MONOCLONAL ANTIBODIES AGAINST THE α -BUNGAROTOXIN-BINDING PROTEIN OF CHICK OPTIC LOBE¹

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Abstract

The nicotinic acetylcholine (ACh) receptor probe α -bungarotoxin (α -Butx) binds with high affinity to a membrane protein of the vertebrate central nervous system. To characterize further this putative neuronal ACh receptor, we have prepared monoclonal antibodies (mAbs) against the α -Butx-binding protein of chick optic lobe. Mice were immunized with affinity-purified protein preparations which were estimated to be 10 to 20% pure. Spleen cells from an immunized mouse were fused with the mouse myeloma cell line X63-Ag 8.653. From this fusion, 14 stable hybridoma lines were isolated which produce mAbs against the chick neuronal α -Butx-binding protein. Most of the antibodies inhibited α -Butx-binding to membrane fractions and/or detergent extracts of chick optic lobe. Some of the mAbs cross-reacted with the α -Butx-binding protein of the rat pheochromocytoma cell line PC12. However, none of the mAbs bound to a significant extent to the nicotinic ACh receptor of chick skeletal muscle or of *Torpedo californica* electric organ. All antibodies specifically isolated a polypeptide of $M_r = 57,000 (\pm 2,000)$ from radiolabeled neuronal protein preparations. The present data show that these mAbs constitute useful tools for the further molecular and functional characterization of the neuronal α -Butx-binding protein.

The biochemistry and physiology of the nicotinic ACh receptor in skeletal muscle and fish electric organ have been the subject of extensive investigation (reviewed in Changeux, 1981; Conti-Tronconi and Raftery, 1982). Also, the regulation and the assembly of this multimeric membrane protein have been studied in detail (Fambrough, 1979; Anderson, 1983). This progress has largely relied on the use of highly selective receptor ligands— α -toxins from the venoms of various elapid snake species. One of these polypeptide toxins, α -bungarotoxin (α -Butx), has been most commonly used and shown to block the function of the muscle ACh receptor by binding to its agonist recognition site (Changeux, 1981; Conti-Tronconi and Raftery, 1982).

α -Butx-binding sites of nicotinic-cholinergic specificity are also found in synaptic regions of the nervous systems of both vertebrates and invertebrates (reviewed in Morley and Kemp,

1981; Oswald and Freeman, 1981). The identification of these neuronal α -Butx-binding sites as nicotinic ACh receptors is still highly controversial. In insect ganglia, and in the goldfish and pigeon visual systems, α -Butx has been shown to block cholinergic receptor function (Gepner et al., 1978; Freeman et al., 1980; Schmidt and Freeman, 1980; Oswald and Freeman, 1981). Thus, the α -Butx-binding protein of these tissues can be classified as a putative neuronal nicotinic ACh receptor. For chick ganglia and the pheochromocytoma cell line PC12, however, convincing evidence exists that the α -Butx-binding component is distinct from the functional nicotinic ACh receptor present on the cell surface (Partick and Stallcup, 1977a, b; Carbonetto et al., 1978). Also, the pharmacology of the currently available binding data indicates considerable similarity, but not identity, of the neuronal α -Butx-binding site with that of the "classical" muscle nicotinic ACh receptor (Vogel and Nirenberg, 1976; Patrick and Stallcup, 1977b; Wang et al., 1978; Oswald and Freeman, 1979; Betz, 1981; Morley and Kemp, 1981; Oswald and Freeman, 1981; Betz and Rehm, 1982).

Due to its low abundance, the biochemistry of the neuronal α -Butx-binding protein is not yet well investigated. Currently, the most extensive data exist for the α -Butx-binding protein of the chick visual system, a tissue comparatively rich in α -Butx-binding sites (Vogel and Nirenberg, 1976; Wang et al., 1978; Betz, 1981; Betz and Rehm, 1982). In particular, the α -Butx-binding function of the chick protein has been localized on a polypeptide of $M_r = 57,000 (\pm 3,000)$, and affinity purification for its isolation have been developed (Betz et al., 1982; Norman et al., 1982). To elucidate further the structure and function of the neuronal α -Butx-binding protein, we have produced specific antibodies against this membrane component by using the

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monoclonal antibody (mAb) technology. Here we describe the preparation of these mAbs and report their characterization by immunoglobulin isotype, binding affinity, and protein-binding region.

Materials and Methods

α -Butx was purchased from Miami Serpentarium (Miami, FL) (for purity see Betz et al., 1982). *N*-Acetylglucosamine, carbamylcholine, mercaptoethanol, hypoxanthine, aminopterin, thymidine, and pristane were from Sigma; wheat germ agglutinin-Sepharose, and protein A-Sepharose were from Pharmacia; polyethylene glycol 4000 was from Merck. [125 I]- α -Butx (initial specific activity, 100 to 150 Ci/mmol), [35 S]methionine (initial specific activity, 1400 Ci/mmol), and carrier-free Na 125 I were obtained from New England Nuclear. Rabbit anti-mouse Ig were from Cappel; rabbit anti-mouse IgG subclass-specific antisera were from Miles; all tissue culture plasticware was from Costar; fetal calf serum was from Boehringer-Mannheim. Penicillin, streptomycin, pyruvate, glutamine, Freund's complete and incomplete adjuvant, and Dulbecco's modified Eagle medium containing 4.5 gm/liter of D-glucose (DMEM-H21) were obtained from Gibco. Purified agar was from Difco. Formalin-fixed *Staphylococcus aureus* cells (Pansorbin) were from Calbiochem-Behring Co.

Optic lobe tissue was obtained from newly hatched chickens, frozen in liquid nitrogen, and stored at -70°C . BALB/c mice and goldfish were obtained from the Max-Planck-Institute (Martinsried, FRG). Frozen *Torpedo californica* electric organ was from Biopacific Marine (Venice, CA).

Agarose beads derivatized with α -Butx to a substitution of about 0.3 mg of toxin bound/ml were prepared as described (Betz et al., 1982). In many experiments, 10 mM Tris/Cl buffer, pH 7.4, containing 10 mM NaCl and 1% (w/v) Triton X-100 (buffer A) was used. When stated, buffers contained the previously introduced "antiprotease mixture" (Betz et al., 1982; Pfeiffer et al., 1982) at the following final concentrations: EDTA, 5 mM; EGTA, 3 mM; benzethonium chloride, 100 μM ; benzamidine, 1 mM; phenylmethylsulfonyl fluoride, 100 μM ; aprotinin, 16 milliunits/ml; mercaptoethanol, 5 mM.

Cell Culture

The nonsecreting myeloma cell line X63-Ag 8.653 (Kearney et al., 1979) was kindly provided by H. Thoenen (Max-Planck-Institute, Martinsried, FRG). Selective growth medium (HAT medium) for hybridoma cells contained 0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 2 mM glutamine, 1 mM pyruvate, 50 μM mercaptoethanol, 15% heat-inactivated fetal calf serum, 100 units/ml of penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin in DMEM-H21. HT medium had the same composition without aminopterin. The growth of myeloma cells and the limiting dilution and mass cultivation of hybrid cells were performed in DMEM-H21 containing fetal calf serum, glutamine, pyruvate, mercaptoethanol, and antibiotics at the concentrations specified for HAT medium. For many of the hybridoma lines, the latter medium was preconditioned for 1 to 3 days by mouse peritoneal macrophages (Galfre and Milstein, 1981). The preparation and the metabolic labeling of cell proteins of monolayer cultures of chick embryo retina cells and of the rat pheochromocytoma cell line PC 12 (Greene and Tischler, 1976) were performed as described (Betz et al., 1982).

Preparation of membrane fractions and detergent extracts from chick optic lobe

P2 membrane fractions were prepared from 10 to 20 gm of optic lobe tissue of newly hatched chickens as described (Betz, 1981) except that the anti-protease mixture was included in all buffers. The membrane pellets were extracted in buffer A containing the antiprotease mixture; unsolubilized material was removed by centrifugation at $100,000 \times g$ for 30 min. The supernatants were used immediately, or portions were frozen in liquid nitrogen and stored at -70°C for up to 6 months. Membrane fractions and detergent extracts of chick skeletal muscle, *Torpedo* electric organ, and goldfish optic lobe tissue were prepared according to published procedures (Betz et al., 1977; Betz, 1981). [125 I]- α -Butx binding to membranes, or detergent extracts, was determined using the previously described cellulose acetate, or diethylaminoethyl paper disc, filtration assays (Klett et al., 1973; Betz, 1981).

Purification of the α -Butx-binding protein

Detergent extract (40 to 50 ml, 100 to 150 pmol of α -Butx-binding sites) was incubated on a roller shaker with 3 to 8 ml of α -Butx-derivatized agarose at 4°C for 2 to 4 hr. The agarose beads were then transferred to one or two glass columns (1×10 cm), and the extract was continuously pumped through the beads for 15 to 18 hr (flow rate 7 to 10 ml/hr). By that time, 80 to 90% of the α -Butx-binding sites were adsorbed to the resin. The columns were washed sequentially with 50 to 100 ml of buffer A containing 1 M NaCl, and with 80 to 100 ml of buffer A. The columns were then filled with buffer A containing 1 M carbamylcholine and eluted by continuous cycling over a wheat germ agglutinin-Sepharose column (0.5 ml) which had been equilibrated with the carbamylcholine buffer. After 16 to 24 hr, the wheat germ agglutinin-Sepharose column was washed with approximately 20 ml of buffer A containing 1 M NaCl, then 20 ml of buffer A, and eluted overnight by continuous cycling with 0.8 to 1.5 ml of 0.5 M *N*-acetylglucosamine in buffer A. Eluates were usually collected in two fractions, the first after 15 to 24 hr, and the second after another 1 to 2 days of cycling with the sugar. The purified receptor fractions were frozen in liquid nitrogen and stored at -70°C . Throughout the purification, the anti-protease mixture was included in all buffers.

Antibody-binding assays

Two types of assays were used to monitor antibody binding to the α -Butx-binding protein.

(i) *Immunoprecipitation of the [125 I]- α -Butx-labeled protein.* [125 I]- α -Butx-labeled detergent extracts were used to detect routinely the presence of mAbs and to determine their binding affinities and titers. Detergent extracts were incubated with 10 nM [125 I]- α -Butx for 2 hr at room temperature. Aliquots (50 μl for mAb screening) were then incubated in 1.5-ml Eppendorf cups at room temperature with 10 to 20 μl of 5% (w/v) Triton X-100 and 100 to 200 μl of culture supernatant (or appropriate dilutions of concentrated mAb fractions). Two hours later, 30 to 50 μl of a 2.5% suspension of fixed *S. aureus* precoated with anti-mouse Ig (Kessler, 1975) were added. After another 2 hr at room temperature, the incubations were diluted with 1 ml of buffer A and centrifuged at $12,000 \times g$ for 2 min. The supernatants were removed; the tubes were filled with 1 ml of buffer A, centrifuged as above, and drained of fluid by suction; and the pellet was counted in a γ -counter. All determinations were performed in duplicate and corrected for unspecific precipitation observed with culture medium (or control mAb $\beta 1$).

(ii) *Inhibition of [125 I]- α -Butx binding.* Antibodies inhibiting α -toxin binding to the α -Butx-binding protein were revealed by incubating 50 μl of chick optic lobe P2 membranes with 100 to 200 μl of culture supernatant or diluted mAb fraction. Control incubations contained culture medium, or mAb $\beta 1$, respectively. After 2 hr at room temperature, [125 I]- α -Butx was added to a final concentration of 5 nM, and the membranes were incubated at 37°C for 30 min. Then the amount of [125 I]- α -Butx bound was determined by filtration on cellulose acetate filters (Betz, 1981). All assays were corrected for unspecific [125 I]- α -Butx binding observed in the presence of 1 μM unlabeled α -Butx. The inhibition of [125 I]- α -Butx binding to detergent extracts was determined using the same protocol, except that all incubations contained 0.8% (w/v) Triton X-100 and the filtration assay of Klett et al. (1973) was used.

Antibody titers and affinities were determined by titrating appropriate dilutions of the mAb fractions with different concentrations of [125 I]- α -Butx-labeled detergent extracts. The cross-reactivity of mAbs with membrane fractions, or detergent extracts, prepared from different tissues was tested using the same antibody-binding assays.

Immunization

Female BALB/c mice were injected intraperitoneally with 400 μl of a 1:1.2 (v/v) mixture of purified α -Butx-binding protein and of Freund's complete adjuvant. In addition, aliquots of the mixture were injected intradermally in the back (total volume 300 to 400 μl). After 3 to 4 weeks, the animals were boosted by a second intraperitoneal injection of an emulsion of the purified α -Butx-binding protein and Freund's

⁴ mAb $\beta 1$ is a monoclonal antibody against the presynaptic neurotoxin β -bungarotoxin (H. Betz and F. Pfeiffer, unpublished experiments).

incomplete adjuvant (1:1, v/v). This injection was repeated twice in 4-week intervals. One week after the first and second boost, blood was taken from the tail vein, and the serum was tested for antibody titer. The mouse exhibiting the highest titer was selected for fusion. Four weeks after the last booster, and 3 days prior to fusion, this mouse received an intraperitoneal injection of 300 μ l of purified α -Butx-binding protein (without adjuvant). The amount of protein given in the different injections varied with different receptor preparations and with each mouse, but it always was between 1.5 and 6 μ g of total protein/injection.

Cell fusion and antibody production

Spleen cells from an immunized mouse were fused with X63-Ag 8.653 myeloma cells according to standard methods (Gooding, 1980; Galfré and Milstein, 1981). Briefly, 10^6 spleen cells suspended in DMEM-H21 adjusted to pH 8.5 were fused with 2×10^7 myeloma cells using 50% (w/w) polyethylene glycol 4000. The fused cells were suspended in 5 ml of HAT medium, 10^7 freshly prepared mouse peritoneal macrophages in 31 ml of prewarmed (37°C) HAT medium were added, and the cells were then mixed with 36 ml of 0.5% (w/v) agar in HAT medium prewarmed to 45°C. Immediately after mixing, 500- μ l aliquots of the cell suspension were distributed in six 24-well tissue culture plates and incubated at 37°C in a humidified 10% CO₂ incubator. After 2 hr, HAT medium was added to each well, and the cells were grown for 10 days. Then the medium was replaced by HT medium, and the wells were inspected for hybrid growth. All wells contained one to six hybridoma colonies. Three days later, supernatants were taken from all wells and screened for mAbs using both antibody-binding assays. Colonies from positive wells were isolated with the aid of a Pasteur pipette, expanded in 2-ml wells on a feeder layer of mouse peritoneal macrophages, and rescreened for mAb production. Positive cell populations were recloned twice by limiting dilution. For mass production of mAbs, cloned hybrids (code OAR) were grown in 150-cm² Costar tissue culture flasks or injected intraperitoneally into pristane-primed BALB/c mice for ascites production (Galfré and Milstein, 1981).

All quantitative assays and immune precipitations described in this paper were performed with 20 to 40 times concentrated fractions of the culture media. These mAb preparations were isolated by 50% ammonium sulfate precipitation and subsequent dialysis against 20 mM Tris/Cl buffer, pH 7.4, containing 150 mM NaCl and 0.1% (w/v) sodium azide. Aliquots were stored at -70°C. Determination of Ig class and subclass was done by immunodiffusion (Ouchterlony, 1967) using anti-mouse IgG and IgG subclass-specific antisera.

Electrophoresis and protein determination

SDS-polyacrylamide gel electrophoresis and autoradiography/fluorography of the dried gels (10% polyacrylamide) were performed as described (Betz et al., 1982). Some gels were stained for protein using the silver staining procedure of Oakley et al. (1980). Protein was determined according to Lowry et al. (1951) using bovine serum albumin as a standard. Dilute protein solutions were concentrated by trichloroacetic acid precipitation and assayed for protein content according to Peterson (1977) as modified by Pfeiffer et al. (1982).

¹²⁵I labeling of the α -Butx-binding protein

Purified α -Butx-binding protein was dialyzed for 4 days against three changes of 10 mM Tris/Cl buffer, pH 7.4, containing the antiprotease mixture. Aliquots (4 to 7 μ g) of the dialyzed protein preparation were then iodinated with 1 mCi of Na¹²⁵I using the lactoperoxidase method (Sutter et al., 1979). Upon gel filtration on Biogel P2, 35 to 40% of the radioactivity migrated in the void volume. SDS-polyacrylamide gel electrophoresis and gel slicing showed that about 3% of the latter radioactivity was incorporated into protein. The remaining radiolabel migrated at the dye front and probably represented ¹²⁵I-labeled Triton X-100 (data not shown).

Immunoaffinity purification of the α -Butx-binding protein on mAb OAR 11b-coated protein A-Sepharose

Protein A-Sepharose (2.5 ml) was incubated with 0.5 ml of mAb OAR 11b in 5 ml of buffer A at 4°C for 4 hr. The beads were transferred to a column (1 $\times$ 10 cm) and washed with 50 ml of buffer A containing 150 mM NaCl. Then 20 ml of detergent extract prepared from optic lobe membranes (70 pmol of α -Butx-binding sites) was pumped through the beads at a flow rate of 1 ml/hr, and fractions of 1 ml were collected.

The column was washed with 50 ml of buffer A containing 200 mM NaCl and then eluted sequentially with 20 ml of 100 mM sodium phosphate buffer, pH 6.0, containing 1% (w/v) Triton X-100, and 20 ml of 100 mM sodium citrate buffer, pH 5.0, containing 1% (w/v) Triton X-100. During the elution, the flow rate was 20 ml/hr, and 2.5-ml fractions were collected in tubes containing 0.2 ml of 1 M Tris/Cl buffer, pH 7.4. Throughout this purification, the antiprotease mixture was included in all buffers.

Immunoprecipitation of radiolabeled cell extracts and of ¹²⁵I-labeled α -Butx-binding protein

Detergent extracts of [³⁵S]methionine-labeled cell cultures were prepared as described (Betz et al., 1982). The extracts were preadsorbed with 100 μ l/ml of fixed *S. aureus* at 4°C for 45 min, and then the extracts were centrifuged at 150,000 $\times$ g for 30 min. Aliquots of the extracts were incubated at 4°C with protein A-Sepharose beads which had been coated with mAb. After 3 hr of gentle shaking, the beads were washed three times with buffer A containing 150 mM NaCl and once with the same buffer without NaCl. The beads were then boiled for 5 min in 200 μ l of SDS-sample buffer (Laemmli, 1970), and the supernatants were used for SDS-polyacrylamide gel electrophoresis. Immunoprecipitation of the ¹²⁵I-labeled α -Butx-binding protein was performed as described for [¹²⁵I]- α -Butx-labeled detergent extracts. In all of these experiments, the antiprotease mixture was included in all buffers.

Results

Purification of the α -Butx-binding protein of chick optic lobe. The α -Butx-binding protein was purified within 3 to 4 days from Triton X-100 extracts of chick optic lobe membranes by sequential affinity chromatography on α -Butx-derivatized agarose and wheat germ agglutinin-Sepharose as detailed under "Materials and Methods." This isolation procedure resulted in a >5000-fold purification of the α -Butx-binding protein, yielding preparations which exhibited specific binding activities of 0.6 to 1 nmol/mg of protein (Table I). Assuming a *M_r* of about 250,000 for the native α -Butx-binding protein (Betz, 1981; Norman et al., 1982) and one or two α -Butx-binding sites per toxin receptor, the pure protein should bind 4 to 8 nmol of α -Butx/mg of protein. Therefore, the preparation was considered to be about 10 to 20% pure. On silver-stained gels (Fig. 1), the purified α -Butx-binding protein preparations exhibited a complex polypeptide pattern. One of the prominent bands migrated at an apparent molecular weight of 56,000 ($\pm$ 2,000), i.e., the previously documented *M_r* of the α -Butx-binding polypeptide of chick optic lobe (Betz et al., 1982). The relative content of this polypeptide in the purified fractions amounted to roughly 10% of the total silver-stained protein (scanning data not shown) and, thus, agreed with the estimate obtained from the [¹²⁵I]- α -Butx-binding data.

Detection of anti- α -Butx-binding protein antibodies in immunized BALB/c mice. Four BALB/c mice were immunized repeatedly with 1.5- to 6- μ g quantities of the affinity-purified

TABLE I

Purification of the α -Butx-binding protein

The purification was performed from 10 gm of chick optic lobe tissue as detailed under "Materials and Methods." [¹²⁵I]- α -Butx binding to the α -Butx-agarose eluate was determined after dialyzing an aliquot against buffer A containing the antiprotease mixture.

Purification Step	¹²⁵ I- α -Butx binding		Purification	Yield
	pmol	pmol/mg of protein	-fold	%
Tissue homogenate	175	0.16	1.0	100
Detergent extract	122	0.43	2.7	70
α -Butx-agarose eluate	ND ^a	402	2512	ND
Wheat germ agglutinin-Sepharose eluate	12	930	5813	7

^a ND not determined.

Figure 1. SDS-polyacrylamide gel electrophoresis of the purified α -Butx-binding protein. The α -Butx-binding protein of chick optic lobe was purified by sequential affinity chromatography on α -Butx-derivatized agarose and wheat germ agglutinin-Sepharose as described under "Materials and Methods." Aliquots of the column eluates (500 μ l) were concentrated by trichloroacetic acid precipitation (Pfeiffer et al., 1982), electrophoresed, and the gel was silver stained. Lane 2, Eluate from α -Butx-derivatized agarose; lane 3, eluate from wheat germ agglutinin-Sepharose. Lane 1, molecular weight markers β -galactosidase ($M_r = 116,000$), phosphorylase B ($M_r = 92,500$), bovine serum albumin ($M_r = 66,200$), glutamate dehydrogenase ($M_r = 53,000$), ovalbumin ($M_r = 45,000$), glyceraldehyde 3-phosphate dehydrogenase ($M_r = 36,000$), carbonic anhydrase ($M_r = 31,000$), and soybean trypsin inhibitor ($M_r = 21,000$). The arrow indicates the position of the putative α -Butx-binding polypeptide.

α -Butx-binding protein emulsified in Freund's adjuvant. After two to three immunizations all four mice exhibited antibody titers against the [125 I]- α -Butx-labeled toxin-binding protein of detergent extracts of chick optic lobe membranes. As demonstrated for one serum in Figure 2, the antibodies bound the specifically labeled protein and showed no cross-reactivity with [125 I]- α -Butx. The mouse exhibiting the highest antibody titer was selected for the production of hybridoma cells. By fusion of the spleen of this mouse with the X63-Ag 8.653 myeloma cell line, many (above 400) hybrid colonies were obtained. Two weeks after the fusion, the culture supernatants of 18 of 144 wells were able to bind the [125 I]- α -Butx-labeled α -toxin-binding protein present in detergent extracts of chick optic lobe. Some of the positive culture supernatants also inhibited α -Butx binding to chick optic lobe membranes. However, no additional hybrids secreting specific antibodies were detected with this second screening procedure. From these 18 positive wells, 14 stable hybridoma cell lines (OAR 1-14) which secreted mAbs against the α -Butx-binding protein were established by cloning and recloning. All cell lines were derived from different wells.

Characterization of mAbs. The mAbs produced by the hybrid cells all were IgG, and they were all shown to react with protein A (data not shown). Eleven of the mAbs were of subclass IgG1, and three were of the IgG2a type (Table II). The antibodies differed significantly with respect to their binding affinity for the detergent-solubilized α -Butx-binding protein. All mAbs exhibited K_D values in the nanomolar range (Table II). However, it should be noted that these K_D values are only approximate estimates of binding affinity (for discussion see Tzartos et al., 1981). Most of the mAbs inhibited [125 I]- α -Butx binding to membrane fractions and/or detergent extracts from chick optic lobe tissue by >80% (Table II). Usually, toxin binding to detergent extracts was inhibited more efficiently than binding to membrane fractions. This was particularly striking in the case of mAbs OAR 1, 11 and 14, where the antibodies had no significant effect on [125 I]- α -Butx binding to membranes but completely blocked soluble α -Butx-binding sites (Fig. 3, and data not shown). Control experiments showed that none of the mAbs bound [125 I]- α -Butx (data not shown).

Cross-reactivity with other tissues. Some of the mAbs cross-

reacted with [125 I]- α -Butx-labeled detergent extracts of the rat pheochromocytoma cell line PC12 (Table II) which exhibits α -Butx-binding sites with properties similar to those of the α -toxin-binding protein of the chick visual system (Patrick and

Stallcup, 1977a, b). However, none of the antibodies bound significant amounts of the [125 I]- α -Butx-binding sites present in detergent extracts of goldfish optic lobe, of skeletal muscle from 13-day-old chick embryos, or of electric organ from *Torpedo californica* (<3% precipitation when using an amount of mAb sufficient to precipitate a 10- to 100-fold excess of chick optic lobe α -Butx-binding sites). Also, the mAbs were ineffec-

Figure 2. Detection by immunoprecipitation of α -Butx-binding protein antibodies in mouse serum. Detergent extract of chick optic lobe membranes was incubated with 10 nM [125 I]- α -Butx in the absence (sample A) or presence (sample B) of 1 μ M unlabeled α -Butx. Aliquots (50 μ l) of the labeled extracts or 50 μ l of 10 nM [125 I]- α -Butx were then incubated with the indicated dilutions of serum of the mouse used for fusion. After 2 hr at room temperature, *S. aureus* was added, and the incubations were processed as described under "Materials and Methods." Incubations were with: sample A (●), sample B (▲), and [125 I]- α -Butx (○).

Figure 3. Inhibition by mAb OAR 14a of [125 I]- α -Butx binding to membrane fractions and detergent extracts of chick optic lobe. Aliquots of membrane fractions (86 fmol of [125 I]- α -Butx-binding sites) or of detergent extracts (82 fmol of [125 I]- α -Butx-binding sites) were incubated with 100 μ l of the indicated dilutions of mAb OAR 14a for 2 hr at room temperature. Then [125 I]- α -Butx binding to the membranes (▲) or detergent extracts (●) was determined.

TABLE II
Properties of mAbs against the α -Butx-binding protein of chick optic lobe

mAb OAR	Ig class ^a	Titer ^b	K_D ^b	Inhibition of [125 I]- α -Butx Binding to:		Cross-reaction with Detergent Extract from PC12 cells ^d
				Membranes ^c	Detergent Extract ^c	
		nM	nM			
1a	IgG1	700	6	—	+	++
2a	IgG2a	2800	3	—	—	—
3a	IgG2a	1200	0.3	—	—	(+)
4b	IgG1	110	1	+	+	(+)
5a	IgG1	440	0.5	+	+	++
6a	IgG1	3	2	—	—	(+)
7a	IgG1	3100	2	+	+	—
8a	IgG1	4000	7	+	+	+
9b	IgG1	1150	0.5	+	+	—
10a	IgG1	2650	3	+	+	—
11b	IgG1	420	0.8	—	+	++
12a	IgG1	650	1	+	+	—
13a	IgG1	3700	ND ^e	+	+	—
14a	IgG2a	10	ND	—	+	—

^a Determined by immunodiffusion.

^b Determined by titrating a constant amount of antibody with five different concentrations of [125 I]- α -Butx-labeled detergent extract.

^c Determined as described in the legend to Figure 3.

^d Detergent extract prepared from 1 to 5 $\times 10^8$ PC12 cells was labeled with [125 I]- α -Butx, and aliquots were tested for cross-reactivity with the different mAbs as described under "Materials and Methods."

^e ND, not determined.

tive in inhibiting [125 I]- α -Butx binding to membrane fractions prepared from chick muscle or *Torpedo* tissues.

Immunoaffinity purification and immunoprecipitation of the α -Butx-binding protein. In initial experiments, it was shown that the mAbs were suitable affinity reagents for purifying the α -Butx-binding protein from detergent extracts of chick optic lobe membranes. Protein A-Sepharose was coated with mAb OAR 11b, and detergent extract was passed through this affinity matrix using a column procedure. After washing of the column, α -Butx-binding sites were recovered by elution at pH 5.0 (Fig. 4). This protocol gave a 100-fold increase in α -Butx-binding activity per milligram of protein. Most (>90%) of the protein in the pH 5.0 eluate was identified as IgG light and heavy chains by SDS-polyacrylamide gel electrophoresis and silver staining (data not shown). To reveal specifically adsorbed polypeptides, subsequent experiments, therefore, involved radioactively labeled sources of the α -Butx-binding protein.

As a first approach, monolayer cultures, prepared from either chick embryo retina cells or from the rat pheochromocytoma cell line PC12, were labeled metabolically using [35 S]methionine. The α -Butx-binding protein was then precipitated from detergent extracts of the radiolabeled cells by using protein A-Sepharose which had been coated with different mAbs. However, all of these experiments gave high levels of unspecific coprecipitation of radiolabeled proteins. Therefore, controls were run in parallel which contained nonspecific antibodies—for example, a mAb against the snake venom protein β -bungarotoxin (mAb β 1). By this approach, a specifically precipitated band was revealed upon SDS-polyacrylamide gel electrophoresis in immunoprecipitates of [35 S]methionine-labeled PC12 extracts which migrated at $M_r = 58,000 (\pm 2,000)$ (Fig. 5). This molecular weight is consistent with previous molecular weight determinations of the major α -Butx-binding polypeptide in PC12 cells (Betz et al., 1982). With immunoprecipitates of extracts from [35 S]methionine-labeled chick retina cultures, no specifically precipitated polypeptides could be detected by SDS-polyacrylamide gel electrophoresis and autoradiography (data not shown). This probably resulted from the much lower metabolic radiolabeling of the retina cultures compared to PC12 cells (Betz et al., 1982).

Therefore, the α -Butx-binding protein purified from chick optic lobe extracts was labeled with [125 I] using the lactoperoxidase method. Under all conditions used, the efficiency of [125 I]

incorporation into protein was low (see "Materials and Methods"). This most likely was due to the presence of Triton X-100 (Froehner et al., 1977). On SDS-polyacrylamide gels, the radiolabeled material exhibited multiple radioactive polypeptide bands (Fig. 6). Incubation of the [125 I]-labeled protein preparation with the different mAbs resulted in the immunoprecipitation of two radiolabeled polypeptides of apparent $M_r = 82,000$ and $57,000$ (Fig. 6, and data not shown). The $M_r = 82,000$ band was also precipitated with control antibodies (mAb β 1) and, therefore, was considered nonspecific. Furthermore, for all except one of the mAbs which inhibit α -Butx binding to detergent extracts of chick optic lobe membranes (mAbs OAR 1a, 4b, 5a, 7a to 9b, and 11b to 14a), the precipitation of the $M_r = 57,000$ band was significantly reduced upon pre-incubation of the radiolabeled material with unlabeled α -Butx (Fig. 7 and Table III). These data demonstrated that the antibody-binding region of these latter mAbs is located within the α -Butx-binding site of the $M_r = 57,000 (\pm 2,000)$ polypeptide.

For some of the mAbs which inhibited α -Butx-binding, immunoprecipitation of the [125 I]-labeled chick α -Butx-binding protein was also performed in the presence of small cholinergic ligands, i.e., the agonist carbamylcholine and the antagonists *d*-tubocurarine and hexamethonium. In these experiments, carbamylcholine and *d*-tubocurarine had no effect (Fig. 7 and Table III). However, hexamethonium reduced the precipitation of the $M_r = 57,000$ band by mAb OAR 7a but not by the other antibodies tested (Table III). Similar results were also obtained in a [125 I]- α -Butx-binding competition assay using chick retina cultures (Fulpius et al., 1980; Betz, 1981). Here, hexamethonium, but not the other ligands, blocked the inhibition of [125 I]- α -Butx binding by mAbs OAR 7a and 8a (data not shown). These findings suggest that the binding region for mAb OAR 7a (and probably OAR 8a) is not identical to that of the other mAbs tested.

Discussion

In this investigation, mAbs have been produced against a low abundance neuronal membrane component, the α -Butx-binding protein of chick optic lobe, by immunization of mice with microgram quantities of an affinity-purified protein preparation which was judged to be about 10% pure. All immunized mice developed antibody titers against the toxin-binding protein; this suggests that this membrane protein is highly immunogenic. By fusion of the spleen cells of an immunized mouse with the nonsecreting mouse myeloma cell line X63-Ag 8.653, 14 stable hybridoma lines were established which secreted mAbs against the α -Butx-binding protein. Eleven of the mAbs were IgG1, the three others of the IgG2a subclass. At present, it cannot be decided whether some of the mAbs may be identical. However, the reported differences in binding affinity, pharmacological properties, and cross-reactivity exclude this possibility for most of the antibodies.

The mAbs were used as probes to identify immunogenic sites of the α -Butx-binding protein. As a first indication of their specificity and nonidentity, most, but not all, of the mAbs (OAR 1a, 4b, 5a, and 7a to 14a) were found to inhibit [125 I]- α -Butx binding to membrane fractions and/or detergent extract of chick optic lobe. Thus, these antibodies probably bind at or close to the presumptive cholinergic ligand-binding region of the neuronal α -Butx-binding site (Betz et al., 1982; Norman et al., 1982). The number of these "blocking" mAbs obtained in our fusion (11 of 14) was high. This contrasts to observations obtained with *Torpedo* ACh receptor as immunogen. Here, usually only a small fraction of the resulting mAbs was directed against the α -toxin-binding site. Most of the mAbs bound to the so-called "main immunogenic region" of the smallest ACh receptor subunit of $M_r = 40,000$ (Fulpius et al., 1980; Tzartos et al., 1981). These results extend the previously documented

Figure 4. Immunoaffinity purification of the α -Butx-binding protein. The α -Butx-binding protein was purified on protein A-Sepharose coated with mAb 11b as described under "Materials and Methods." Immediately after elution of the column, the fractions were analyzed for [125 I]- α -Butx binding (○) and protein (▲).

Figure 5. SDS-polyacrylamide gel electrophoresis of mAb immunoprecipitates from detergent extracts of [35 S]methionine-labeled PC12 cells. PC12 cells were labeled with [35 S]methionine and lysed in detergent, and the extract was used for immunoprecipitation as described under "Materials and Methods." Aliquots of the detergent extract and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1, Detergent extract. Lanes 2 to 5, Immunoprecipitates obtained with mAb OAR 1a (lane 2), mAb OAR 5a (lane 3), mAb OAR 11b (lane 4), and mAb β 1 (lane 5).

differences between the neuronal α -Butx-binding protein and the classical ACh receptor (reviewed in Morley and Kemp, 1981; Oswald and Freeman, 1981). Furthermore, they suggest that in the neuronal membrane protein the main immunogenic and the α -toxin-binding regions are closely associated.

An interesting finding of the [125 I]- α -Butx-binding inhibition experiments was that all blocking mAbs inhibited [125 I]- α -Butx binding to the solubilized neuronal protein more potently than to membrane fractions. In particular, mAbs OAR 1a, 11b, and 14a blocked toxin binding to only detergent extracts, but not to membranes. This finding suggests that the antigenic region(s) recognized by these latter antibodies is (are) inaccessible in the protein's native membrane environment. Thus, this region(s) most likely is (are) close to the transmembrane portion of the protein and somehow different from the binding domain(s) of mAbs OAR 4b, 5a, 7a, 8a, 9b, 10a, 12a, and 13a. Furthermore, the immunoprecipitation experiments in the

presence of hexamethonium suggest that the binding region of mAb OAR 7a (and probably 8a; see "Results") is not identical to that of mAbs OAR 4b, 5a, 9b, 10a, 12a, and 13a. Together with the group of the nonblocking mAbs OAR 2a, 3a, and 6a, we conclude that our mAbs discriminate at least four different antigenic regions on the α -Butx-binding protein of chick optic lobe (Fig. 8).

A puzzling finding from our experiments was that all the blocking mAbs produced were detected in an antibody-screening assay which used [125 I]- α -Butx-labeled detergent extracts as antigen. One possible explanation is that α -Butx and mAb binding are not mutually exclusive. For sterical reasons, binding of the large IgG molecule could inhibit the binding of α -Butx ($M_r = 8,000$) more efficiently than vice versa. The fact that even very high concentrations of α -Butx only partially prevented the immunoprecipitation of the $M_r = 57,000$ polypeptide lends some support to this hypothesis. Alternatively, the α -

Butx- and/or mAb-binding sites may be represented more than once on the native protein. At present, we cannot distinguish between these possibilities. However, the latter interpretation is consistent with recent investigations which have suggested that the neuronal α -Butx-binding protein may be a homopolymer of one type of subunit (Seto et al., 1981; Norman et al., 1982).

Using the antibody-binding assay procedures developed for the screening of the hybridoma supernatants, mAbs OAR 1a, 4b, and 11b were found to cross-react strongly with the α -Butx-binding protein of the rat pheochromocytoma cell line PC12. Therefore, these mAbs may be useful to study the α -Butx-binding protein of the rat nervous system. None of our mAbs cross-reacted to a significant extent with the α -Butx-binding site of goldfish optic lobe or with nicotinic ACh receptor of chick skeletal muscle and of *Torpedo* electric organ. Also, in our hands none of 22 mAbs specific for different immunogenic regions and/or subunits of the *Torpedo* ACh receptor (kindly provided by S. Tzartos and J. Lindstrom; see Tzartos et al., 1981) was able to precipitate the α -Butx-binding protein from detergent extracts of chick optic lobe (H. Betz, unpublished experiments). Similarly, the distribution in chick brain of binding sites for these mAbs against the fish ACh receptor has recently been shown to differ from that of [125 I]- α -Butx binding (Swanson et al., 1983). All of these observations are consistent with previous notions that there is little immunological cross-reactivity between the ACh receptor of target organs and the neuronal α -Butx-binding protein (reviewed in Morley and Kemp, 1981). Furthermore, even after repeated injections of the affinity-purified α -Butx-binding protein, none of our mice developed myasthenic syndromes, as is usually observed after

injection of *Torpedo* ACh receptor (Patrick et al., 1973). Taken together, these results further strengthen the view that the neuronal α -Butx-binding site is considerably different from the muscle or fish ACh receptor.

When evaluating the cross-reactivity data presented here, however, it should be considered that the sensitivity of the assay methods used may not have been sufficient for detecting weakly cross-reacting material for the following reasons: (i) The number of antibody-binding sites present on the bacterial immunoadsorbent is limited. Thus, only comparatively low mAb concentrations could be used. (ii) The affinity of [125 I]- α -Butx to peripheral ACh receptor is very high (K_D around 1 pM; see Wang et al., 1978). Thus, low affinity competing antibodies may be difficult to detect. Presently, our data cannot exclude low levels of cross-reactivity of the mAbs OAR with the peripheral ACh receptor. A more cautious interpretation of our results also appears to be justified from the following findings. (i) A few polyclonal antisera against *Torpedo* or skeletal muscle ACh receptor have been found to bind the neuronal α -Butx-binding protein from mouse or chick (Block and Billiar, 1979; Betz, 1981; Norman et al., 1982). (ii) Preliminary experiments employing a highly sensitive enzyme-linked immunosorbent assay developed for the ACh receptor (Watters and Maelicke, 1983) demonstrate that most of the blocking mAbs OAR against the chick neuronal α -Butx-binding protein described in this study can, albeit weakly, bind to affinity-purified *Torpedo* ACh receptor (G. Fels, personal communication). These results suggest that, despite their biochemical and immunological differences, the peripheral ACh receptor and the α -Butx-binding protein of the nervous system have at least some antigenic similarity within their (presumptive) ligand-binding regions.

Figure 6. SDS-polyacrylamide gel electrophoresis of mAb immunoprecipitates of the [125 I]-labeled α -Butx-binding protein. Aliquots of the [125 I]-labeled α -Butx-binding protein (10 μ l in buffer A containing the antiprotease mixture) were used for immunoprecipitation by mAbs OAR 1a to 14a and β 1, respectively. The immunoprecipitates and an aliquot of the [125 I]-labeled α -Butx-binding protein were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Only some of the gel lanes are shown. Lane 1, [125 I]-labeled α -Butx-binding protein. Lanes 2 to 8, Immunoprecipitates obtained with mAbs OAR 2a, 7a, 11b, 12a, 13a, 14a, and mAb β 1. Lane 9, Precipitate obtained with culture medium only.

Figure 7. Effect of α -Butx and cholinergic ligands on the immunoprecipitation of the ^{125}I -labeled α -Butx-binding protein by mAb OAR 4b. The ^{125}I -labeled α -Butx-binding protein was immunoprecipitated in the absence or presence of α -Butx or cholinergic ligands as described in the legend to Figure 6. The immunoprecipitates then were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. *Lane 1*, Immunoprecipitate obtained in the absence of ligand; *lane 2*, in the presence of $3\ \mu\text{M}$ α -Butx; *lane 3*, in the presence of $300\ \mu\text{M}$ *d*-tubocurarine; *lane 4*, in the presence of $1\ \text{mM}$ hexamethonium; *lane 5*, in the presence of $10\ \text{mM}$ carbamylcholine.

TABLE III

Effect of cholinergic ligands on the immunoprecipitation of the $M_r = 57,000$ polypeptide

Immunoprecipitation by mAbs OAR 1a to 14a of the ^{125}I -labeled α -Butx-binding protein was performed in the absence or presence of 3 μM α -Butx, 300 μM *d*-tubocurarine, 1 mM hexamethonium, or 30 mM carbamylcholine as described in the legend to Figure 7. After SDS-polyacrylamide gel electrophoresis and autoradiography, the $M_r = 57,000$ band was cut from gel and counted in a gamma counter. The data were corrected for background and normalized to the amount of radioactivity present in the $M_r = 57,000$ band after immunoprecipitation in the absence of cholinergic ligand.

mAb OAR	Immunoprecipitation of $M_r = 57,000$ Polypeptide in the Presence of:			
	α -Butx	<i>d</i> -Tubo- curarine	Hexa- methonium	Carba- mylcholine
	percentage of control ^a			
1a	26	ND ^b	ND	ND
2a	83	ND	ND	ND
3a	82	ND	ND	ND
4b	24	106	103	101
5a	26	ND	ND	ND
6a	82	ND	ND	ND
7a	44	114	51	88
8a	49	112	95	96
9b	48	120	110	106
10a	87	115	114	92
11b	41	ND	ND	ND
12a	27	99	94	87
13a	61	107	92	92
14a	18	ND	ND	ND

^a Control immunoprecipitates contained 400 to 500 cpm.

^b ND, not determined.

Figure 8. Model of the postulated immunogenic regions on the $M_r = 57,000$ polypeptide of the α -Butx-binding protein of chick optic lobe. The area marked B represents the α -Butx-binding region, the area marked H the hexamethonium subsite, and the area marked M the transmembrane region of the protein. For an explanation see the "Discussion."

Immunoprecipitation of [^{35}S]methionine-labeled PC12 cell extracts and of the ^{125}I -labeled α -Butx-binding protein of chick optic lobe showed that all mAbs OAR bound to the previously identified neuronal α -Butx-binding polypeptide of $M_r = 57,000$ (Betz et al., 1982). In the case of all except one of the mAbs which inhibit α -Butx-binding, the precipitation of this polypeptide was reduced in the presence of α -Butx. These findings indicate that the $M_r = 57,000$ polypeptide contains not only the α -toxin and cholinergic ligand-binding site (Betz et al., 1982; Normal et al., 1982) but also the major immunogenic region of this neuronal membrane protein. However, they do not exclude the existence of polypeptides other than that of $M_r = 57,000$. In a previous study, we have presented evidence for two additional polypeptides, of $M_r = 35,000$ and $25,000$, being associated with the α -Butx-binding site of chick retina (Betz et al., 1982). In the experiments reported here, such polypeptides may have been lost by proteolysis and/or dissociation during immunoprecipitation, purification, and/or iodination of the protein. On the other hand, the lower molecular weight polypeptides may have been proteolytic fragments of the $M_r = 57,000$ component. Clearly, additional biochemical studies are required to elucidate the subunit composition and stoichiometry of the neuronal α -Butx-binding protein. More importantly, the enigma of its function, i.e., its relationship and/or identity to a neuronal cholinergic receptor protein, has to be solved. The data presented here indicate that the mAbs we have produced may provide useful tools for pursuing these questions. In addition, the antibodies also may help to study the regulation of the α -Butx-binding protein (Betz, 1983) at the level of protein metabolism.

References

- Anderson, D. J. (1983) Acetylcholine receptor biosynthesis: From kinetics to molecular mechanism. *Trends Neurosci.* 6: 169-171.
- Betz, H. (1981) Characterization of the α -bungarotoxin receptor in chick-embryo retina. *Eur. J. Biochem.* 117: 131-139.
- Betz, H. (1983) Regulation of α -bungarotoxin receptor in chick retina cultures: Effects of membrane depolarization, cyclic nucleotide derivatives, and Ca^{2+} . *J. Neurosci.* 3: 1333-1341.
- Betz, H., and H. Rehm (1982) Characterization of a putative nicotinic acetylcholine receptor in chick retina. In *Neuroreceptors*, F. Hucho, ed., pp. 165-171, Walter de Gruyter & Co., Berlin.
- Betz, H., J. P. Bourgeois, and J. P. Changeux (1977) Evidence for degradation of the acetylcholine (nicotinic) receptor in skeletal muscle during the development of the chick embryo. *FEBS Lett.* 77: 219-224.
- Betz, H., D. Graham, and H. Rehm (1982) Identification of polypeptides associated with a putative neuronal nicotinic acetylcholine receptor. *J. Biol. Chem.* 257: 11390-11394.
- Block, G. A., and R. B. Billiar (1979) Immunological similarities between the hypothalamic α -bungarotoxin receptor and the *Torpedo californica* nicotinic cholinergic receptor. *Brain Res.* 178: 381-387.
- Carbonetto, S. T., D. M. Fambrough, and K. J. Muller (1978) Non-equivalence of α -bungarotoxin receptors and acetylcholine receptors in chick sympathetic neurons. *Proc. Natl. Acad. Sci. U. S. A.* 75: 1016-1020.
- Changeux, J. P. (1981) The acetylcholine receptor: An "allosteric" membrane protein. *Harvey Lect.* 75: 85-254.
- Conti-Tronconi, B. M., and M. A. Raftery (1982) The nicotinic cholinergic receptor: Correlation of molecular structure with functional properties. *Annu. Rev. Biochem.* 51: 491-530.
- Fambrough, D. M. (1979) Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* 59: 165-227.
- Freeman, J. A., J. T. Schmidt, and R. E. Oswald (1980) Effect of α -bungarotoxin on retinotectal synaptic transmission in the goldfish and the toad. *Neuroscience* 5: 929-942.
- Froehner, S. C., C. G. Reiness, and Z. W. Hall (1977) Subunit structure of the acetylcholine receptor from denervated rat skeletal muscle. *J. Biol. Chem.* 252: 8589-8596.
- Fulpius, B. W., R. Miskin, and E. Reich (1980) Antibodies from

- myasthenic patients that compete with cholinergic agents for binding to nicotinic receptors. *Proc. Natl. Acad. Sci. U. S. A.* 77: 4326-4330.
- Galfr, G., and C. Milstein (1981) Preparation of monoclonal antibodies: Strategies and procedures. *Methods Enzymol.* 73: 3-46.
- Gepner, J. I., L. M. Hall, and D. B. Sattelle (1978) Insect acetylcholine receptors as a site of insecticide action. *Nature* 276: 188-190.
- Gooding, J. W. (1980) Antibody production by hybridomas. *J. Immunol. Methods.* 39: 285-308.
- Greene, L. A., and A. S. Tischler (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 73: 2424-2428.
- Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123: 1548-1550.
- Kessler, S. W. (1975) Rapid isolation of antigens from cells with a staphylococcal protein A-adsorbent: Parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 115: 1617-1624.
- Klett, R. P., B. W. Fulpius, D. Cooper, M. Smith, E. Reich, and L. D. Possani (1973) The acetylcholine receptor. *J. Biol. Chem.* 248: 265-275.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227: 680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Morley, B. J., and G. E. Kemp (1981) Characterization of a putative nicotinic acetylcholine receptor in mammalian brain. *Brain Res. Rev.* 3: 81-104.
- Norman, R. I., F. Mehraban, E. A. Barnard, and J. O. Dolly (1982) Nicotinic acetylcholine receptor from chick optic lobe. *Proc. Natl. Acad. Sci. U. S. A.* 79: 1321-1325.
- Oakley, B. R., D. R. Kirsch, and N. R. Morris (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105: 361-363.
- Oswald, R. E., and J. A. Freeman (1979) Characterization of the nicotinic acetylcholine receptor isolated from goldfish brain. *J. Biol. Chem.* 254: 3419-3426.
- Oswald, R. E., and J. A. Freeman (1981) Alpha-bungarotoxin binding and central nervous system nicotinic acetylcholine receptor. *Neuroscience* 6: 1-14.
- Ouchterlony, O. (1967) Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology*. D. M. Weir, ed., pp. 655-706. Blackwell, Oxford.
- Patrick, J., and W. B. Stallcup (1977a) Immunological distinction between acetylcholine receptor and the α -bungarotoxin binding component on sympathetic neurons. *Proc. Natl. Acad. Sci. U. S. A.* 74: 4689-4692.
- Patrick, J., and W. B. Stallcup (1977b) α -Bungarotoxin binding and cholinergic receptor function on a rat sympathetic nerve line. *J. Biol. Chem.* 252: 8629-8633.
- Patrick, J., J. Lindstrom, B. Culp, and J. McMillan (1973) Studies on purified acetylcholine receptor and antiacetylcholine receptor antibody. *Proc. Natl. Acad. Sci. U. S. A.* 70: 3334-3338.
- Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* 83: 346-356.
- Pfeiffer, F., D. Graham, and H. Betz (1982) Purification by affinity chromatography of the glycine receptor of rat spinal cord. *J. Biol. Chem.* 257: 9389-9393.
- Schmidt, J. T., and J. A. Freeman (1980) Electrophysiological evidence that retinotectal synaptic transmission in the goldfish is nicotinic cholinergic. *Brain Res.* 187: 129-142.
- Seto, A., Y. Arimatsu, and T. Amano (1981) Subunit structure of α -bungarotoxin binding component in mouse brain. *J. Neurochem.* 37: 210-216.
- Sutter, A., R. J. Riopelle, R. M. Harris-Warnick, and E. M. Shooter (1979) Nerve growth factor receptors: Characterization of two distinct classes of binding sites on chick embryo sensory ganglion cells. *J. Biol. Chem.* 254: 5972-5982.
- Swanson, L. W., J. Lindstrom, S. Tzartos, L. C. Schmued, D. D. M. O'Leary, and W. M. Cowan (1983) Immunohistochemical localization of monoclonal antibodies to the nicotinic acetylcholine receptor in chick midbrain. *Proc. Natl. Acad. Sci. U. S. A.* 80: 4532-4536.
- Tzartos, S. J., D. E. Rand, B. L. Einarson, and J. M. Lindstrom (1981) Mapping of surface structures of electrophorus acetylcholine receptor using monoclonal antibodies. *J. Biol. Chem.* 256: 8635-8645.
- Vogel, Z., and M. Nirenberg (1976) Localization of acetylcholine receptors during synaptogenesis in retina. *Proc. Natl. Acad. Sci. U. S. A.* 73: 1806-1810.
- Wang, G. K., S. Molinaro, and J. Schmidt (1978) Ligand responses of α -bungarotoxin binding sites from skeletal muscle and optic lobe of the chick. *J. Biol. Chem.* 253: 8507-8512.
- Watters, D., and A. Maelicke (1983) Organization of ligand binding sites at the acetylcholine receptor: A study with monoclonal antibodies. *Biochemistry* 22: 1811-1819.
- Weinberg, C. G., and Z. W. Hall (1979) Antibodies from patients with myasthenia gravis recognize determinants unique to extrajunctional acetylcholine receptors. *Proc. Natl. Acad. Sci. U. S. A.* 76: 504-508.