLKWKPS
K57	ETNLWL**G**QIWNDYKLKWKPS
Q58	ETNLWLK**G**IWNDYKLKWKPS
I59	ETNLWLKQ**G**WNDYKLKWKPS
W60	ETNLWLKQI**G**NDYKLKWKPS
N61	ETNLWLKQIW**G**DYKLKWKPS
D62	ETNLWLKQIWN**G**YKLKWKPS
Y63	ETNLWLKQIWND**G**KLKWKPS
K64	ETNLWLKQIWNDY**G**LKWKPS
L65	ETNLWLKQIWNDYK**G**KWKPS
K66	ETNLWLKQIWNDYKL**G**WKPS
W67	ETNLWLKQIWNDYKLK**G**KPS
K68	ETNLWLKQIWNDYKLKW**G**PS
P69	ETNLWLKQIWNDYKLKWK**G**S
S70	ETNLWLKQIWNDYKLKWKP**G**

TABLE 1. Scatchard Analysis of Single Amino Acid Substitution Analogs of the Sequence Segment α3(51-70).

Peptide	Kd (nM)	Relative to Native (fold increase in Kd)
Native	326 (65)	1.0
E51	984 (53)	3.0
T52	317 (67)	1.0
N53	391 (85)	1.2
L54	905 (42)	2.8
W55	420 (39)	1.3
L56	1,263 (166)	3.9
K57	530 (87)	1.6
Q58	304 (171)	0.9
I59	158 (122)	0.5
W60	720 (91)	2.2
N61	281 (120)	0.9
D62	1,645 (202)	5.0
Y63	1,570 (194)	4.8
K64	492 (106)	1.5
L65	316 (113)	1.0
K66	420 (64)	1.3
W67	723 (98)	2.2
K68	378 (75)	1.2
P69	341 (111)	1.0
S70	771 (120)	2.4

Kds were obtained by Scatchard analysis of data points in triplicate for each concentration, and the average Kd of three experiments is given with the standard deviations are in parentheses.

Identification of Sequence Segments Forming the α-Bungarotoxin Binding Sites on Two Nicotinic Acetylcholine Receptor α Subunits from the Avian Brain

ABSTRACT

The relationship between neuronal α-bungarotoxin binding proteins (αBGTBPs)[1] and nicotinic acetylcholine receptor (nAChR) function in the brain of higher vertebrates has remained controversial for over a decade. Recently, the cDNAs for two homologous putative ligand binding subunits, designated αBGTBP α1 and αBGTBP α2, have been isolated on the basis of their homology to the N-terminus of an αBGTBP purified from chick brain. In the present study, a panel of overlapping synthetic peptides corresponding to the complete chick brain αBGTBP α1 subunit and residues 166-215 of the αBGTBP α2 subunits were tested for their ability to bind ^{125}I-αBGT. The sequence segments corresponding to αBGTBP α1(181-200) and αBGTBP α2(181-200) were found to consistently and specifically bind ^{125}I-αBGT. The ability of these peptides to bind αBGT was significantly decreased by reduction and alkylation of the Cys residues at positions 190/191, whereas oxidation had little effect on αBGT binding activity. The relative affinities for αBGT of the peptide sequences αBGTBP α1(181-200) and αBGTBP α2(181-200) were compared to those of peptides corresponding to the sequence segments *Torpedo* α1(181-200) and chick muscle α1(179-198). In competition assays, the IC_{50} for αBGTBP α1(181-200) was 20-fold higher than that obtained for the other peptides (~ 2 μM versus 40 μM). These results indicate that αBGTBP α1 and αBGTBP α2 are ligand binding subunits able to bind αBGT at sites homologous with nAChR α subunits, and that these subunits may confer differential ligand binding properties on the two αBGTBP subtypes of which they are components.

INTRODUCTION

The α-neurotoxins, isolated from elapid snake venoms, such as α-bungarotoxin (αBGT) from *Bungarus multicinctus,* bind to the muscle-type nicotinic acetylcholine receptor (nAChR) with subnanomolar affinity. The α-neurotoxins were used to purify nAChRs from *Torpedo* electric organ and vertebrate muscle, making possible the determination of the complete amino acid sequence of their four subunits (α1, β1, γ, and δ) (Raftery et al., 1980; Conti-Tronconi et al., 1982; Noda et al., 1982,1983a,b). In addition to the nAChR of skeletal muscle, receptors that bind αBGT have also been found in numerous regions of the mammalian and avian brain and autonomic ganglia (reviewed by Quik and Geertsen, 1988). αBGT binding to these sites displays typical nicotinic pharmacology -- i.e., d-tubocurare, nicotine and carbamylcholine compete with ^{125}I-αBGT binding (Patrick and Stallcup, 1977; Clarke et al., 1985; Marks et al., 1986; Lukas et al., 1986a,b). Heterogeneity of αBGT binding receptors is suggested by reports of high and low affinity binding components in the rat brain (Lukas, 1984; Meeker et al., 1986).

A few nicotinic cholinergic responses in the mammalian brain are inhibited by αBGT, including cholinergic responses in the carotid sinus (Dinger et al., 1985) and cerebellum (de la Garza et al., 1987), and the effects of light on circadian rhythms (Zatz and Brownstein, 1981; Rusak and Bina, 1990). The majority of reponses elicited by nicotinic agonists in the brain, however, are insensitive to αBGT (Lipton et al., 1987; Loring and Zigmond, 1988; Vidal and Changeux, 1989; Calabresi et al., 1989; Schulz and Zigmond, 1989; de la Garza et al., 1989; Wong and Gallagher, 1989; Mulle and Changeux, 1990). A functional distinction has also been made between a neuronal αBGT binding protein (αBGTBP) and an αBGT-insensitive nAChR, which are both present on neurons of autonomic ganglia (Bursztajn and Gershon, 1977; Patrick and Stallcup, 1977; Carbonetto et al., 1978; Kemp and Edge, 1987; Whiting et al., 1987; McLane et al., 1990a). The nAChRs of the brain and autonomic ganglia are predominantly inhibited by κ-bungarotoxin (κBGT) (Chiappinelli, 1983, 1985), also referred to as Toxin F (Loring et al., 1984), bungarotoxin 3.1 (Halvorsen and Berg, 1986), and neuronal bungarotoxin (Lindstrom et al., 1987).

Neuronal αBGT-binding receptors, comprising two to four subunits, have been purified from chick optic lobe and brain (Norman et al., 1982; Mehraben et al., 1984; Betz and Pfeiffer, 1984; Conti-Tronconi et al., 1985) and rat brain (Kemp et al., 1985; Wonnacott, 1986; Whiting and Lindstrom, 1987) using α-neurotoxins as affinity ligands. The N-terminal amino acid sequence of a chick brain αBGT binding protein subunit is homologous to the α subunit of the muscle and *Torpedo* nAChRs (Conti-Tronconi et al., 1985). A distinct class of neuronal nAChRs that do not bind αBGT are composed of two subunits and have been isolated by immunoaffinity chromatography (Whiting and Lindstrom, 1986, 1987; Whiting et al. 1987). This αBGT-insensitive class of nAChRs is now well characterized, as a result of the isolation of cDNAs for several different nAChR subunits from avian and rodent neurons. The deduced amino acid sequences for four neuronal nAChR α subunits (α2, α3, α4 and α5) and three β subunits (β2, β3, and β4; also designated "non-α" or "structural subunits") have been reported, and other homologous subunits may exist (Wada et al., 1988; Boulter et al., 1986, 1990; Goldman et al., 1987; Nef et al., 1988; Schoepfer et al., 1988; Boyd et al., 1988; Deneris et al., 1988, 1989; Duvoisin et al., 1989). Functional neuronal nAChRs have been formed by some of these subunits in *Xenopus* oocytes by coexpression of the α2, α3 or α4 subunits with the β2 or β4 subunits (Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1988; Ballivet et al., 1988; Duvoisin et al., 1989; Papke et al., 1989; Luetje et al., 1990a). The nAChRs formed by the α3 and α4 subunits are sensitive to κBGT, when coexpressed with the β2 subunit, whereas none of these nAChRs are blocked by αBGT (reviewed in Luetje et al., 1990b). Interestingly, the α3β4 complex is insensitive to κBGT (Duvoisin et al., 1989) indicating that the β subunit is also able to affect the ligand binding characteristics of the neuronal nAChR. It is possible that the other α and β subunits that have not been successfully expressed in *Xenopus* oocytes require other, yet unidentified subunits for functional expression, and that one of these complexes is an αBGT-sensitive nAChR.

Two additional subunits, designated αBGTBP α1 and αBGTBP α2, from the avian brain have recently been isolated using an oligonucleotide probe corresponding to the N-terminal

sequence of the purified chick brain αBGT binding protein (Schoepfer et al., 1990). The αBGTBP α1 subunit is present in >90% of all αBGTBPs in chicken brains, whereas the αBGTBP α2 subunit is present in combination with the αBGTBP α1 subunit in a minor subtype comprising ~15% of the total αBGTBPs. A cDNA identical to αBGTBP α1 was subsequently reported by Couturier et al. (1990), and was designated α7 by this group. They observed that when this cDNA was expressed in *Xenopus* oocytes, it produced a cation channel gated by acetylcholine and nicotine, and competitively inhibited by αBGT. In addition, a bacterial fusion protein corresponding to residues 124-239 of the α7 subunit detectably bound ^{125}I-αBGT. Comparison of the deduced amino acid sequences of chick brain αBGTBP α1 and α2 subunits with other nAChR α subunits from muscle and brain indicates that they share only ~40% identity, and are distinct from the α5 subunit, which may represent another αBGTBP subtype (Boulter et al., 1990; McLane et al., 1990b, 1991). In particular, the sequence segment believed to contain the putative αBGT binding site, i.e. amino acid residues 170-200, is highly divergent, when compared to the muscle nAChR α1 sequences. This region is highly conserved between muscle nAChR α1 subunits of different species, and is divergent in the neuronal nAChR α subunits, which do not bind αBGT (Ohana and Gershoni, 1990; McLane et al., 1990b). The αBGTBP subunits, however, share structural features with the α subunits of other nAChRs -- in particular, their sequences contain two adjacent cysteines at positions 190 and 191, which is the hallmark of nAChR α subunits. Evidence that a binding site for acetylcholine is located near the homologous residues at positions Cys 192 and 193 of the *Torpedo* α1 subunit has come from studies showing their selective labelling by cholinergic affinity ligands (Kao et al., 1984; Dennis et al., 1988). The binding of conventional nicotinic ligands (Blanchard et al., 1979) and cholinergic affinity reagents (Kao et al., 1984; Wilson et al., 1984) is competitive with αBGT, indicating that this region contains a shared site for neurotransmitter and αBGT binding. The αBGTBPs are affinity labelled by the same reagents that label Cys 192 and 193 of the *Torpedo* nAChR α1 subunit (Norman et al., 1982; Kemp et al., 1985). For this reason, we were interested in testing the ability of the

αBGTBP α1 and α2 subunits to bind αBGT, and in comparing their αBGT binding properties with the chick muscle α1 subunit.

In the present study, overlapping synthetic peptides corresponding to the complete αBGTBP α1 subunit and residues 166-215 of the αBGTBP α2 subunit were tested for their ability to directly bind ^{125}I-αBGT in toxin blot assays, and to compete for αBGT binding with native membrane-bound *Torpedo* nAChR using a competition assay. The results were compared to those obtained using a chick α1 muscle sequence between residues 179-198 and the *Torpedo* sequence segment 181-200, which both bind αBGT with relatively high affinity, and with a negative control sequence corresponding to residues 181-200 of the cobra α1 subunit (Burden et al., 1975; Conti-Tronconi et al., 1990; McLane et al., 1991). The results indicate that peptides corresponding to the sequence segments αBGTBP α1(181-200) and αBGTBP α2(181-200) are both able to bind αBGT, though with somewhat different affinities. These results support the concepts that αBGTBP α1 and α2 subunits form the αBGT binding sites of neuronal αBGTBPs, and that these binding sites are formed by a region of the subunit homologous to a cholinergic binding site of muscle nAChR α1 subunits.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Characterization

Peptides, 19-21 amino acids long, were synthesized by manual parallel synthesis (Houghten, 1985). The purity of the peptides was assessed by reverse phase HPLC (high pressure liquid chromatography) using a C18 column (Ultrasphere ODS) and an acetonitrile/water gradient (5-70%) containing 0.1% trifluoroacetic acid. A major peak was consistently present, which accounted for 65-85% of the total absorbance at 214 nm. The amino acid composition of all peptides, determined by derivatization of amino acid residues released by acid hydrolysis with phenylisothiocyanate, followed by separation on a reverse phase HPLC column (PICO.TAG) as described by Heinrickson and Meredith (1984), yielded a satisfactory correspondence between experimental and theoretical values. The sequence and purity of peptides corresponding to

sequences 181-200 of different α subunits, and other randomly selected peptides, were verified by gas-phase sequencing (Applied Biosystems), indicating that contamination by truncated peptides was less than 5-15%. The sequence and codes of the peptides are reported in Fig. 1.

Modification of Cysteine/Cystine Residues

Synthetic peptides (0.5 mg/ml) in 100 mM potassium phosphate buffer, pH 8.5, were treated with either 2-iodosobenzoic acid (IOBA) (0.1 mM) or dithiothreitol (DTT) (1.5 mM) for five hours at room temperature. Samples of untreated, oxidized and reduced peptides were alkylated with iodoacetamide (IAA) (6 mM) overnight at 4°C. The peptides were separated from the low molecular weight reactants on a desalting column (Pierce Excellulose GF-5). Samples of peptides were chromatographed using a BioRad P6 column (1.8 x 47 cm) for determination of the ratio of monomer/dimer. Both columns were equilibrated and eluted with 10 mM potassium phosphate buffer (pH 7.4). The P6 column was calibrated with bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), human growth hormone releasing factor (5041 daltons) and blue dextran 2000 (Sigma). Carboxymethylation of free sulfhydryl groups was assessed by amino acid composition analysis, as described above. The carboxymethyl cysteine (CM-Cys) derivative was calibrated with a standard (Pierce) and elutes after the Asp/Asn and Glu/Gln peaks, and prior to the Ser peak in 140 mM sodium acetate buffer (pH 6.4) containing 0.0~% triethylamine and 6% acetonitrile. The HPLC profiles corresponding to **IOBA/IAA** treatment indicated that oxidation of reduced peptides is essentially complete under the reaction conditions.

Preparation and Calibration of Radiolabelled αBGT

αBGT was isolated from *Bungarus multicinctus* venom (Biotoxins Inc.) by the method of Clark et al. (1972). For some experiments, commercially prepared αBGT (Biotoxins Inc., Lot # ABT 88A) was used. The purity of αBGT, as assessed by gas phase sequencing, indicated that contaminating sequences, if present, were below the level of detectability (< 3-5%). αBGT was radiolabelled with carrier-free ^{125}I (Lindstrom et al., 1981) and calibrated as described by

Blanchard et al. (1979). The specific activity of the ^{125}I-αBGT preparations used in experiments was 4-120 Ci/mmol.

Toxin Blots and Scatchard Analysis

Nitrocellulose strips (MSI, Inc.) were spotted with 1 µl of each peptide solution (0.25 mg/ml in 10 mM potassium phosphate buffer, pH 7.4 (KP buffer)) and allowed to dry at room temperature. The strips were blocked by incubation with 10 mg/ml cytochrome c (Sigma) in KP buffer for 2 hrs. at room temperature. Cytochrome c (5 mg/ml), which shares similar charge properties to αBGT, was added to solutions of ^{125}I-αBGT to reduce nonspecific binding. The blocked nitrocellulose strips were incubated with 0.1-10 µM ^{125}I-αBGT in PBS (10 mM sodium phosphate, 100 mM NaCl, pH 7.2) for 2-4 hours at room temperature. The time course of association of ^{125}I-αBGT to peptides has previously been reported and indicates that equilibrium binding is attained under these conditions (Conti-Tronconi et al., 1990). The toxin blots were washed 8 times with 3 ml PBS containing 0.1% Tween-20 for 2.5 min/wash, mounted for autoradiography using Kodak X-Omat film and exposed at -70°C. The concentration dependence of ^{125}I-αBGT binding was determined by counting triplicate samples (5 mm squares) of nitrocellulose in a Beckman 5500 gamma counter. The apparent background binding of ^{125}I-αBGT binding to nitrocellulose as assessed by autoradiography varied between strips. In order to obtain a value for the specific binding to peptides at each concentration of ^{125}I-αBGT, the nonspecific binding of ^{125}I-αBGT was determined by preincubation of replicate strips with 100 µM unlabelled αBGT for 2 hours at room temperature prior to addition of ^{125}I-αBGT, and was found to be linear with the concentration of ^{125}I-αBGT (5-25% of the specifically bound ^{25}I-αBGT). The binding of ^{125}I-αBGT to peptides did not exceed 3% of the total radioactivity added. The relative affinities of peptides bound to nitrocellulose were compared by calculating an apparent Kd using the programs EBDA and LIGAND (Munson and Rodbard, 1980; McPherson, 1983). Given the solid phase nature of these assays and the nonsaturating conditions used, the Kd and Bmax values ~~calulated~~ only approximate the true values. These values are reproted in McLane et al. (1991).

Competitive Inhibition of ^{125}I-αBGT Binding by Peptides

Peptides (5-500 μg/ml) were preincubated with ^{125}I-αBGT (0.5-2 pmoles) in 10 mM potassium phosphate buffer (pH 7.4) containing 10 mg/ml cytochrome c overnight at 4°C. To 100 μl of the peptide/toxin solution, 0.2-1 pmoles of membrane bound *Torpedo* nAChR (Neubig et al., 1979; Elliot et al., 1980) was added. After incubation for 3 min. at room tempertaure, the assay tubes were centrifuged at 14,000 x g for 45 min., washed with PBS, and recentrifuged. The pellet was counted in a Beckman 5500 gamma counter. Nonspecific binding (< 5%) was determined by preincubation of *Torpedo* nAChR with 20 nM unlabelled αBGT for 10 min. prior to addition to the radiolabelled toxin solutions. Binding of ^{125}I-αBGT in the presence of peptides was compared with values obtained with toxin preincubated with only buffer. The IC_{50} values were determined by logit-log analysis (Rodbard and Frazier, 1975), using the computer program EBDA (McPherson, 1983). The results are presented graphically as a Hofstee plot (Molinoff et al., 1981), in which the IC_{50} values can be directly compared as the negative slopes of the linear regression lines.

RESULTS

Rationale

Proteolytic fragments (Wilson et al., 1984,1985; Pederson et al., 1986; Neumann et al., 1986a), synthetic peptides (Neumann et al., 1986b; Ralston et al., 1987; Wilson et al., 1988; Wilson and Lentz, 1988; Conti-Tronconi et al., 1988, 1989, 1990), and biosynthetic peptides (Barkas et al., 1987; Aronheim et al., 1988; Ohana and Gershoni, 1990) have been used to locate continuous sequence segments of the *Torpedo* nAChR α subunit able to form independent αBGT binding sites, i.e. "prototopes". We have used synthetic peptides to define an αBGT binding site on the Torpedo nAChR α1 and rat α5 subunits (Conti-Tronconi et al., 1990; McLane et al., 1990b) and a κBGT binding site on the rat α3 nAChR subunit (McLane et al., 1990a).

In the present study, we compared the ability of synthetic peptides corresponding to the homologous sequence segments of the chick muscle α1 subunit, and the chick brain αBGTBP α1

and α2 subunits to bind ^{125}I-αBGT. The *Torpedo* α1(181–200) and the cobra muscle α1(181-200) sequences were used as positive and negative controls, respectively. In addition, we have included two unrelated peptides to control for nonspecific electrostatic effects on αBGT binding--a peptide corresponding to amino acid residues 61-78 of the α subunit of the glycine receptor (Greeningloh et al., 1987), which is negatively charged (pI 2.6), and a positively charged peptide, corresponding to amino acid residues 331-350 of the rat nAChR β2 subunit (pI 12.5). One might predict that αBGT, with a pI of 9.2 (Clark et al., 1972), could bind nonspecifically to negatively charged peptide sequences and/or that a positively charged peptide could mask negatively charged groups, which might contribute to forming an αBGT-binding site on the native *Torpedo* nAChR. As reported below, however, these effects of nonspecific electrostatic interactions on ^{125}I-αBGT binding were not detected in this study.

Peptides Corresponding to Chick Brain αBGTBP α1(181-200) and αBGTBP α2(181-200) Bind ^{125}I-αBGT in Toxin Blot Assays

Peptides (0.25 mg/ml) were spotted onto nitrocellulose strips and blocked with cytochrome c (10 mg/ml) prior to incubation with ^{125}I-αBGT (0.5-4 μM). After washing, the strips were dried and mounted, and the ability of a peptide to bind αBGT was determined by autoradiography. The results of typical qualitative experiments is shown in **Fig. 2A** (n=4), where the binding of ^{125}I-αBGT (2μM) to the chick muscle α1, and αBGTBP α1and α2 is compared with control peptides. A strong signal was consistently observed for *Torpedo* α1(181-200), chick muscle α1(179-198), and chick BGTBP α2(181-200). A weaker, but positive signal was also observed for the chick BGTBP α1(181-200) peptide. Background levels of ^{125}I-αBGT binding were observed for the negative control peptides, glycine α(61-78), rat nAChR β2(331-350), cobra muscle nAChR α1(181-200), and for other overlapping peptides of the chick brain αBGTBP α1 and α2 subunits ---αBGTBP α1(171-189), α1(185-204), α2(166-185) and α2(196-215). In addition, peptides αBGTBP α1(166-175) and α1(196-215) did not bind ^{125}I-αBGT (data not shown). These results indicated that peptides corresponding to residues 181-200 of the αBGTBP α1 and α2 subunits were able to form prototopes that bind ^{125}I-αBGT.

The specificity of ^{125}I-αBGT binding to peptides *Torpedo* α(181-200), chick muscle α1(179-198), chick brain αBGTBP α1(181-200) and chick brain αBGTBP α2(181-200) was assessed by blocking the nitrocellulose strips with cytochrome c (10 mg/ml) in the presence or absence of 100 μM unlabelled αBGT prior to addition of ^{125}I-αBGT (0.8 μM). The ability of unlabelled αBGT to block ^{125}I-αBGT binding was determined by autoradiography (data not shown) and by counting single peptide blots in a gamma counter. The results of a typical experiment are shown in **Fig. 2B**. In four independent experiments, preincubation with unlabelled αBGT substantially reduced (76-84% inhibition) the total ^{125}I-αBGT bound to peptides in the absence of unlabelled αBGT. Unlabelled κBGT (80 μM) did not inhibit ^{125}I-αBGT binding (data not shown).

Other classic nicotinic cholinergic ligands (d-tubocurare, nicotine and carbamylcholine), at concentrations up to 10 mM, did not inhibit the binding of αBGT to peptides. Difficulties in defining the pharmacological profile of αBGT binding to small peptides corresponding to the *Torpedo* and human α1 subunit have previously been reported, and IC_{50} values, when attainable, were in the range of 10-500 mM (eg., Neumann et al., 1986; Griesmann et al., 1990). Wilson and Lentz (1988), however, using a 32-mer peptide corresponding to residues 173-204 of the *Torpedo* α1 subunit, convincingly demonstrated that d-tubocurare inhibits αBGT binding (IC_{50} ~100 μM). Studies using monoclonal antibodies that recognize different epitopes within the ligand binding domain of the *Torpedo* α1 subunit also indicate that there is only partial overlap between the sequence segments that are recognized by carbamylcholine, αBGT and d-tubocurare (Watters and Maelicke, 1983; Mihovilovic and Richman, 1987). Thus, the peptides used in the present study may not contain the residues required for binding of other cholinergic ligands, in addition to αBGT.

The concentration dependence of αBGT binding was assessed over a range of 0.1-10 μM ^{125}I-αBGT as described in "Experimental Procedures" (data not shown). The apparent Kds for peptides*Torpedo* α1(181-200) and chick muscle α1(179-198) have previously been reported, i.e. Kd ~1-2 μM (McLane et al., 1991). In assays conducted under the same conditions, the chick

brain αBGTBP α2(181-200) was shown to have approximately the same affinity for ^{125}I-αBGT *Torpedo* α1(181-200) and chick muscle α1(179-198), whereas peptide αBGTBP α1(181-200) was found to bind ^{125}I-αBGT with 3-4-fold lower affinity. Differences observed in the binding of ^{125}I-αBGT to these peptides in toxin blot assays could not be accounted for by differences in the approximate Bmax values calculated by this method.

Peptides Corresponding to Chick Brain BGTBP α1(181-200) and BGTBP α2(181-200) Compete with Native Torpedo nAChR for ^{125}I-αBGT Binding

We previously used a competition assay to define an αBGT binding site on the *Torpedo* nAChR α1 subunit (Conti-Tronconi et al., 1991) and on the rat brain α5 subunit (McLane et al., 1990b). This assay tests the ability of peptides in solution to sequester ^{125}I-αBGT during a preincubation period, thus preventing αBGT from binding to native membrane-bound *Torpedo* nAChR when it is later added. **Fig. 3** illustrates the results obtained from one of several consistent experiments (n=8). ^{125}I-αBGT is preincubated with peptides (100 μg/ml) and compared to the positive control (100% binding), i.e., ^{125}I-αBGT was preincubated with buffer only. Peptides *Torpedo* α1(181-200) and chick muscle α1(179-198) inhibit the binding of ^{125}I-αBGT to native *Torpedo* nAChR by 84% and 86%, respectively. Inhibition of ^{125}I-αBGT binding by negative control peptides, glycine α(61-78), rat nAChR β2(331-350), and cobra α1(181-200), was less than 10%. Preincubation of *Torpedo* nAChR with unlabelled αBGT prior to addition to ^{125}I-αBGT resulted in >98% inhibition. Peptides chick brain αBGTBP α1(181-200) and α2(181-200) inhibit the binding of ^{125}I-αBGT to native *Torpedo* nAChR by 79% and 83%, respectively. These results confirm that peptides corresponding to amino acid residues 181-200 of the chick brain αBGTBP α1 and α2 subunits form prototopes able to bind αBGT.

The concentration dependence of the inhibition of ^{125}I-αBGT binding was compared for peptides *Torpedo* α1(181-200), chick muscle α1(179-198), chick brain αBGTBP α1(181-200) and αBGTBP α2(181-200). The results of a typical experiment are shown in **Fig. 4,** in which the negative slopes of the linear regression lines represent the the IC_{50}s. The IC_{50} values determined from different experiments (n=5) are summarized in **Table I.** The corresponding K_I

values are not given, as such analysis is inappropriate given that the assay is not performed under equilibrium conditions. The IC_{50} analyses of the relative affinities of peptides for αBGT agree with those results obtained from Scatchard analyses -- i.e., the peptides corresponding to sequences *Torpedo* α1(181-200), chick muscle α1(179-198), and chick brain αBGTBP α2(181-200) are comparable in their potencies (~ 3-5 μg/ml, or ~ 1-2 μM) as competitors for ^{125}I-αBGT binding with native *Torpedo* nAChR. These values are similar to those which we previously obtained for the peptides corresponding to the rodent muscle nAChR α1(182-201) and brain α5(180-199) sequences (~1 μM and ~4 μM, respectively). A ten-fold greater concentration of peptide αBGTBP α1(181-200) is required for equivalent inhibition in the competition assay (~50-100 μg/ml, or ~ 20-40 μM). This level of inhibition is similar to the values that we obtained for peptides corresponding to the sequence segments 181-200 of the human and calf muscle α1 subunits, which bind αBGT with relatively low affinity, when compared to peptides corresponding to the *Torpedo* α1 subunit sequence (Wilson and Lentz, 1988; Ohana and Gershoni, 1990; McLane et al., 1991).

Scanning of the Complete Chick Brain αBGTBP α1 Sequence for Other αBGT Binding Sequence Segments

In order to determine if other sequence segments of the chick brain αBGTBP α1 subunit are involved in forming the αBGT binding site, a panel of 38 overlapping peptides were synthesized corresponding to the complete subunit, as shown in Fig. 1. These peptides were tested using toxin blot assays and *Torpedo* nAChR competition assays for their ability to bind ^{125}I-αBGT. The results obtained in one of several experiments (n=4) are shown in **Fig. 5**. Only peptide αBGTBP α1(181-200), at a concentration of 200 μg/ml, efficiently inhibited the binding of ^{125}I-αBGT to *Torpedo* nAChR (78-88% inhibition of total ^{125}I-αBGT binding), i.e., levels of inhibition comparable to those found for inhibition by pretreatment of *Torpedo* nAChR with 20 nM unlabelled αBGT. Therefore, peptide αBGTBP α1(181-200) represents a principal prototope forming the αBGT binding site on the chick brain αBGTBP α1 subunit.

Effect of Modification of Cysteine/Cystine Residues on αBGT Binding

In order to assess the importance for αBGT binding of the redox state of cysteine residues at positions 190/191 of the αBGTBP α subunits, we modified peptides *Torpedo* α1(181-200), cobra muscle α1(181-200), chick muscle α1(179-198), chick αBGTBP α1(181-200) and α2(181-200) with sulfhydryl reagents. The peptides were treated in the following ways: 1) oxidation with iodosobenzoic acid and alkylation of remaining free sulfhydryls with iodoacetamide (**IOBA/IAA**), 2) reduction with dithiothreitol, followed by alkylation of free sulfhydryls with iodoacetamide (**DTT/IAA**), and 3) alkylation with iodoacetamide of free sulfhydryl groups available in untreated peptide (**IAA**). The latter treatment was used to determine the proportion of peptide in the reduced and oxidized state under normal assay conditions. The products of the reactions were assessed by amino acid composition analysis following acid hydrolysis and derivatization with phenylisothiocyanate, as described in "Experimental Procedures".

The carbamide derivative of cysteine, the product of IAA treatment, is converted to carboxymethyl cysteine (**CM-Cys**) by acid hydrolysis prior to derivatization, and the yield of **CM-Cys** for peptides alkylated with or without prior reduction was used to estimate the proportion of peptide in reduced and oxidized states. The estimated percentage of peptide molecules with free sulfhydryl groups was estimated to be 76% for chick muscle α1(179-198) and 45% for chick αBGTBP α1(181-200). These results are typical of peptides homologous to *Torpedo* α1(181-200) from the muscle nAChR α1 subunits of different species, which we have found to be 40-80% reduced (McLane et al., 1991). In contrast, the peptide chick αBGTBP α2(181-200) appeared to be >90% in the oxidized form, as evidenced by the lack of a CM-Cys peak after IAA treatment without prior reduction, as shown in **Fig. 6**.

In order to determine if the oxidized forms of the peptides were in the form of monomers or dimers, samples of peptides were analyzed by gel permeation chromatography on a BioRad P6 column. The chromatograms for the untreated peptides are shown in **Fig.7A**. The principle peaks for chick muscle α1(179-198), chick αBGTBP α1(181-200) and αBGTBP α2(181-200) chromatographed as peptides of molecular weights 2.2 ± 0.2 kDa, 2.7 ± 0.2 kDa, and 2.7 ± 0.2 kDa, respectively. A small peak at ~ 5.0 kDa, corresponding to a dimer, was observed for both

chick αBGTBP peptides and accounted for < 10-20% of the absorbance at 280 nm. Similar results were obtained with the peptides which had been oxidized, reduced and/or alkylated, and with peptides corresponding to *Torpedo* α1(181-200) and cobra muscle α1(181-200) (data not shown). Therefore, the predominant form of the peptides under the conditions of the assays used in this study is the monomer.

Peptides modified by reduction, oxidation, and/or alkylation were tested for their ability to inhibit ^{125}I-αBGT binding to *Torpedo* nAChR using the competition assay described in **Fig. 3.** The results of a typical experiment (n=3) are shown in **Fig. 7B**, and are compared with control peptides *Torpedo* α1(181-200) and cobra muscle α1(181-200). The cobra peptide does not compete for ^{125}I-αBGT in untreated form, or when reduced, oxidized and/or alkylated. The *Torpedo* α1(181-200) peptide, which is ~ 70% reduced under assay conditions, shows reduced ability to bind αBGT when alkylated with or without prior reduction, whereas oxidation either slightly improves αBGT binding or has no effect (Conti-Tronconi et al., 1990, McLane et al. 1991). The results for different treatments of the peptides chick muscle α1(179-198) and chick αBGTBP α1(181-200) show the same pattern of inhibition as the *Torpedo* peptide. The chick αBGTBP α2(181-200) peptide has a similar pattern of inhibition for oxidation and reduction/alkylation conditions, but alkylation with IAA without prior reduction does not alter the ability of the peptide to inhibit ^{125}I-αBGT binding to *Torpedo* nAChR. These results are consistent with the HPLC analysis, which indicate that the peptide is >90% oxidized and that few free sulfhydryl residues are available in the untreated peptide for alkylation.

DISCUSSION

The results of these studies demonstrate that peptides corresponding to sequence segments of the αBGTBP α1 and α2 subunits are able to bind αBGT. The relative affinities of peptides αBGTBP α1(181-200) and αBGTBP α2(181-200) were compared by IC_{50} analysis (**Fig. 4, Table I**). The IC_{50}s, determined using a competition assay, also indicated that αBGTBP α1(181-200) bound αBGT with relatively low affinity (IC_{50} ~40 μM), whereas the peptide αBGTBP

α2(181-200) was able to compete for αBGT as efficiently as *Torpedo* α1(181-200) and chick muscle α1(179-198) -- IC_{50}s of ~1-2 μM. The functional subunit aggregate formed by expression of the αBGTBP α1 (α7) subunit in *Xenopus* oocytes is blocked by αBGT with an IC_{50} of 0.7 nM (Couturier et al., 1990), indicating a much higher affinity for αBGT than that found for peptide αBGTBP α1(181-200). The relative differences in affinities previously reported for αBGT binding to synthetic or biosynthetic peptides, when compared to native muscle and *Torpedo* nAChRs, are of the same order of magnitude (Wilson et al., 1985, 1988; Neumann et al., 1986b; Gotti et al., 1987; Griesmann et al., 1990; Aronheim et al., 1988; Ohana and Gershoni, 1990; Conti-Tronconi et al., 1990). αBGT binds to synthetic peptides with an affinity that compares with that of the isolated and denatured *Torpedo* α1 subunits (Kd ~10^{-7} M) (Haggerty and Froehner, 1981; Gershoni et al., 1983), but with much lower affinity compared to the native *Torpedo* nAChR (<10^{-9} M), where other sequence segments of the α, γ and δ subunits may contribute to the formation of the ligand binding site (Kurosaki et al., 1987; Sumikawa and Miledi, 1989; Blount and Merlie, 1989; Pederson and Cohen, 1990; Conroy et al., 1990; Saedi et al., 1991).

The adjacent cysteine residues at positions 190/191 of the αBGTBP α1 and α2 subunits are a hallmark of the nAChR α subunits, and are believed to form a vicinal disulfide bond in the native *Torpedo* α1 subunit (Kao et al., 1984; Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988; Kellaris et al., 1989). The effects of cysteine/cystine modification of the peptides αBGTBP α1(181-200) and αBGTBP α2(181-200) on αBGT binding were tested using the competition assay (Fig. 7B), and these experiments indicated that oxidation of the peptides had little effect on αBGT binding, whereas alkylation of free sulfhydryl groups drastically reduced the ability of peptides to compete for αBGT with native *Torpedo* nAChR. We have previously observed that reduction and alkylation of peptides corresponding to the *Torpedo* α1 subunit sequence α1(172-205) (Ralston et al., 1987), and the *Torpedo* and muscle nAChR α1 subunit sequences α1(181-200) of different species (Conti-Tronconi et al., 1990; McLane et al., 1991) reduced their αBGT binding activity. The convergence of these results indicates that although oxidation of Cys 190/191 to form a vicinal disulfide bond may not be critical for αBGT binding, one or both cysteines are

involved in forming the interface of this sequence segment with αBGT, as reflected in the reduced ability of the αBGT to bind to the peptides modified by cysteine alkylation.

Recent studies have demonstrated that αBGT competes for affinity labelling of Tyr 93, as well as Tyr190, Cys192 and Cys193 of the *Torpedo* nAChR α1 subunit (Galzi et al., 1990). In previous studies, we have also found that in addition to the sequence segment containing the vicinal cysteine pair at positions 192/193 relative to the *Torpedo* α subunit sequence, and spanning residues 181-200, other more N-terminal sequence segments contribute to forming the αBGT binding site on the *Torpedo* nAChR α subunit (Conti-Tronconi et al., 1990) and the rat neuronal α5 subunit (McLane et al., 1990b). The corresponding sequences of the αBGTBP α1 subunit, however, share only 20-35% homology with these other αBGT binding sequences on the *Torpedo* α1 and α5 subunits. In the present study, overlapping synthetic peptides scanning the complete αBGTBP α1 subunit were tested for their ability to bind ^{125}I-αBGT directly and to compete for αBGT with native *Torpedo* nAChR, but we were only able to detect αBGT binding to the sequence segment αBGTBP α1(181-200). Therefore, if other sequence segments contribute to the formation of a high affinity αBGT binding site on the αBGTBP α1 subunit, they are either incapable of forming a prototope or have not been properly represented by our panel of synthetic peptides. Given the high degree of homology of the putative extracellular segments of the αBGTBP α1 and α2 subunits, it is likely that a panel of peptides corresponding to the αBGTBP α2 subunit would yield similar results.

The highly divergent nature of the avian brain αBGTBP α subunit sequences at positions 181-200 offers a unique opportunity to determine the amino acid residues that are most critical to the interaction with αBGT, and can best be appreciated by comparing homologous sequences of α subunits from nAChRs that bind αBGT with those that do not (as shown in Fig. 8). Inspection of these sequences reveals that seven amino acids are characteristic of all α subunits regardless of αBGT binding activity, and therefore cannot uniquely confer the ability to bind αBGT; i.e., G183 (or the conservative substitution Ala in the *Drosophila* neuronal α subunits), Tyr190, Cys192, Cys193, Asp195 (or the conservative substitution of Glu in α subunits of neuronal origin),

Tyr198 and Asp200. All α subunits that bind αBGT have Tyr189 (or the conservative substitution Phe) and Pro197, whereas Lys189 and Ile197 (with the exception of the cobra α1 subunit) are characteristic of all subunits that do not bind αBGT. Therefore, comparison of naturally occuring α sequences suggests that Tyr189 and, perhaps P197, may be critical for conferring the ability to bind αBGT. Single amino acid residue substitutions by Gly or Ala of the *Torpedo* α1(181-200) peptide indicates that both Tyr189 and Tyr190 are critical for αBGT binding, whereas Pro197 is not (Conti-Tronconi et al., 1991). The importance of Tyr189 to αBGT binding is also indicated by natural substitutions, such as the replacements by Phe189 in the calf and mouse α1 sequences, and Thr189 in the human α1 sequence, which reduce the affinity for αBGT (Wilson and Lentz, 1988; Ohana and Gershoni, 1990; McLane et al., 1991). Of the five amino acids that differ between the αBGTBP α1 and α2 subunit between residues 181-200, the replacement of Ser/Phe at positions 188/189 of the α1 subunit by Leu/Tyr in the α2 sequence, may account for the observed decrease in affinity of the peptide corresponding αBGTBP α1(181-200). The binding surface of αBGT is believed to involve primarily hydrophobic and hydrogen bonding residues, and a few charged amino acids, including Arg and/or Lys side chains (Low, 1979; Karlsson, 1979; Dufton and Hider, 1983; Love and Stroud, 1986; Garcia-Barron et al., 1987). It is easy to imagine that an aromatic residue at position 189 of the nAChR α subunit would interact with a hydrophobic pocket on the αBGT, whereas a positive charge, induced by Lys189 of the neuronal α subunits, would disrupt the binding.

Thus, although the αBGTBP α subunits contain αBGT binding sites which share only sparing homology with the conventional αBGT binding muscle α1 subunits, certain conserved structural aspects that are reflected in their primary amino acid sequence may account for the ability of these subunits to bind αBGT. On the other hand, αBGT has been shown to exist in at least two different low energy conformations (Inagaki et al., 1982; Love and Stroud, 1986; Basus et al., 1988; Kosen et al., 1988), and such flexibility may account for the ability of αBGT tc accomodate small structural differences between nAChRs of different species, as well as highly divergent proteins, such as the αBGTBP α1 and α2 subunits. Finally, it has been demonstrated that a single

amino acid substitution that destroys the function of a protein molecule, can be compensated by simultaneous nonconservative substitutions of other amino acid residues (Raines et al., 1986; Blacklow and Knowles, 1990). Thus, the few amino acids that are conserved between other αBGT binding α subunits and the αBGTBP α1 and α2 sequences may only adumbrate common structural features.

The αBGTBP α1 subunit was cloned using an oligonucleotide probe based on the N-terminal sequence of a protein isolated by αBGT affinity chromatography (Conti-Tronconi et al., 1985), whereas αBGTBP α2 was isolated on the basis of its homology to αBGTBP α1 (Schoepfer et al., 1990). Monoclonal antibodies against fusion proteins containing unique sequences of the αBGTBP α1 and α2 subunits immunoprecipitate native αBGT binding proteins from the chick brain, indicating that their gene products contribute to the formation of receptor complexes, presumably nAChRs, that are able to bind αBGT. The results reported here indicate that synthetic peptides corresponding to sequence segments of the αBGTBP α1 and α2 subunits, which are homologous to a cholinergic binding site on muscle and neuronal nAChRs, form prototopes able to bind αBGT. Although it has long been known that αBGTBPs have nicotinic pharmacological properties, and we have now shown that two αBGTBP subunits bind αBGT, the endogenous ligand and functional role of αBGTBPs in the brain remain to be determined.

REFERENCES

Aronheim, A., Eshel, Y., Mosckovitz, R., and Gershoni, J. M. (1988) *J. Biol. Chem.* **263**, 9933-9937

Ballivet, M., Nef, P., Couturier, S., Rungger, D., Bader, C. R., Bertrand, D., and Cooper, E. (1988) *Neuron* **1**, 847-852

Barkas, T., Mauron, A., Roth, B., Alliod, C., Tzartos, S.J., and Ballivet, M. (1987) *Science* **235**, 77-80

Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., and Kuntz, I. D. (1988) *Biochemistry* **27**, 2772-2775

Betz, H., and Pfeiffer, F. (1984) *J. Neurosci.* **4**, 2095-2105

Blacklow, S. C., and Knowles, J. R. (1990) *Biochemistry* **29**, 4099-4108.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H.-P. H., and Raftery, M. A. (1979) *Biochemistry* **18**, 1875-1883

Blount, P., and Merlie, J. (1989) *Neuron* **3**, 349-357

Bossy, B., Ballivet, M., and Spierer, P. (1988) *EMBO J.* **7**, 611-618

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., and Patrick, J. (1986) *Nature* **319**, 368-374

Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D.,Heinemann, S., Patrick, J. (1990) *J. Biol. Chem.* **265**, 4472-4482

Boyd, R. T., Jacob, M. H., Couturier, S., Ballivet, M., and Berg, D. K. (1988) *Neuron* **1**, 495-502

Burden, S. Y., Hartzell, M. C. and Yoshikami, D. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 3245-3249.

Bursztajn, S., and Gershon, M. D. (1977) *J. Physiol.* **269**, 17-31

Calabresi, P., Lacey, M. G., and North, R. A. (1989) *Br. J. Pharmacol.* **98**, 135-140

Carbonetto, S., Fambrough, D., and Muller, K. (1978) *Proc. Natl. Acad. Sci. U. S. A. 75*, 1016-1020

Chiappinelli, V. A. (1983) *Brain Res.* **277**, 9-21

Chiappinelli, V. A. (1985) *Pharmac. Ther.* **31**, 1-32

Clark, D. G., Macmurchie, D. D., Elliot, E., Wolcott, R. G., Landel, A. M., and Raftery, M. A. (1972) *Biochemistry* **11**, 1663-1668

Clarke, P. B. S., Rochelle, D., Schwartz, R. D., Paul, S. M., Pert, C. B., and Pert, A. (1985) *J. Neurosci.* **5**, 1307-1315

Conroy, W., Saedi, M., and Lindstrom, J. (1990) *J. Biol. Chem.* **265**, 21642-21651

Conti-Tronconi, B. M., Gotti, C. M., Hunkapillar, M. W., and Raftery, M. A. (1982) *Science* **218**, 1227-1229

Conti-Tronconi, B. M., Dunn, S. M. J., Barnard, E. A., Dolly, J. O., Lai, F. A., Ray, N., and Raftery, M. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5208-5212

Conti-Tronconi, B. M., Tzartos, S., Spencer, S. R., Kokla, A., Tang, F., and Maelicke, A. (1988) In *Nicotinic Acetylcholine Receptors in the Nervous System*, (Clementi, F., Gotti, C., and Sher, E., eds.) NATO-ASI, series H, Vol. 25, pp. 119-136, Springer-Verlag, Berlin

Conti-Tronconi, B. M., Fels, G., McLane, K., Tang, F., Bellone, M., Kokla, A., Tzartos, S., Milius, R. and Maelicke, A. (1989) In *Molecular Neurobiology of Neuroreceptors and Ion Channels*, (Maelicke, A., ed.) NATO-ASI, series H, Vol. 32, pp. 291-310, Springer-Verlag, Berlin

Conti-Tronconi, B. M., Tang, F., Diethelm, B. M., Spencer, S. R., Reinhardt-Maelicke, S., and Maelicke, A. (1990) *Biochemistry* **29**, 6221-6230

Conti-Tronconi, B. M., Diethelm, B. M., Wu, X., Tang, F., Bertazzon, T., Schroder, B., Reinhardt-Maelicke, S., and Maelicke, A. (1991) *Biochemistry* **30**, 2575-2584

Couturier, S., Bertrand, D., Matter, J.-M., Hernandez, M.-C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Ballivet, M. (1990) *Neuron* **5**, 847-856.

de la Garza, R., McGuire, T. J., Freedman, R. and Hoffer, B. J. (1987) *Neuroscience* **23**, 887-891

de la Garza, R., Freedman, R., and Hoffer, B. J. (1989) *Neurosci. Lett.* **99**, 95-100

Deneris, E. S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L. W., Patrick, J., and Heinemann, S. (1988) *Neuron* **1**, 45-54

Deneris, E. S., Boulter, J., Swanson, L. W., Patrick, J., and Heinemann, S. (1989) *J. Biol. Chem.* **264**, 6268-6272

Dennis, M., Giraudat, J., Kotzba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chretien, M., and Changeux, J.-P. (1988) *Biochemistry* **27**, 2346-2357

Dinger, B., Gonzalez, C., Yoshizaki, K., and Fidone, S. (1985) *Brain Res.* 339, 295-304

Dufton, M. J., and Hider, R. C. (1983) *CRC Crit. Rev. Biochem.* **14**, 113-171

Duvoisin, R. M., Deneris, E. S., Patrick, J., and Heinemann, S. (1989) *Neuron* **3**, 487-496

Elliot, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P., Racs, J., and Raftery, M. (1980) *Biochem. J.* **185**, 667-677

Galzi, J.-L., Revah, F., Balck, D., Goeldner, M., Hirth, C., and Changeux, J.-P. (1990) *J. Biol. Chem.* **265**, 10430-10437

Garcia-Borron, J.-C., Bieber, A. L., and Martinez-Carrion, M. (1987) *Biochemistry* **28**, 4295-4303

Gershoni, J. M., Hawrot, E., and Lentz, T. L. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 4973-4977

Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., and Heinemann, S. (1987) *Cell* **48**, 965-973

Gotti, C., Mazzola, G., Longhi, R., Fornasari, D., and Clementi, F. (1987) *Neurosci. Lett.* **82**, 113-119

Grenningloh, G., Rienitz, A., Schmidt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E. D., and Betz, H. (1987) *Nature* **328**, 215-220

Griesmann, G. E., McCormick, D. J., De Aizpurua, H. J., and Lennon, V. A. (1990) *J. Neurochem.* **54**, 1541-1547

Haggerty, J. G., and Froehner, S. C. (1981) *J. Biol. Chem.* **256**, 8294-8297

Halvorsen, S., and Berg, D. (1986) *J. Neurosci.* **6**, 3405-3412

Heinrickson, S., and Meredith, B. (1984) *Anal. Biochem.* **136**, 65-74

Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5131-5135

Inagaki, F., Clayden, N. J., Tamiya, N., and Williams, R. J. P. (1982) *Eur. J. Biochem.* **123**, 99-104

Kao, P. N., Dwork, A. J., Kaldany, R. J., Silver, M. L., Wideman, J., Stein, S., and Karlin, A. (1984) *J. Biol. Chem.* **259**, 11662-11665

Kao, P. N., and Karlin, A. (1986) J. Biol. Chem. 261, 8085-8088

Karlsson, E. (1979) *Handbook Exp. Pharmacol.* **52**, 159-212

Kellaris, K. V., Ware, D. K., Smith, S., and Kyte, J. (1989) *Biochemistry* **28**, 3469-3482

Kemp, G., and Edge, M. (1987) *Mol. Pharmacol.* **32**, 356-363

Kemp, G., Bentley, L., McNamee, M. G., and Morley, B. J. (1985) *Brain Res.* **347**, 274-283

Kosen, P. A., Finer-Moore, J., McCarthy, M. P., and Basus, V. J. (1988) *Biochemistry* **27**, 2775-2781

Kurosaki, T., Fukuda, K., Kono, T., Yasuo, M., Ken-ichi, T., Mishina, M., and Numa, S. (1987) *FEBS Lett.* **214**, 253-258

Lindstrom, J., Einarson, E., and Tzartos, S. (1981) In *Methods in Enzymology* Vol. 74, (Langone, J. J., and van Vunakis, H. eds.) pp. 432-460, Academic Press, New York

Lindstrom, J., Schoepfer, R., and Whiting, P. (1987) *Mol. Neurobiol.* **1**, 281-337

Lipton, S. A., Aizenmann, E., and Loring, R. H. (1987) *Pflugers Archiv.* **410**, 37-43

Loring, R. H., Chiappinelli, V. A., Zigmond, R. E., and Cohen, J. B. (1984) *Neuroscience* **11**, 989-999

Loring, R. H., and Zigmond, R. E. (1988) *Trends Neurosci.* **11**, 73-78

Love, R. A., and Stroud, R. M. (1986) *Protein Eng.* **1**, 37-46

Low, B. A. (1979) *Handbook Exp. Pharmacol.* **52**, 213-257

Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., and Patrick, J. (1990a) *J. Neurochem.* **55**, 632-640

Luetje, C. W., Patrick, J., and Seguela, P. (1990b) *FASEB J.* **4**, 2753-2760

Lukas, R. J. (1984) *Biochemistry* **23**, 1160-1164

Lukas, R. J. (1986a) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5741-5745

Lukas, R. J. (1986b) *J. Neurochem.* **47**, 1768-1773

Marks, M. J., Stitzel, J. A., Romm, E., Wehner, J. M., and Collins, A. C. (1986) *Mol. Pharmacol.* **30**, 427-436

McLane, K. E., Tang, F., and Conti-Tronconi, B. M. (1990a) *J. Biol. Chem.*, **265**, 1537-1544

McLane, K. E., Wu, X., and Conti-Tronconi, B. M. (1990b) *J. Biol. Chem.* **265**, 9816-9824

McLane, K. E., Wu, X., Diethelm, B. and Conti-Tronconi, B. M. (1991) *Biochemistry* in press

McPherson, G. A. (1983) *Computer Prog. Biomed.* **17**, 107-111.

Meeker, R. B., Michels, K. M., Libber, M. T., and Hayward, J. N. (1986) *J. Neurosci.* **6,** 1866-1875

Mehraban, F., Kemshead, J. T., and Dolly, J. O. (1984) Eur. *J. Biochem.* **138**, 53-61

Mihovilovic, M., and Richman, D. P. (1987) *J. Biol. Chem.* **262**, 4978-4986

Mosckovitz, R., and Gershoni, J. M. (1988) *J. Biol. Chem.* **263**, 1017-1022

Mulle, C., and Changeux, J.-P. (1990) *J. Neurosci.* **10**, 169-175

Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220-239

Nef, P., Oneyser, C., Alliod, C., Couturier, S., and Ballivet, M. (1988) *EMBO J.* **7**, 595-601

Neubig, R. R., Krodel, E. K., Boyd, N. D. and Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 690-694

Neumann, D., Barchan, D., Safran, A., Gershoni, J. M., and Fuchs, S. (1986a) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3008-3011

Neumann, D., Barchan, D., Fridkin, M., and Fuchs, S. (1986b) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9250-9253

Neumann, D., Barchan, D., Horowitz, M., Kochva, E., and Fuchs, S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7255-7259

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1982) *Nature* **299**, 793-797

Noda, M. , Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T., and Numa, S. (1983a) *Nature* **302**, 528-532

Noda, M., Furutani, Y.,Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S., and Numa, S. (1983b) *Nature* **305**, 818-823

Norman, R. I., Mehraban, F., Barnard, E. A. , and Dolly, J. O. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 1321-1325

Ohana, B., and Gershoni, J. M. (1990) *Biochemistry* **29**, 6409-6415

Papke, R. L., Boulter, J., Patrick, J., and Heinemann, S. (1989) *Neuron* **3**, 589-596

Patrick, J., and Stallcup, W. B. (1977) *J. Biol. Chem.* **252**, 8629-8633

Pedersen, S. E., Dreyer, E. B., and Cohen, J. B. (1986) *J. Biol. Chem.* **261**, 13735-13742

Pederson, S. E., and Cohen, J. B. (1990) *Proc. Natl. Acad. Sci. U. S. A. 87*, 2785-2789

Quik, M., and Geertsen, S. (1988) *Can. J. Physiol. Pharmacol.* **66**, 971-979

Raftery, M. A., Hunkapillar, M. W., Strader, C. D., and Hood, L. E. (1980) *Science* **208**, 1454-1457

Raines, R.T., Sutton, E. L., Straus, D. R., Gilbert, W., and Knowles, J. R. (1986) *Biochemistry* **25**, 7142-7154

Ralston, S., Sarin, V., Thanh, H. L., Rivier, J., Fox, L., and Lindstrom, J. (1987) *Biochemistry* **26**, 3261-3266

Rodbard, D., and Frazier, G. R. (1975) In *Methods in Enzymology*, Vol. 37 (Part B) (O'Malley, B.W., and Hardman, J. G. eds.) pp. 3-22, , Academic Press, New York

Rusak, B., and Bina, K. G. (1990) *Ann. Rev. Neurosci.* **13**, 387-401

Saedi, M., Conroy, W., and Lindstrom, J. (1991) *J. Cell Biol.* 112, 1007-1015

Sawruk, E., Schloss, P., Betz, H., and Schmitt, B. (1990) *EMBO J.* **9**, 2671-2677

Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S., and Lindstrom, J. (1988) *Neuron* **1**, 241-248

Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M., and Lindstrom, J. (1990) *Neuron* **4**, 35-48

Schultz, D. W., and Zigmond, R. E. (1989) *Neurosci. Lett.* **98**, 310-316

Sumikawa, K., and Miledi, R. (1989) *Molec. Brain Res.* **5**, 183-192

Vidal, C., and Changeux, J.-P. (1989) *Neuroscience* **29**, 261-270

Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E.S., Swanson, L.W., Heinemann, S., and Patrick, J. (1988) *Science* **240**, 330-334

Watters, D., and Maelicke, A. (1983) *Biochemistry* **22**, 1811-1819

Whiting, P. J., and Lindstrom, J. M. (1986) *Biochemistry* **25**, 2082-2093

Whiting, P. J., and Lindstrom, J. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 595-599

Whiting, P. J., Schoepfer, R., Swanson, L. W., Simmons, D. M., Lindstrom, J. M. (1987) *Nature* **327**, 515-518

Wilson, P. T., Gershoni, J. M., Hawrot, E., and Lentz, T. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2553-2557

Wilson, P. T., Lentz, T. L., and Hawrot, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8790-8794

Wilson, P. T., Hawrot, E., and Lentz, T. L. (1988) *Mol. Pharmacol.* **34**, 643-651

Wilson, P. T., and Lentz, T. L. (1988) *Biochemistry* **27**, 6667-6674

Wong, L. A., and Gallagher, J. P. (1989) *Nature* **341**, 439-442

Wonnacott, S. (1986) *J. Neurochem.* **47**, 1706-1712

Zatz, M., and Brownstein, M. J. (1981) *Brain Res.* **213**, 438-442

FOOTNOTES

[1] Abbreviations used include αBGT (alpha-bungarotoxin), αBGTBP (alpha-bunagrotoxin binding protein), κBGT (kappa-bungarotoxin), nAChR (nicotinic acetylcholine receptor), IAA (iodoacetamide), DTT (dithiothreitol), IOBA (2-iodosobenzoic acid), HPLC (high pressure liquid chromatography), CM-Cys (S-(carboxymethyl)cysteine), PBS (phosphate-buffered saline).

FIGURE LEGENDS

Fig. 1. **Peptide Sequences and Codes**

Peptides were synthesized and characterized, as described in "Experimental Procedures". For each peptide, the species, receptor, subunit and subtype are given. Negative control peptides included: 1) a negatively charged peptide, represented by the glycine receptor α1 subunit amino acids 61-78 (Grenningloh et al., 1987); 2) a positively charged peptide, represented by the rat brain β2 subunit sequence between residues 331-350 (Deneris et al., 1988); and 3) the cobra muscle α1 sequence between residues 181-200 (Neumann et al., 1989). Positive control sequences included the *Torpedo* electric organ nAChR α subunit (referred to as α1) sequence between residues 181-200 (Noda et al., 1982), and the homologous chick muscle α1 sequence between residues 179-198 (Nef et al., 1988). Overlapping peptides corresponding to the complete chick brain αBGTBP α1 subunit sequence and the sequence including amino acids residues 166-215 of the chick brain αBGTBP α2 subunit (Schoepfer et al., 1990) were tested in these studies for αBGT binding. These peptide sequences are highlighted, and the amino acid residues are given.

Fig. 2. **Qualitative Assessment of ^{125}I-αBGT Binding to Peptides by Toxin Blot Assay: Specific Binding to Sequences 181-200 of αBGTBP α1 and α2.**

(**A**) Peptides solutions (1 μl) were spotted onto nitrocellulose strips and allowed to dry at room temperature The strips were blocked by incubation with 10 mg/ml cytochrome c for 2 hours, followed by incubation with ^{125}I-αBGT (1 μM, 4.4 Ci/mmole) in the presence of 5 mg/ml cytochrome c for 4 hours at room temperature with agitation. The strips were washed as described in "Experimental Procedures". Autoradiographs were exposed for 16 hours at -70°C. The autoradiograms of two different nitrocellulose strips are shown, which accounts for the different backgrounds observed.

(**B**) Toxin blot assays were performed essentially as in (**A**), except nitrocellulose strips were incubated with or without unlabelled αBGT (100 μM) during the blocking period, and incubations

with ^{125}I-αBGT (1 μM, 4.5 Ci/mmole) were performed for 1 hour. Strips were washed as described in "Experimental Procedures" and counted in a gamma counter. The results are presented as the mean of triplicate determinations, and the error bars are standard deviations.

Fig. 3. **Competition of Peptides for ^{125}I-αBGT with Native Membrane Bound *Torpedo* nAChR**

Peptides (40 μM) were incubated overnight at 4°C. with ^{125}I-αBGT (20 nM, 186 cpm/fmole) in a final volume of 100 μl 10 mM potassium phosphate buffer, pH 7.4. containing 10 mg/ml cytochrome c. *Torpedo* membrane-bound nAChR (5 nM) was added and after mixing the solution was centrifuged at 14,000 x g for 45 min. at 10°C. The pellet was washed with PBS and counted in a gamma counter. The results are expressed as the means of triplicate deteminations, and the error bars are standard deviations. "BGT" refers to the binding of ^{125}I-αBGT to *Torpedo* nACHR that has been preincubated with 20 nM unlabelled αBGT for 10 min. prior to its addition to ^{125}I-αBGT, and the positive control is the ^{125}I-αBGT binding to *Torpedo* nAChR in the absence of peptide ("No Peptide"). The peptides correspond to **Glycine** α(61-78), the **Rat nAChR** β2(331-350) ***Torpedo*** α1(181–200), **Cobra** α**1**(181-200), **Chick muscle** α**1**(179-198), **Chick αBGTBP** α**1**(181-200), and **Chick αBGTBP** α**2**(181-200).

Figure 4. Concentration Dependence of Peptide Competition for ^{125}I-αBGT

Torpedo competition assays were performed as described in "Experimental Procedures" and in Figure 3, except that the concentration of peptide was varied between 5-500 μg/ml (~2-200 μM). The assays were performed on different days and contained 20 nM of ^{125}I-αBGT (100-150 cpm/fmole) and 2-3 nM of membrane-bound *Torpedo* nAChR. The results are expressed as the mean of triplicate determinations at each concentration of peptide. The "0% Displaced" values for peptides, Torpedo α1(181-200), chick muscle α1(179-198), chick αBGTBP α1(181–200) and chick αBGTBP α2(181-200) are 18,475, 24,652, 27,581, and 24,818 cpm, respectively. The results are presented as a Hofstee plot (Molinoff et al., 1981), in which the IC_{50} values can be

directly compared as the negative slopes of the linear regression lines. The IC_{50} values obtained by analysis using the computer analysis program EBDA (McPherson, 1983), and correlation coefficients (in parenthese) from linear regression are: ***Torpedo*** **α1**(181–200) IC_{50} 0.83 (-0.87), **Chick muscle α1**(179-198) IC_{50} 1.84 (-0.98), **Chick αBGTBP α1**(181-200) IC_{50} 21 (-0.95), and **Chick αBGTBP α2**(181-200) IC_{50} 1.20 (-0.96).

Fig. 5. ***Torpedo*** **Competition Assay of Peptides for the Complete Chick Brain αBGTBP α1 Sequence**

Torpedo competition assays were performed as described in "Experimental Procedures" and in Figure 4. The results are expressed as the means of triplicate deteminations, and the error bars are standard deviations. "BGT" refers to the binding of ^{125}I-αBGT to *Torpedo* nACHR that has been preincubated with 20 nM unlabelled αBGT for 10 min. prior to its addition to ^{125}I-αBGT (198 cpm/fmole), and the positive control is the ^{125}I-αBGT binding to *Torpedo* nAChR in the absence of peptide ("No Peptide").

Fig. 6. **Carboxymethylation Analysis of Peptide αBGTBP α2(181-200)**

The reaction conditions used for oxidation, reduction and/or alkylation of peptides, and the preparation of peptides for amino acid analysis are described in "Experimental Procedures". The phenylisothiocyanate derivatives of the amino acids released by acid hydrolysis were analyzed on a reverse phase HPLC column (PICO.TAG). The results represent the partial elution profile in 140 mM sodium acetate buffer (pH 6.4) containing 0.05% triethylamine and 6% acetonitrile. The **arrow** marks the expected elution time of the phenylisothiocyanate derivative of carboxymethyl-Cys (**CM-Cys**). The other markers are the derivatives of other amino acid derivatives, **D,N** = Asp/Asn, **G** = Gly, **E** = Glu. For explanation of the abbreviations used for the different treatments (**IAA Only**, and **DTT/IAA**), please refer to the text.

Fig. 7. **Effect of Cysteine/Cystine Modification on αBGT Binding**

(A) P6 Chromatography of Peptides

Untreated peptides and peptides oxidized, reduced and/or alkylated were separated from reactants on a 5 ml Pierce Excellulose GF5 coulumn, and samples of peptides (~100 μg) were applied to P6 columns (1.8 x 47 cm). Both columns were equilibrated and eluted with 10 mM potassium phosphate buffer (pH 7.4). The P6 column was calibrated with bradykinin (1060 daltons), porcine renin substrate tetradecapeptide (1759 daltons), human β-endorphin (3465 daltons), human growth hormone releasing factor (5041 daltons) and blue dextran 2000 (Sigma). The results presented are for untreated peptides, but similiar chromatograms were obtained for the treated peptides.

(B) *Torpedo* Competition Assay Peptides

Untreated peptides and peptides oxidized, reduced and/or alkylated were separated from reactants on a 5 ml Pierce Excellulose GF5 coulumn. *Torpedo* competition assays were performed as described in "Experimental Procedures" and Figure 4. Peptides (~100 μg/ml, 20 μM) were incubated with 5 nM ^{125}I-αBGT (150 cpm/fmole) for 4 hrs. at room temperature, prior to addition of 2 nM *Torpedo* nAChR. "BGT" refers to the binding of ^{125}I-αBGT to *Torpedo* nACHR that has been preincubated with 20 nM unlabelled αBGT for 10 min. prior to its addition to ^{125}I-αBGT, and the positive control is the ^{125}I-αBGT binding to *Torpedo* nAChR in the absence of peptide ("No Peptide").

Fig. 8. **Comparison of Sequences from nAChRs that Bind αBGT with Those that Cannot.**

Homologous sequences from α subunits are aligned according to the *Torpedo* nAChR α1 sequence numbering. Amino acids present in all α sequences, or at positions where there are only occasional conservative substitutions are indicated by a **black** background. Similarly, amino acids characteristic only of those sequences that bind αBGT are indicated by a **dotted** background, whereas those characteristic of only those sequences that cannot bind αBGT are indicated by a

hatched background. The sources of the sequences shown are: *Torpedo* α1 (Noda et al., 1982), chick α1, α2, α3, and α4 (Nef et al., 1988), rat α2 (Wada et al., 1988), rat α3 (Boulter et al., 1986), rat α4 (Goldman et al., 1987), chick αBGTBP α1 and α2 (Schoepfer et al., 1990), *Drosophila* ALS (Bossy et al., 1988) and *Drosophila* ARD (Sawruk et al., 1990), and cobra muscle α1 (Neumann et al., 1989).

Table I: Competition for ^{125}I-αBGT Binding to Torpedo nAChR: IC_{50} Analyses of Peptides

Peptide	IC50 (μM)*
Torpedo α1(181-200)	1.9 (0.2)
Chick Muscle α1(179-198)	2.1 (0.7)
Chick Brain αBGTBP α1(181-200)	37 (10)
Chick Brain αBGTBP α2(181-200)	1.8 (0.6)

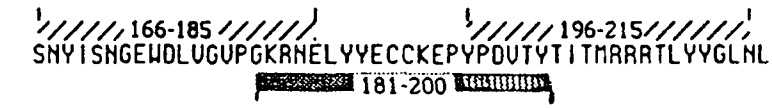
* Standard deviations (n=5 experiments) are given in parentheses. IC50s values were determined using the computer program EBDA (McPherson, 1983).

TOP

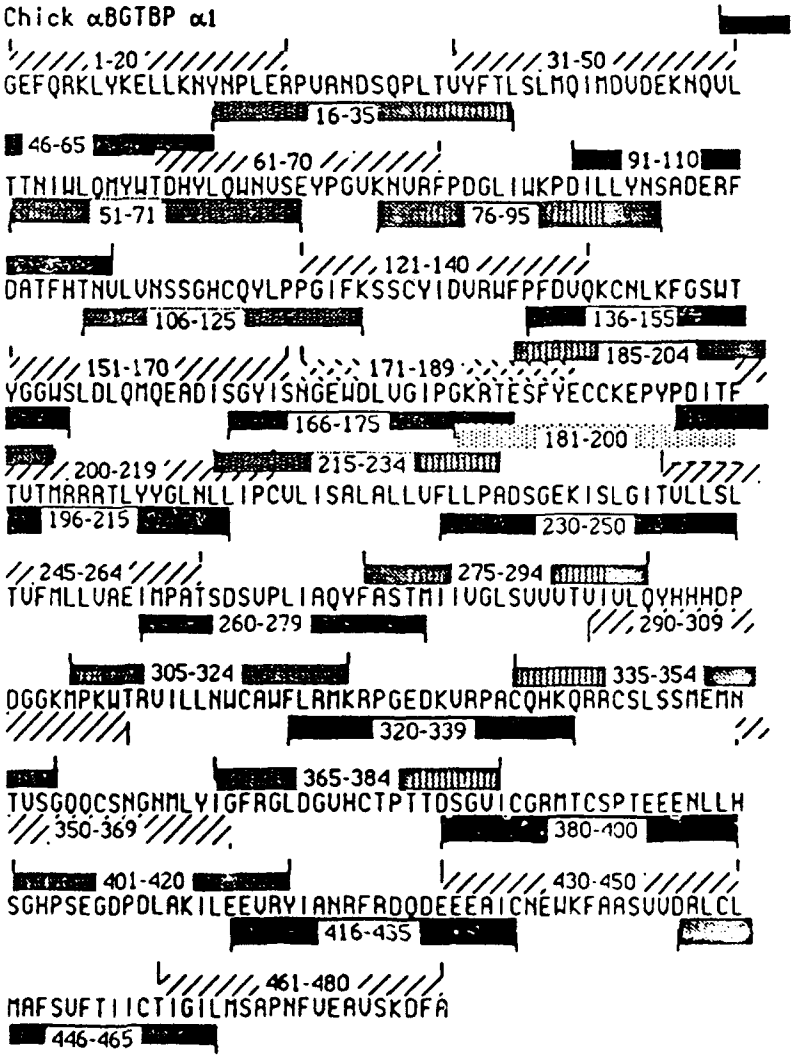
Fig. 1

Glycine α(61-78)	NEYPDDSLDLDPSMLDSI
Rat nAChR β2 (331-350)	ARQRLALRRRQREREGEAV
Torpedo nAChR α (181-200)	YRGWKHWVYYTCCPDTPYLD
Cobra Muscle nAChR α1(181-200)	YRGFWHSVNYSCCLDTPYLD
Chick Muscle nAChR α1(179-198)	YRGWKHWVYYACCPDTPYLD

Chick αBGTBP α2



Chick αBGTBP α1



TOP

A

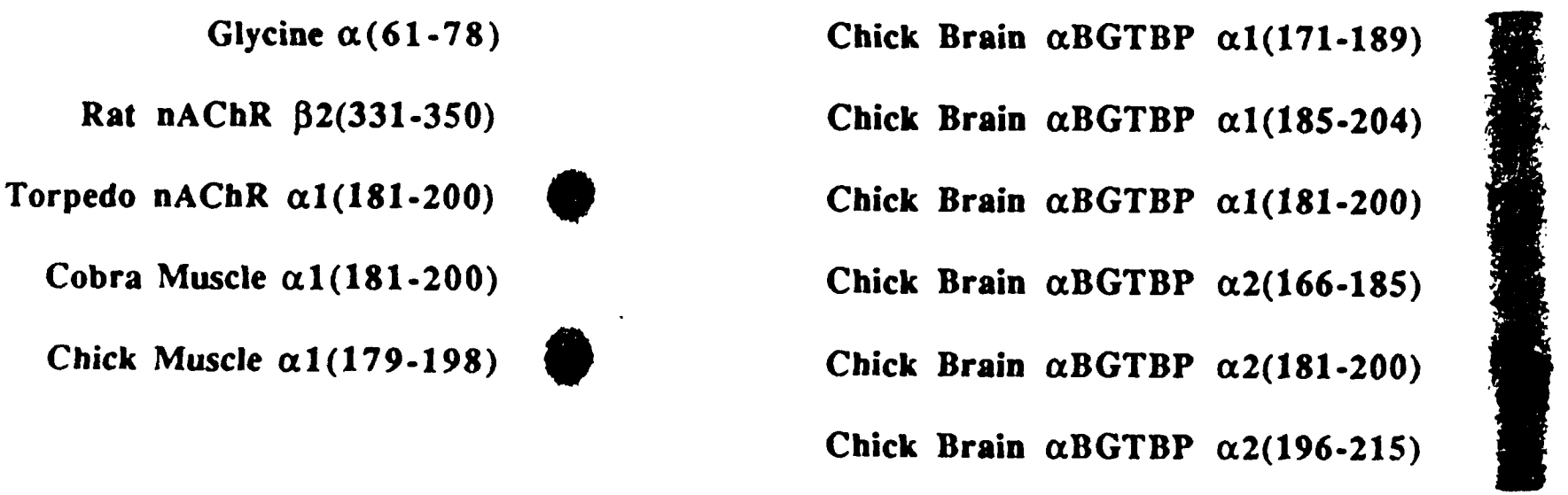
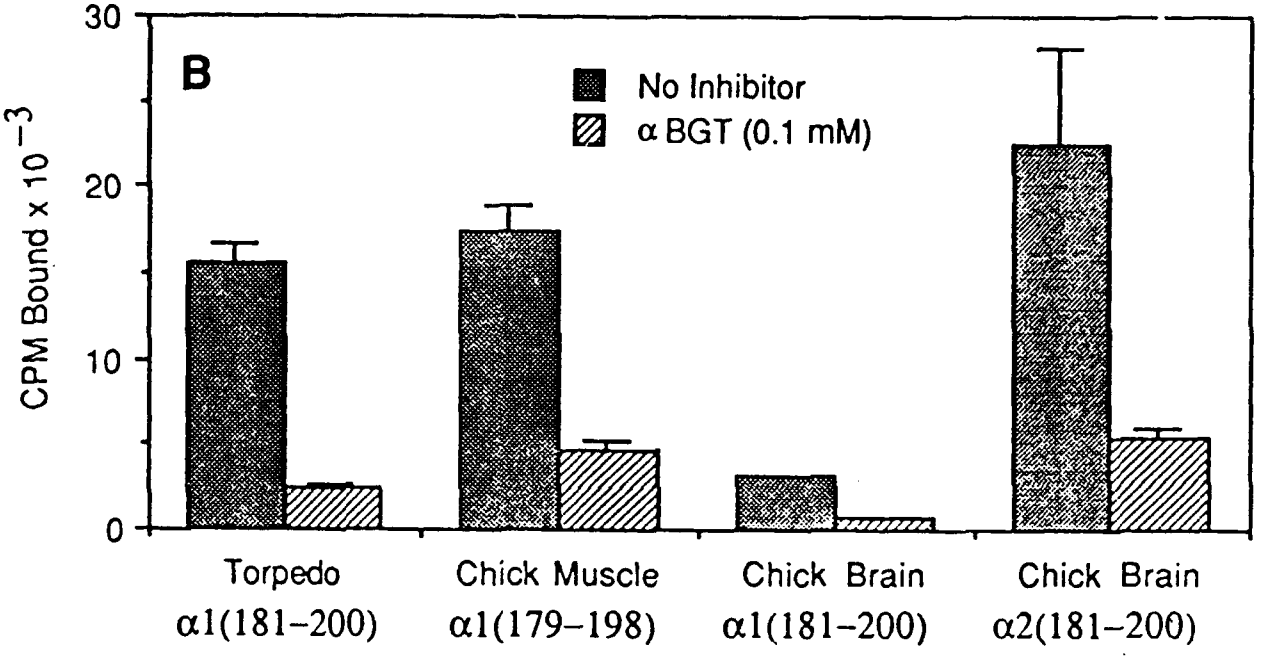

Glycine α(61-78)
Rat nAChR β2(331-350)
Torpedo nAChR α1(181-200)
Cobra Muscle α1(181-200)
Chick Muscle α1(179-198)
Chick Brain αBGTBP α1(171-189)
Chick Brain αBGTBP α1(185-204)
Chick Brain αBGTBP α1(181-200)
Chick Brain αBGTBP α2(166-185)
Chick Brain αBGTBP α2(181-200)
Chick Brain αBGTBP α2(196-215)

Fig. 2A

Fig. 2B


B
No Inhibitor
α BGT (0.1 mM)
CPM Bound x 10^{-3}
30
20
10
0
Torpedo
α1(181–200)
Chick Muscle
α1(179–198)
Chick Brain
α1(181–200)
Chick Brain
α2(181–200)

TOP

Fig. 3

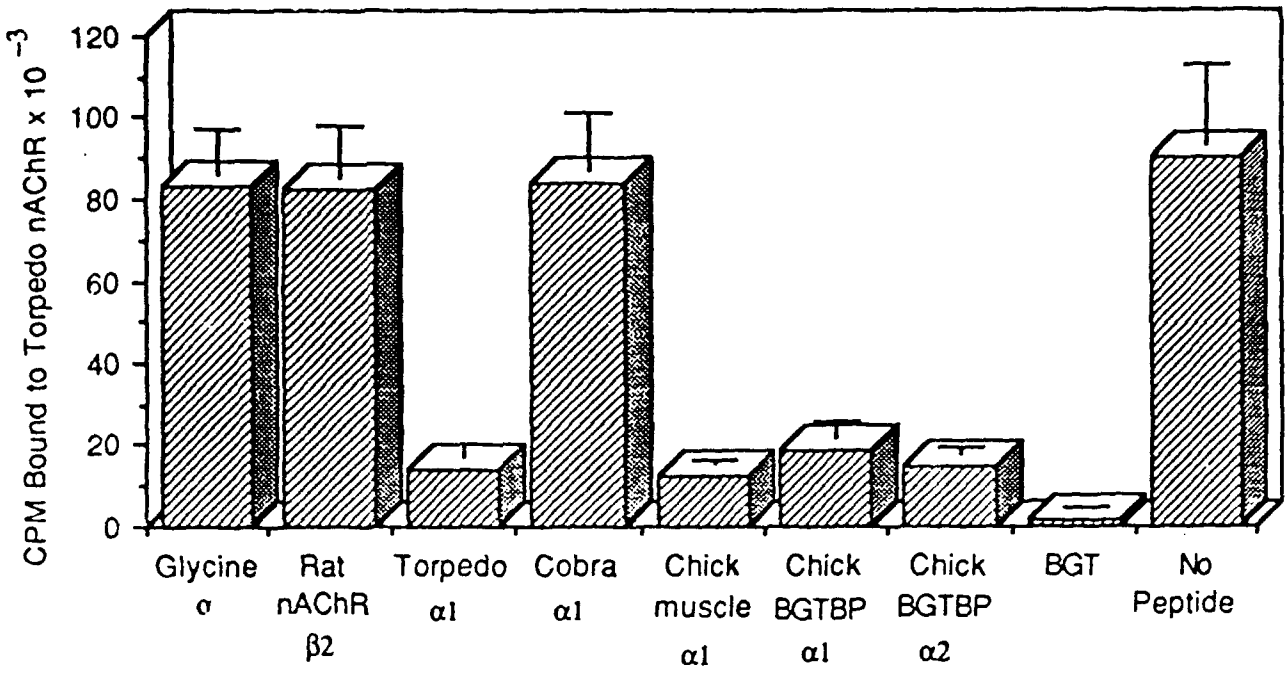
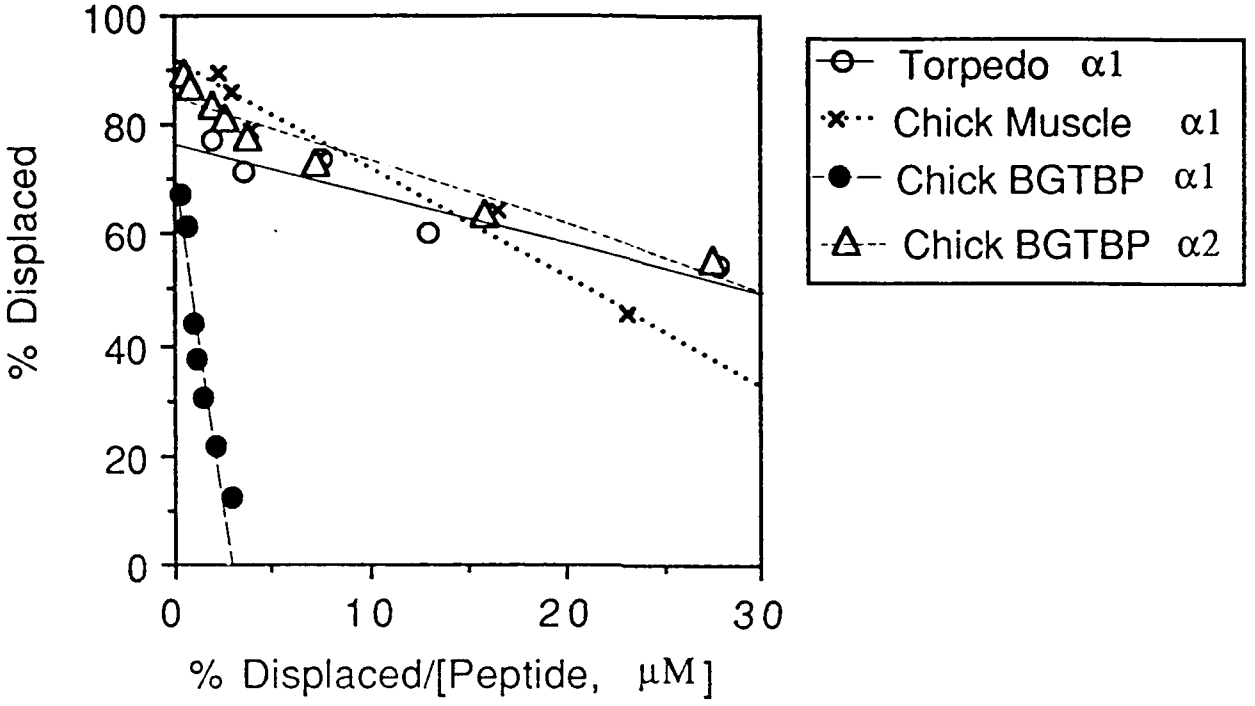

CPM Bound to Torpedo nAChR x 10 -3
120
100
80
60
40
20
0
Glycine α
Rat nAChR β2
Torpedo α1
Cobra α1
Chick muscle α1
Chick BGTBP α1
Chick BGTBP α2
BGT
No Peptide

Fig. 4


100
80
60
40
20
0
% Displaced
0
10
20
30
% Displaced/[Peptide, μM]
Torpedo α1
Chick Muscle α1
Chick BGTBP α1
Chick BGTBP α2

TOP

Fig. 5

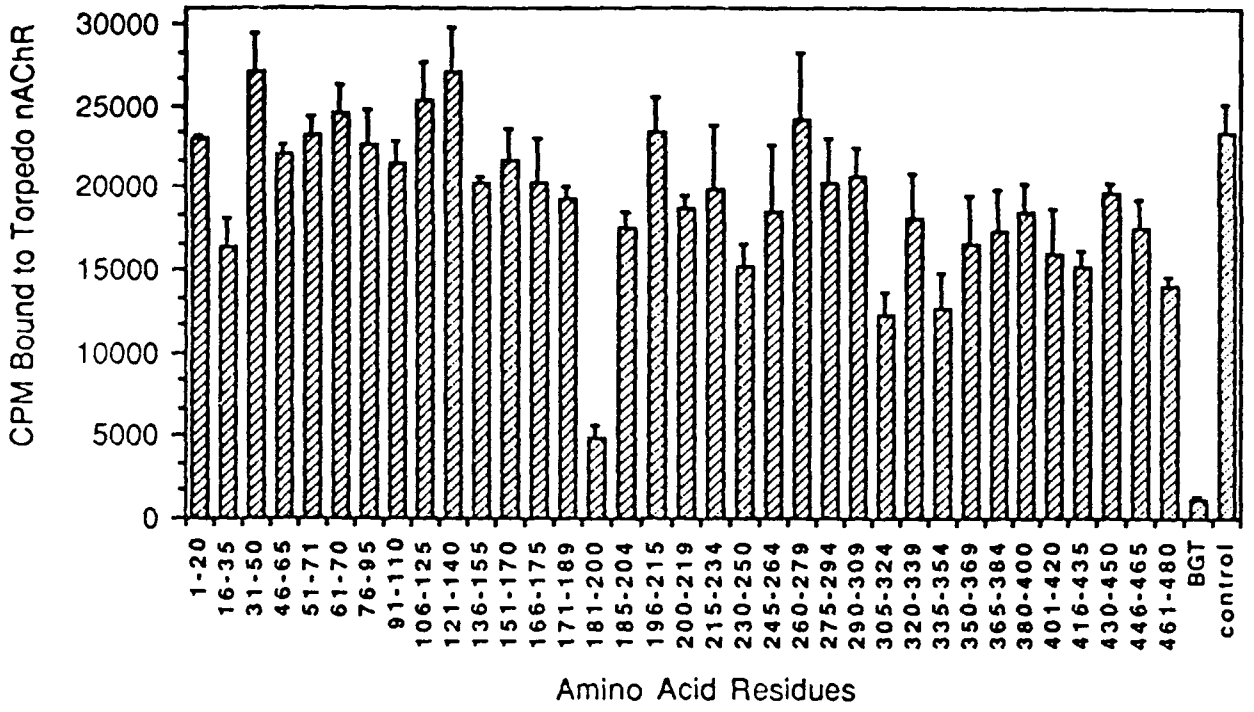
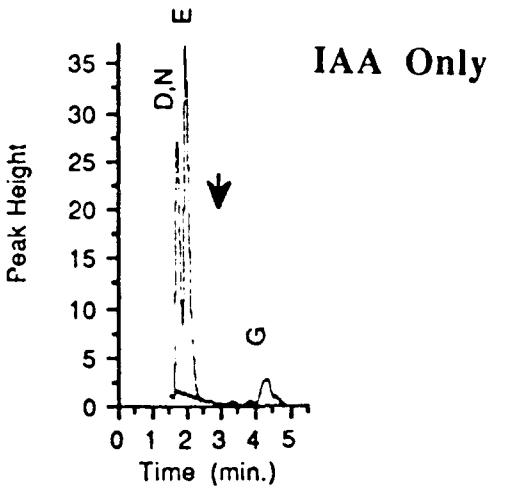

CPM Bound to Torpedo nAChR
30000
25000
20000
15000
10000
5000
0
1-20
16-35
31-50
46-65
51-71
61-70
76-95
91-110
106-125
121-140
136-155
151-170
166-175
171-189
181-200
185-204
196-215
200-219
215-234
230-250
245-264
260-279
275-294
290-309
305-324
320-339
335-354
350-369
365-384
380-400
401-420
416-435
430-450
446-465
461-480
BGT
control
Amino Acid Residues

Fig. 6

Chick αBGTBP
α2(181-200)



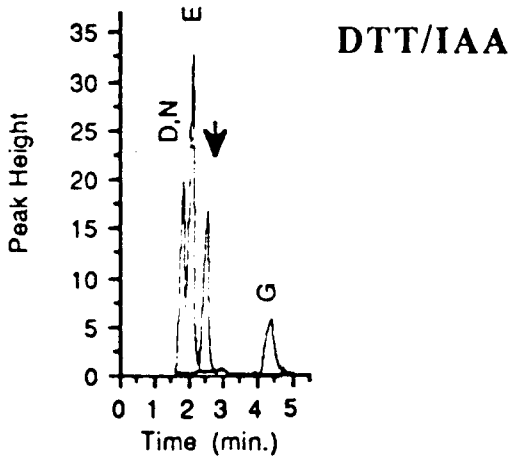


Fig. 7

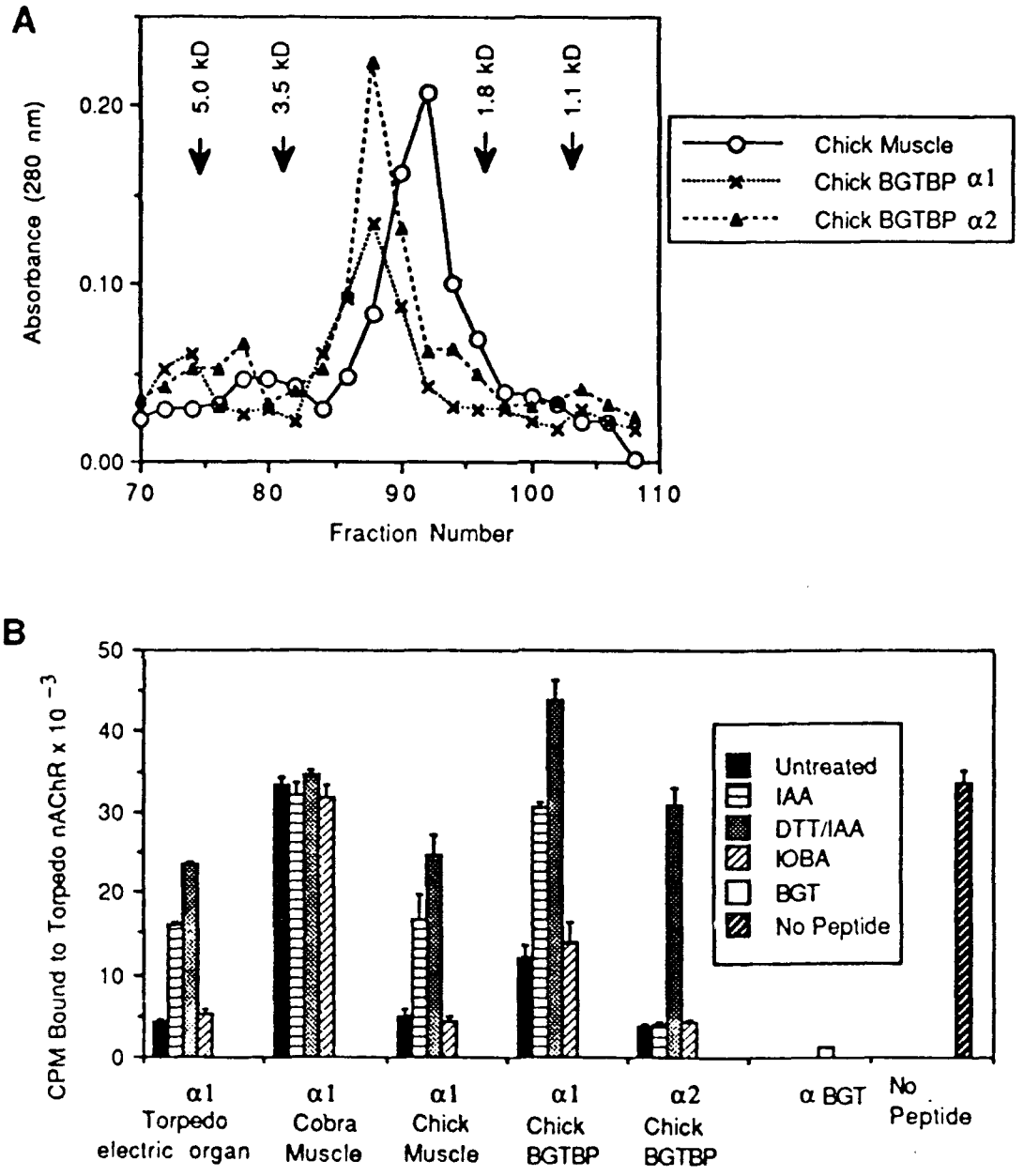
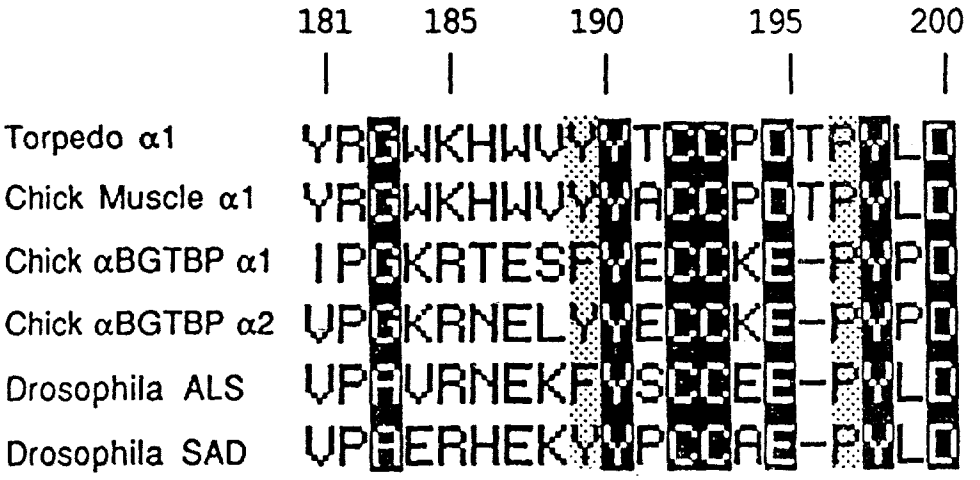
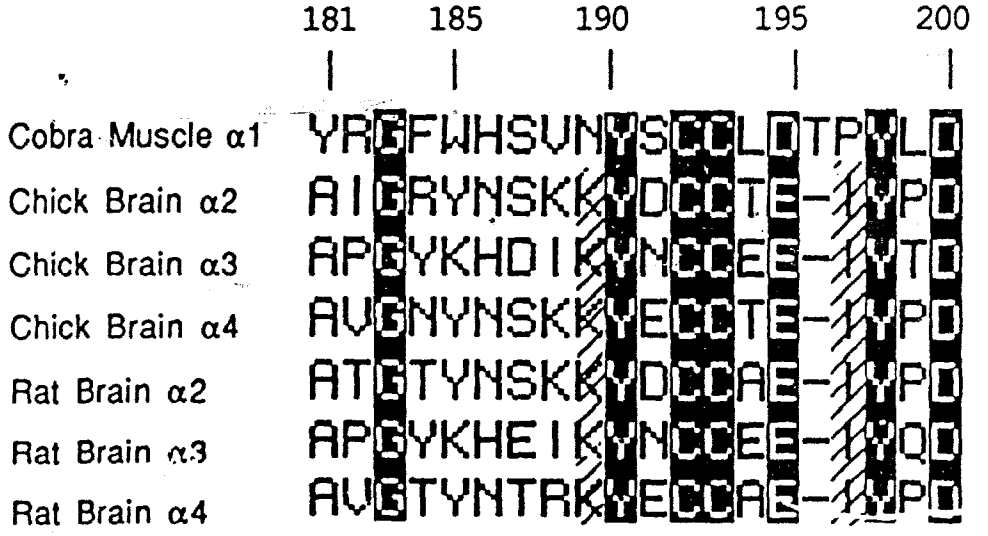

A
5.0 kD
3.5 kD
1.8 kD
1.1 kD
Absorbance (280 nm)
0.20
0.10
0.00
70
80
90
100
110
Fraction Number
Chick Muscle
Chick BGTBP α1
Chick BGTBP α2
B
CPM Bound to Torpedo nAChR x 10 -3
50
40
30
20
10
0
Untreated
IAA
DTT/IAA
IOBA
BGT
No Peptide
α1 Torpedo electric organ
α1 Cobra Muscle
α1 Chick Muscle
α1 Chick BGTBP
α2 Chick BGTBP
α BGT
No Peptide

Fig. 8

nAChR α Subunit Sequences that Bind αBGT



nAChR α Subunit Sequences that Do Not Bind αBGT



END