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NICOTINIC ACETYLCHOLINE RECEPTORS: AN OVERVIEW

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A. INTRODUCTION

Nicotinic acetylcholine receptors (AChR) mediate chemical communication at synapses in many parts of the nervous system of vertebrates, including neuromuscular junctions, autonomic ganglia and certain sites in the brain [1]. This is achieved by an interaction of neurally-released acetylcholine (ACh) with its recognition sites on the AChR of the postsynaptic membrane (Section B), which activates a gated cation-channel and, thereby, gives rise to a transient change in the permeability of that membrane. Transfer of the signal from the nerve to the effector cell is rapid (~ 1 msec) because the channel, which is selective for Na^+ and K^+, is an integral part of the AChR molecule. This has been demonstrated unequivocally for AChR of fish electric organ [reviewed in 2] and, indirectly in the case of vertebrate skeletal muscle [3]. In fact, the state of knowledge of AChR is at the most advanced stage for any type of receptor; hence it is a prototype. AChR from these peripheral tissues is known to be a multisubunit glycoprotein; its properties have been established in detail in the membrane-bound and purified states (Sections B and C). Recent application of the powerful techniques of molecular biology has resulted in further advances: the successful translation of m-RNA *in vitro* to produce functional AChR, and hence the cloning of the genes coding for the AChR subunits together with the elucidation of their complete amino acid sequences, as reviewed in Section D below. Other exciting advances include the identification of the AChR protein as an antigen in an auto-immune disease known as myasthenia gravis [4]; this has resulted in the development of more effective treatments for this muscle weakness condition. More recently, monoclonal antibodies have been prepared against AChR of muscle [5] and fish electric organ [6]; these are proving to be invaluable tools for identifying myasthenogenic determinants, investigating structure/activity relations (Section E), isolating m-RNA (coding for AChR) for molecular genetic studies (Section D) and, finally, for use as templates of structure facilitating recognition of homologous AChR in brain (Section F).

Much of the aforementioned progress on AChR can be attributed to (i) the use of snake α-neurotoxins, particularly α-bungarotoxin (α-BuTX), as highly selective, polypeptide probes that bind pseudo-irreversibly to the AChR; (ii) the highly enriched AChR content of *Torpedo* electric organ; (iii) the ability of detergents to solubilise AChR from the membrane with retention of biological activity and (iv) the application of sophisticated techniques of molecular biology, including the translation system of the *Xenopus* oocyte, shown to be particularly appropriate and informative in the case of a receptor protein. Although experimentation on the electroplaque model system has yielded much useful information, this is not always directly applicable to AChR of muscle or, particularly, brain. It is obvious that studies on mammalian tissues are much more relevant clinically; however, the sparsity of receptor sites in the latter, together with the presence of high levels of proteolytic enzymes, make experimentation with these very difficult [7]. Nevertheless, significant progress has been achieved with skeletal muscle AChR in the last decade and, thus, it warrants emphasis in this article. Since comprehensive reviews of electric fish AChR have appeared in recent years [2, 8], reference will be made to this only when information on muscle AChR is lacking. From a point of view of identity and function, there is much interest in the AChR types present in the central nervous system. In this case, research is hampered because α-BuTX does not normally inhibit nicotinic transmission there except in the optic tectum of lower vertebrates [reviewed in 9]; nevertheless, a specific α-BuTX binding component (BTBC) exists in brain. To ascertain whether this BTBC represents a functional receptor is, therefore, a challenging and important research problem which necessitates the use of additional probes. We discuss here evidence for the existence of an homologous, putative AChR protein in chick brain and highlight progress on the use of monoclonal antibodies against muscle AChR in facilitating further studies of this elusive neuronal molecule.

B. IN SITU STUDIES ON PERIPHERAL AChR IN THE NATIVE MEMBRANE-BOUND STATE

Detection. In addition to electrophysiological measurements, AChR can be recognised by means of α-neurotoxins labelled with a radioactive, fluorescent or enzyme marker [7]. α-BuTX from *Bungarus multicinctus* is normally used because of the irreversible nature of its binding to AChR [10] and the ease with which the aforementioned derivatives can be prepared, without appreciable loss of biological

Abbreviations: ACh, acetylcholine; AChR, nicotinic ACh receptor; α-BuTX, α-bungarotoxin; Con A, concanavalin A; BTBC, α-bungarotoxin binding component; MB(P)TA, 4-(*N*-maleimeido)-benzyl (or phenyl) trimethylammonium iodide.

activity. Most conveniently α-BuTX can be tritiated with N-succinimidyl [2,3^{3}H]propionate [11] or radioiodinated with ^{125}I [12] to high levels of specific radioactivity, followed by separation from the unlabelled toxin. Fluorescent derivatives of α-BuTX, prepared by reaction with tetramethyl-rhodamine isothiocyanate, are most convenient for studies on muscle, but fluorescein conjugates prepared in a similar manner have also been used successfully [13]. Lastly, physiologically active conjugates of α-BuTX and peroxidase or anti-α-BuTX antibodies, used in conjunction with a second antibody labelled with an appropriate enzyme, allow AChR to be localised using both light and electron-microscopy [13].

Influence of innervation on AChR distribution and content. In adult skeletal muscle AChR can be localised predominantly in the endplate regions using autoradiography [13] or fluorescent microscopy (Fig. 1A); the synaptic location, which can be confirmed by histochemical staining of the same preparation for ACh esterase (Fig. 1B), agrees with the electrophysiological demonstration that the maximum sensitivity to applied ACh is present on the post-synaptic membrane [1]. In notable contrast, non-innervated embryonic or cultured muscle cells have AChR distributed all along the sarcolemmal membrane [7]. Also, quantitation of the radioactivity (by scintillation counting or autoradiographic techniques) in these tissues following labelling with radioactive α-BuTX has shown that their AChR content is much higher than innervated muscle, the level being comparable to that of the electric organ from *Electrophorus* (Table 1). Such extra-synaptic AChR is synthesised *de novo* after denervation [14] and is responsible for the denervation super-sensitivity observed electrophysiologically [1]. Interestingly, these more abundant receptors have a much faster rate of turnover than the synaptic variety (Table 1). Thus, innervation appears to stabilise the synaptic form, but the molecular basis of this neural influence remains unknown. In this regard, it is notable that AChR on the synaptic membrane of muscle is known to be immobile. In contrast, lateral motion of AChR in non-innervated myotubes has been detected with a photobleaching technique, following labelling of the receptors with α-BuTX rhodamine conjugate [15]. Additionally, the rotational mobility of *Torpedo* AChR, as measured by phosphorescence relaxation rates in membrane fragments, is increased after alkaline extraction of an extrinsic protein with a molecular weight of 43,000 [16]. Interaction of AChR molecules with each other or with the latter protein (and possibly others) may be responsible for its stabilisation [2]. Phosphorylation of the AChR, which is known to occur in membranes of electric organ, has also been implicated in these phenomena [8].

Ultrastructural location. Electron microscope autoradiography of muscles or electroplax pretreated with ^{3}H- or ^{125}I-labelled α-BuTX has demonstrated that AChR resides on the post-synaptic membrane [13]. Labelling by means of a peroxidase conjugate of α-BuTX has provided evidence for the existence of some sites on the pre-synaptic membrane but the extent and significance of these remains to be established. In addition, autoradiographic studies

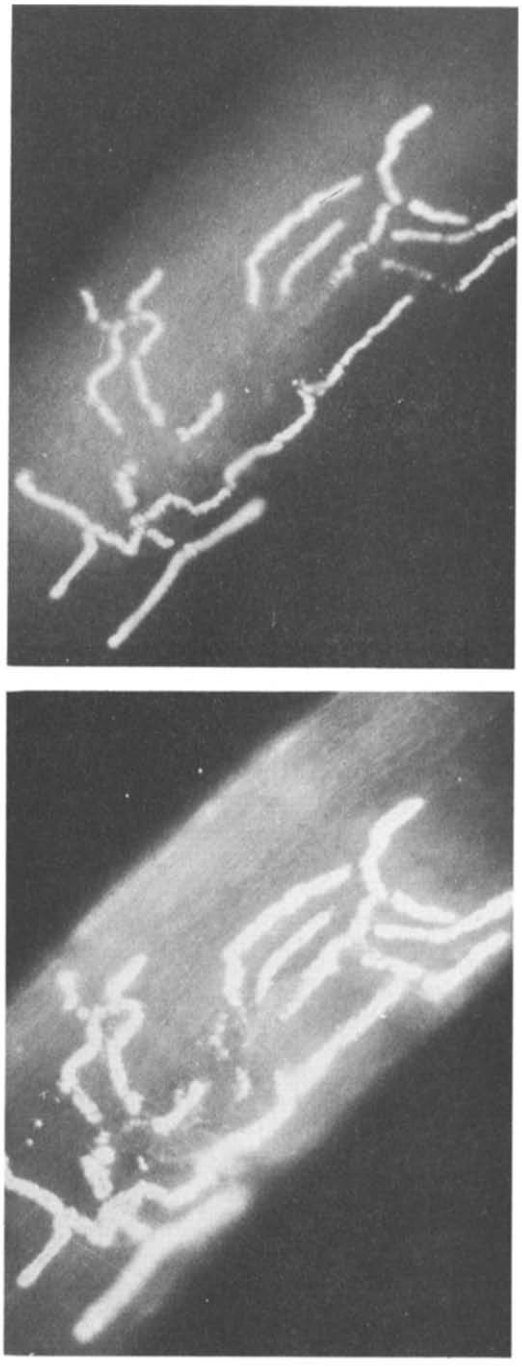

Fig. 1. Fluorescent labelling of AChR at frog neuromuscular junction. Sartorius muscle was treated in Ringer with 1 μM rhodamine-α-BuTX (a homogeneous 2:1 conjugate which is biologically active) for 1 hr at 23°, washed for 2 hr and fixed for 2 hr in 4% paraformaldehyde/0.1 M sodium cacodylate buffer, pH 7.2/0.44 M sucrose. Single fibres of the muscle were teased apart and examined by fluorescent microscopy. (A; upper) The fluorescence pattern seen in a single fibre viewed at 590 nm after excitation with 545 nm. (B; lower) The same fibre was stained cytochemically for ACh esterase, using the acetylthiocholine-copper method of Karnovsky [see 13], and viewed in dark-field illumination. Note that the patterns in (A) and (B) are very similar except that enzymic product occupies a wider area possibly due to diffusion. Preparation made in this laboratory by Dr. A. Le.

Table 1. Properties of membrane-bound AChR

	Skeletal muscle			Electric organ	
	Innervated	Denervated	Embryonic	*Torpedo*	*Electrophorus*
Tissue content* (pmoles of α-BuTX bound/g wet wt.)	0.5–0.8	14–31	27–36	1100–1500	10–20
Density of α-BuTX binding sites/μm^2 of postsynaptic membrane†	20,000–30,500 (at crests of the folds)	—	3000–4000 (in clusters)	27,000‡	49,600‡
Radiation inactivation target size§	—	290,000 (cat)	301,000 (chick)	303,000	—
Turnover rate‖ (half-life)	5–8 days (2–3 days)¶	7–24 hr	28–32 hr (chick muscles)	—	—

* Values are cited for chick muscles (Sumikawa *et al.*, 1982) [21], *Torpedo marmorata* (Unpublished data of R. G. Shorr in this laboratory) and *Electrophorus electricus* (Karlin, 1975) [72].

† Results of a range of autoradiographic measurements are presented for innervated muscle of frog (Matthew-Bellinger and Salpeter, 1978) [73] and mouse (Fertuck and Salpeter, 1976 [74]; Porter and Barnard, 1976 [75]) together with a value published for rat myotubes (Land *et al.*, 1977) [76].

‡ Data of J. Cavanagh (from this laboratory) and Bourgeois *et al.* (1978) [77] for *Torpedo* and *Electrophorus*, respectively.

§ Taken from Lo *et al.* (1982) [18].

‖ References cited in [14].

¶ A second population of AChR, with a somewhat faster turnover rate, appear at muscle synapses following denervation but the overall density remains constant (Loring and Salpeter, 1980) [78].

have deciphered the precise distribution and density of AChR on the post-synaptic membrane. In innervated muscle, AChR is concentrated on the crests of the membrane folds at a maximum density of ~25,000/μm^2 (Table 1), the density at the depths of the folds being very much lower. It is thought that this differential location of AChR maximises the interaction with ACh, which is postulated to be released from sites in the active zone regions directly opposing the AChR-rich crests of the folds [13, 17]. As expected from other measurements cited above, AChR density in extra-synaptic membrane of innervated muscle is ~10^3-fold lower [13, 14]; although this increases after denervation, it never reaches the value at the synapse, which remains constant. Likewise, the average AChR density in embryonic muscle is low, but clusters with higher values (Table 1) are recorded. A comparable density to that of muscle synapses has been observed for *Torpedo*, though the single value reported for *Electrophorus* is significantly higher (Table 1). From the available data, it is apparent that the maximum AChR density is fairly constant for all the muscles studied from a number of species; in contrast, the number of α-BuTX binding sites calculated for muscle ranges from 30 to 87 million per endplate, depending on the species and muscle fibre size [17]. The latter authors have, therefore, postulated that AChR density (rather than total number per synapse) determines the maximum ACh sensitivity, which is also found experimentally to be relatively constant.

Oligomeric size in the membrane. Although several methods are available for determination of the molecular size of AChR after its solubilisation, few techniques are applicable to the AChR in muscle membranes where the content is low (Table 1). However, radiation inactivation of the preparation with high energy electrons using a linear accelerator, coupled to measurement of the loss of α-BuTX binding activity, has been used successfully for this purpose [18]. According to target theory, proportionality exists between the size of the molecule and the amount of energy required to destroy its biological activity. Thus, provided adequate care is taken with the experimental conditions and standard proteins of known molecular weight are added as markers, reliable values for the size of AChR can be obtained from the resultant calibration curves. Using freeze-dried membrane preparations from cat denervated muscle and chicken embryo, the target size obtained for the α-BuTX binding component was ~300,000 (Table 1) with a molecular volume of ~360 nm^3. This finding is in close agreement with that found (270,000) for cross-linked solubilised AChR using SDS gel electrophoresis (see below) and with the value determined by gene sequencing of *Torpedo* AChR subunits (see Section D). The similar target size seen for *Torpedo* AChR indicates that the protein exists as a monomer in the membrane or if dimers are present, as indicated from other investigations, the constituent molecules must be inactivated independently. In accord with this suggestion, successful reconstitution into artificial membranes has been reported for the monomeric and dimeric forms of *Torpedo* AChR [19]. It has been known from other measurements that each AChR monomer con-

tains at least two α-BuTX and ACh binding sites [reviewed in 2]; now, it can be concluded that these reside *in situ* in a single oligomer because all of the α-toxin binding activity is destroyed in a single event.

C. PROPERTIES OF AChR PURIFIED FROM MUSCLE

Solubilisation and purification. AChR is an integral membrane protein; in the case of *Torpedo californica* all of its constituent subunits have been shown by trypsinisation and antibody-labelling experiments to span the membrane ([cf. 2]; see Section D). Efficient solubilisation of AChR is achieved by the use of non-denaturing detergents (e.g. Triton X-100, Lubrol, octylglucoside). Due to the serious problems created by proteolysis [20], maximum precautions must be taken, i.e. including a cocktail of protease inhibitors (Table 2) in all of the solutions used. For denervated muscles, which have an elevated level of proteases [20], it is preferable to perfuse the tissues *in situ* with the anti-proteases prior to their dissection. Extraction of crude membrane preparations with 1–2% Triton X-100/50 mM phosphate buffer, pH 8, for 1 hr at 5°C, with the protease inhibitors present throughout, gives optimal solubilisation of AChR (80–90%) with minimal proteolysis.

For purification, affinity chromatography on α-neurotoxin gel with biospecific ligand elution is routinely used. For this purpose, α-neurotoxin from *Naja n. siamensis* is used because its more reversible binding to AChR affords higher recoveries [7]. The toxin is coupled to Sepharose 4B, following activation with cyanogen bromide, at a concentration of ~0.1 mg/ml of gel bed; this density of ligand is optimal for muscle AChR [20]. Incubation of muscle detergent extract with affinity gel for 3 hr at 5°C results in adsorption of the majority of the AChR present (Table 2). To remove unwanted protein the gel is washed extensively, initially with 50 mM phosphate buffer, pH 8, containing 1 M NaCl and finally with buffer of low ionic strength (25 mM) and minimal detergent concentration (~0.1%). AChR is then eluted at 5°C for 5 hr with 1 M carbamylcholine in the latter medium. These elution conditions minimise the risk of proteolysis and give reasonable recovery (Table 2); yields from overnight elution are somewhat increased. Prior to assaying eluted AChR, carbamylcholine must be removed by gel filtration or chromatography on hydroxylapatite [21]; the latter step also serves to concentrate the AChR. The outlined scheme produces a purification of several thousand fold, and gives material that is close to homogeneity; normally, some contaminants still remain. For complete purification, the sample can be re-chromatographed on α-toxin resin or subjected to density gradient centrifugation [21]. Alternatively, the carbamylcholine eluate can be adsorbed directly onto lentil lectin Sepharose (i.e. removing the receptor via its carbohydrate moieties), washed exhaustively with buffer containing protease inhibitors and eluted with glycosides [21]. For samples eluted from the α-toxin column with relatively low specific activity, this step will achieve an appreciable improvement in specific activity, as illustrated with AChR from chick denervated muscle (Table 2); however, it is a time-consuming procedure and the risk of proteolysis is increased, particularly if the lectin preparation contains any proteases that are resistant to the inhibitors used.

Although variations of these outlined procedures have been employed with reasonable success (Table 3) in other laboratories for muscle AChR of rat [22], human [23, 26], calf [24] and rabbit [25], yields of recovered AChR can be improved considerably by using immuno-affinity chromatography [26]. This utilised a monoclonal antibody, raised against AChR of *Torpedo* and cross-reactive towards human muscle AChR, immobilised on agarose at a concentration of 1 mg/ml. Procedural details are similar to those described for α-toxin gel except that elution was with 1–2 M NaCl, as AChR activity was sensitive to elution buffer of extreme pH values. With innervated human muscle, a purification of 55,000-fold (specific activity of 0.8 nmoles/mg protein) was obtained with a high recovery of 32%. An ion-exchange chromatography step was necessary to produce a maximum further purification of ~7-fold, which is somewhat lower than that found when this step was used following passage through the α-toxin column (Table 3). Nevertheless, it is likely that the specific activity obtained can be improved by selection of an antibody that is optimal for this procedure, from the battery of monoclonal antibodies that are now available [27, 28], particularly those against muscle AChR [5]. Additionally, monospecific antibodies with selectivity for a particular form of AChR could prove extre-

Table 2. Purification of AChR from chick denervated muscle

Stage	(%) Applied AChR (bound or eluted)	(%) Yield overall	Specific activity (pmole/mg)
Triton X-100 extract	100	100	1–2
α-Toxin-Sepharose	61–90	—	—
1 M Carbamylcholine eluate	20–30	13–40	1100–4100
Lentil lectin–Sepharose	60–95	—	—
Glycoside eluate (0.4 M Methyl-glycoside and methyl mannoside)	30–50	3–10	~6000

Values are taken from 4 to 6 representative preparations; the weight of pectoral muscle used in each preparation was 80–160 g. For the preparation of a membrane fraction and extraction of AChR, the buffers used contained the following protease inhibitors: 1 mM EDTA, 0.5 mM phenylmethanesulphonyl fluoride, 0.1 mM benzamidine, 5 μg/ml soybean trypsin inhibitor, 10 μg/ml bacitracin, 5 μg/ml of leupeptin and pepstatin; in addition, 2 mM iodoacetamide was included during isolation of the membrane fraction only [35].

Table 3. Properties of muscle purified AChR

	Species*				
	Chick (innervated, denervated and embryonic)	Human (normal)	Calf (foetal)	Rabbit (inner. and den.)	Rat (inner. and den.)
Specific activity (nmoles toxin/mg protein)	6.0 (den. and emb.)	7.1	6.0	4.1 (den.)	8–10 (den.)
$s_{20,w}$	9S	9S	9.2S	9S	9S
pI (α-BuTX complex)	5.0	5.0	5.2(6.1)‡	5.4	5.2 (inner.) 5.5 (den.)
Reactivity with lectins	Con-A Lentil	Con-A	Con-A	Con-A	Con-A
Myasthenogenic	+ (den.)	+	ND	ND	—
Subunit composition ($M_r \times 10^{-3}$)	40–43†	44	42	42	45(49)
	50	53	49	52	51
	53	56	55	(58)	56
	57	61	58	68	62
		66			
Affinity-labelled subunit ($M_r \times 10^{-3}$)					
Bromo ACh	40§ (den. and emb.)	44(32)	42		
MB(P)TA				42(den.)	45 + 49

* Data on chick is from Sumikawa *et al.* (1982) [21, 35], Barnard and Dolly [9] and Barnard *et al.* [31]; human from Momoi and Lennon [26]; calf from Gotti *et al.* [24] and Conti-Tronconi *et al.* [30]; rabbit from Gotti *et al.* [25]; rat from Froehner *et al.* [79] and Nathanson and Hall [22]. Similar properties have been summarised in a recent report by Turnbull *et al.* [80] for another preparation from human muscle.

† This 4-subunit pattern has been found with denervated muscle.

‡ A second, less abundant species.

§ An additional, minor component of slightly larger size was labelled also but appeared to be convertible to the other subunit by periodate oxidation.

N.D., not determined; ( ) a minor component.

mely useful for isolation of sub-populations from a mixture; such homogeneous species of AChR are required to establish the specificities of anti-AChR antibodies found in human myasthenic serum, which exhibit very different reactivities with the antigens from various muscles (e.g. ocular and leg [29]). They may be particularly important for identification of AChR forms in diseased [26] or developing muscle, in the isolation of m-RNA coding for AChR and for monitoring *in vitro* translation of receptor m-RNA, under various experimental conditions (e.g. in presence of inhibitors of glycosylation or using m-RNA preparations coding for less than the normal complement of AChR subunits).

Criteria of purity. The maximum specific activities obtainable range from 4 to 10 nmoles α-BuTX binding/mg protein for AChR from several species (Tables 2 and 3). Considering the difficulties in measuring the small amounts of protein obtained and, also, problems in accurately determining the specific radioactivities of the labelled α-BuTX preparations used [11], these values are fairly close to the theoretical figure of 7.5 nmoles/mg of protein; the latter is calculated on the basis of an oligomer with a molecular weight of ~267,000 and containing two α-toxin sites (see below). In addition, some inactivation of AChR activity unavoidably occurs, particularly when handling solutions of such low protein concentrations. Finally, the values of specific activity for these membraneous AChR proteins are based on protein determinations made with a soluble standard, serum albumin, which may not behave appropriately in the Folin–Lowry reaction.

Gel isoelectric focusing of the native AChR on an analytical scale, followed by protein staining, was shown to yield a single band with pI ~ 5.0 for AChR purified from cat denervated muscle [20]: parallel, preparative electrofocusing of the same sample, with measurement of α-BuTX binding in extracts of the gel slices, gave one peak of activity with a pI of ~5.0, corresponding to that of the single protein band. However, electrofocusing of AChR preparations in a narrow pH gradient can reveal microheterogeneity. Often, the majority of analytical methods applied to AChR preparations [24–26] rely on detection of α-BuTX binding activity; it must be emphasised that these cannot establish the presence of contaminants but, of course, they can reveal if multiple forms of AChR exist in a given preparation. For example, purified AChR from foetal calf [24] or human muscle with ischaemic vascular disease [26] showed multiple peaks when complexed to ^{125}I-α-BuTX and subjected to isoelectric focusing. Thus, for a more meaningful analysis of purity and to increase the sensitivity of detection, preparations can be ^{125}I-iodinated to label most, if not all, the material present; care must be taken to ensure that the iodination conditions employed do not alter the state of the AChR. Sedimentation analysis of such samples, performed in the absence and presence of antibodies made against homogeneous AChR, offers an excellent criterion of purity [21]. Thus, with purified chick AChR a single radioactive peak ($s_{20,w}$=9S) was obtained on sucrose gradient centrifugation; after incubation with anti-*Torpedo* AChR antibodies, practically all of the radioactivity was shifted to higher S values (Fig. 2),

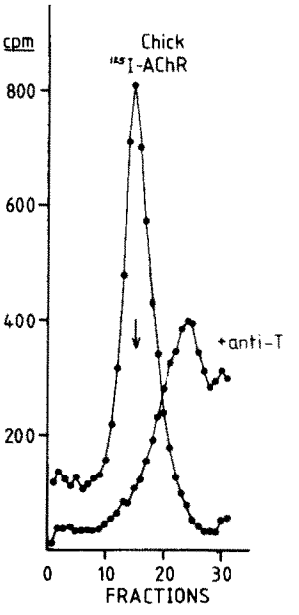

Fig. 2. Immuno-reactivity of ^{125}I-labelled AChR as a criterion of its purity. AChR, isolated from chick denervated muscle by affinity chromatography on α-toxin Sepharose, was ^{125}I-iodinated, purified further by re-chromatography on the latter resin and the major 9S peak isolated by sucrose gradient centrifugation (as outlined below). This radiolabelled material was incubated overnight at 4° with an excess of rabbit antibodies against pure AChR from *Torpedo* electric organ (anti-T) or control serum. The samples were then subjected to sedimentation analysis on 5–20% sucrose gradients; after fractionation of the gradients, the resultant fractions were analyzed for ^{125}I by γ-counting. Molecular sizes were determined by calibration with a set of molecular weight standards (bovine serum albumin-4S, catalase-11.3S) and the two oligomeric forms, 9 and 13S, of *Torpedo* AChR-^{125}I-α-toxin complex. The position (arrow) of the 9S form of *Torpedo* AChR coincided with the major peak of chick AChR-toxin complex that was incubated with non-immune serum; in contrast, incubation with specific anti-AChR antibodies resulted in a near complete shift of the radioactivity to higher S values. Modified from Sumikawa *et al.* (1982) [21].

indicating that all the protein present in the sample represented AChR. Similarly, this test can be applied by complexing the AChR to α-BuTX and then adding anti-toxin antibodies; in this case, the position in the gradient of biologically-inactive AChR or of any extraneous material present would not be altered by the antibodies. Reactivity of ^{125}I-labelled preparations with immobilised α-neurotoxin (with and without the presence of α-BuTX) can be used in a related fashion to quantitate the fraction of the total material present representing AChR [21,22]; for purified AChR preparations from chick denervated, embryonic and innervated muscle, these values were high; 99, 96 and 85%, respectively [21]. Finally, gel electrophoresis in non-denaturing conditions of radio-iodinated samples of AChR can give an informative assessment of purity; all the latter purified preparations gave one peak [21]. Thus, these comprehensive tests confirm that the purification method used for chick muscle AChR (Table 2) gives

material that is homogeneous with respect to charge and size and fully active, as judged by its interaction with α-BuTX and specific anti-AChR antibodies. Of course, the ultimate test of the physiological activity of a pure AChR preparation, and to ascertain if it contains the ion-translocating activity, is to reconstitute it into artificial membranes; to date, this has not been achieved with pure AChR from muscle, unlike AChR from electric organ [2, 19]. As muscle AChR protein is now known to be comprised of homologous subunits (see below), an elaborate criterion of chemical purity (but not necessarily biological activity) recently used [30, 31] is to show that the polypeptides present in a preparation have similar N-terminal amino acid sequences (Fig. 3). However, this requires prior availability of extensive information on the AChR being studied and needs relatively large amounts of sample together with micro-sequencing instrumentation.

Oligomeric properties. Muscle AChR isolated from several species is an acidic (pI ~5) glycoprotein that binds avidly to certain plant lectins, e.g. concanavalin-A (Con A) and lentil lectin (Table 3). On sedimentation analysis, AChR preparations show an $s_{20,w}$ value of ~9S in the presence of detergent (Table 3). In some muscles (e.g. cat [20], chick [21] or human diseased muscle [26]), one or more additional minor forms of lower size (4-5S) have been detected which bind α-BuTX and bromo^{3}H-ACh, but these have been attributed to proteolysis; this can necessitate the use of density gradient centrifugation as a final purification step to produce the predominant 9S form [21, 22]. In the case of the fish AChR, after correcting by the D_2O balance method for the effect of bound detergent, it has been deduced that the 9S AChR species represents a molecular weight of 250,000 [2]. Cross-linking of AChR purified from cat denervated muscle gave a M_r = 270,000 on SDS gel electrophoresis [18]; this is very close to the true size of the monomeric form of fish AChR (see Section D) and reasonably close to the target size in the membrane as obtained by irradiation inactivation (Table 1). The purified AChR protein from chick, human (Table 3), rat [32] or cat [33] muscle was found to be a potent antigen and capable of inducing several symptoms typical [4] of experimental autoimmune myasthenia gravis in rats, rabbits and mice.

Ability to bind nicotinic ligands is retained after purification of muscle AChR; this is reflected in near-theoretical values discussed above for the specific activity of α-BuTX binding (Table 3). Affinity constants measured for the binding of a series of nicotinic agonists and antagonists to chick muscle AChR correspond to their pharmacological potencies (Table 4); the measurements are not as extensive as those performed with fish AChR and, thus, data on the existence of corresponding forms with differing ligand affinities is limited. However, affinity alkylation of purified muscle AChR with bromo[^{3}H]ACh (Fig. 4) or 4-(N-maleimeido)-^{3}H-benzyl or phenyl trimethylammonium iodide (^{3}H-MBTA or ^{3}H-MPTA) has been achieved after reduction of disulphides in the ACh recognition sites (Table 3). All of this labelling was prevented by α-BuTX. In the specific case of bromo-[^{3}H]ACh, it has further been demonstrated [34] that in both muscle and *Torpedo* AChR the stoichiometry of binding is 1 : 1 for α-toxin and bromo-ACh.

AChR subunits and their function. Analysis of purified muscle AChR from several species by SDS gel electrophoresis, followed with detection of the protein bands by Coomassie (Fig. 5) or silver staining have shown that multiple subunits are present (Table 3). Radiolabelling [22, 23, 35] of the preparations with ^{3}H or ^{125}I followed by fluorography or autoradiography [35] is often used to increase the sensitivity of detection. Preparations whose purity has been established from species where it has been feasible to minimise proteolysis, contain 4 major polypeptides. Their molecular weights (apparent M_r, by relative mobilities) can be grouped arbitrarily and named as follows for convenience, in analogy to the 4 different sizes of subunits established for fish AChR [2, 8]: α (40–45,000), β (49–53,000), γ (53–56,000) and δ (57–68,000). When the presently available maximum precautions (see above) are taken against proteolysis during AChR preparation, they are successful with muscles of most species, but not in certain cases such as the cat [20] or rabbit [25, 36] or damaged human [23, 26] muscles, as judged by very low stoichiometries of some of the heavier subunits obtained there. With the exception of the α-polypeptide, which can be recognised by affinity labelling (see below), the identifications of corresponding subunits seen in

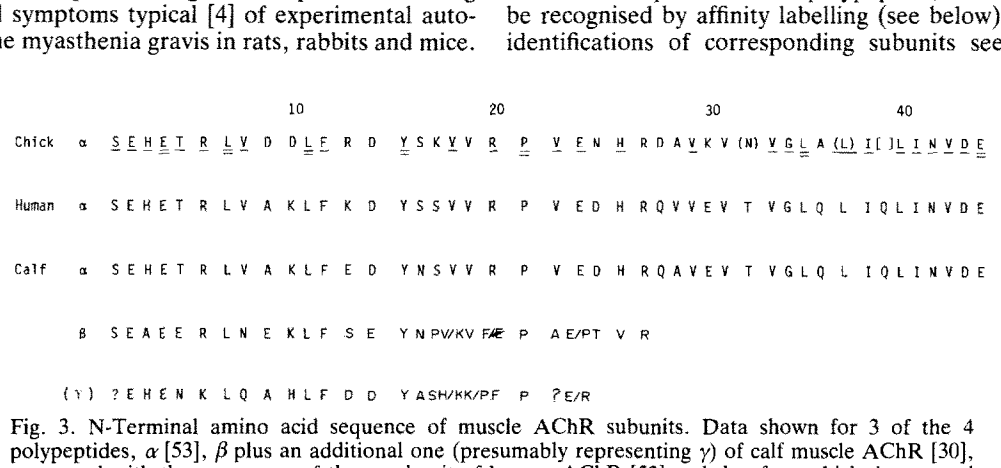

Fig. 3. N-Terminal amino acid sequence of muscle AChR subunits. Data shown for 3 of the 4 polypeptides, α [53], β plus an additional one (presumably representing γ) of calf muscle AChR [30], compared with the sequences of the α-subunit of human AChR [53] and that from chick denervated muscle [31]. Single underline: residue identical in these 3 α-subunits and *Torpedo* α-subunit. Double underline: residue identical in all AChR subunits yet sequenced [ ] = gap inserted. ?, residue present but not identified definitely. ( ), identification less certain.

Table 4. Some properties of muscle AChR and BTBC from optic lobe and brain

Source	Isoelectric point	Lectins bound*	% Antibody cross-reactivity†	ACh-site subunit(kd)‡§	K_d for α-BuTX (pM)‖	K_i for ligands (μM)¶			
						Nic	Deca	Curare	Carb
Optic lobe, chick	5.4	Lentil; ConA	1	54	130	0.3	340	0.9	146
Brain,** chick,		Lentil; ConA	1	54					
rodent††	4.9	Lentil; ConA		(51)	300	0.1	500	1.0	130
Muscle, chick	5.0	Lentil; ConA	4.3	40	1.0	1.4	0.02	0.2	9

Modified from Barnard and Dolly [9]. Where no values are shown, they have not been determined under comparable conditions.

* The strong binding of these proteins to lentil lectin and to ConA lectin denotes that they are glycosylated, with accessible mannosyl or glucosyl groups.

† Reactivity towards polyclonal antiserum, raised to a pure preparation of AChR from cat muscle, measured under conditions of optimal reaction in each case and expressed as a percentage of the titre of that antiserum against its own antigen. The titration curves for the BTBC from optic lobe and brain against this antiserum coincide *completely*.

‡ Apparent molecular weights in kilodaltons.

§ The subunit seen as a labelled polypeptide after reaction of the reduced preparation with bromo[^{3}H]ACh. The parentheses denote that in the case of the mouse brain the identification was only by the chemical cross-linking of ^{125}I-α-BuTX to the protein.

‖ K_d = dissociation constant of the complex; note that for the muscle (as for electric organ) AChR, the reaction is virtually irreversible, whereas with the optic lobe and brain protein the K_d is much higher because of a relatively fast dissociation rate.

¶ Inhibition constant in the reaction with mono-^{125}I-α-BuTX under comparable conditions, a measure of K_d for the ligand; Nic = nicotine, Deca = decamethonium, curare = d-tubocurarine, carb = carbamylcholine.

** BTBC of whole brain (other than chick optic lobe).

††Data for others for rat or mouse brain. The values for K_d and K_I (rat brain) are seen to be very similar to those for optic lobe, allowing for the species variation, and different from those of muscle.

AChR preparations from different sources can be confusing because of the variety of electrophoretic conditions used in their separation and the different M_r values which hence are reported. Moreover, additional protein bands are often detected; for example, two apparent iso-forms of α have been observed with chick and rat muscle AChR. Calf [24] and rabbit [25] pure AChR preparations contain also an actin polypeptide (M_r = 44,000) and a polypeptide with M_r = 66–67,000 is seen in preparations from human and chick muscle (Table 3; Fig. 5). Only in the case of calf and chick muscle AChR have N-terminal amino acid sequences been obtained (as detailed below) to establish, beyond doubt, that the 4 major subunits are homologous (like their counterparts from fish AChR [37]) and, therefore, constituents of the AChR. *In situ* reaction of AChR in rat muscle membranes with a photoactivatable derivative of α-BuTX labelled the 5 subunits found in AChR purified from that tissue [38]; this strengthened the conclusion that all of these polypeptides are the constituents of AChR. It has not been excluded yet, however, if one or more of the smaller polypeptides might be derived from a larger subunit and, therefore, not a natural component of the AChR. This is likely as preliminary data on the stoichiometry of subunits in calf foetal muscle AChR has given a ratio of ~2:1:1:1 (in order of increasing size) [30]; this would yield a pentamer of ~9S as observed (Table 3) and agrees with the oligomeric structure well established for the monomeric form of fish AChR [2, 8].

As mentioned earlier, the α-subunit contains the ACh recognition site, as it is labelled selectively by bromo[^{3}H]ACh or ^{3}H MB(P)TA (Table 3). This has been established for AChR from several muscles, including both synaptic and extra-synaptic forms of cat AChR (Fig. 4). In the case of chick AChR both of the α-polypeptides can be affinity-labelled with bromo[^{3}H]ACh, but the 41,000 M_r component is usually minor and the proportions of the α-isoforms present can be altered by periodate oxidation [35], suggesting possible differences in their carbohydrate moieties. It is unclear how this minor α-subunit relates to the second, larger polypeptide from rat AChR which has been reported to be alkylated with ^{3}H-MBTA [22].

The roles of the other subunits have not been identified but some (at least) of these must constitute the ion-channel, since the 9S oligomer contains both the ACh recognition and ion-translocation activities, as found from reconstitution experiments with fish AChR. Use of monospecific anti-AChR antibodies as functional probes may allow progress in assignment of subunit function (see later). Recently, it has been possible, using gas phase micro-sequencing techniques [37], to obtain the amino acid sequence of portions of the N-terminal region of α-subunit of AChR from calf foetal [30] and chick denervated [31] muscle (Fig. 3). In addition, N-terminal sequences of the β, γ and δ subunits of calf muscle [30] or chick muscle (unpublished data) AChR were determined. These sequences reveal striking homologies in 4 of the polypeptides, strongly indicating that they have evolved from a common precursor by gene duplication, as already proposed for the homologous subunits of *Torpedo* and *Electrophorus* [2, 37] AChR. In this regard it is intriguing that evidence indicative of functional low-affinity sites for ACh (in addition to the normal pair of high-affinity ACh sites) has

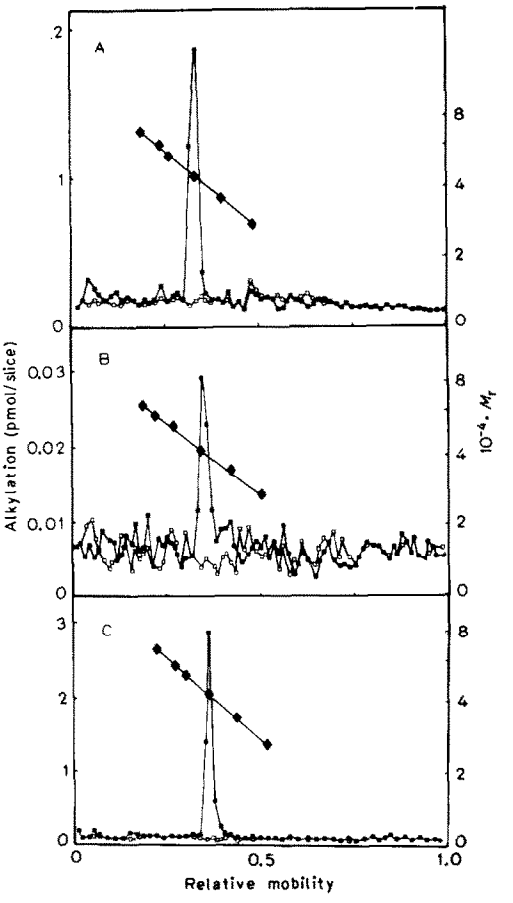

Fig. 4. SDS gel electrophoretograms of synaptic and extra-synaptic AChR from muscle after affinity labelling with bromo[^{3}H]ACh. Crude membranes from cat denervated (A) and innervated (B) muscle were reduced at 22° for 45 min with 0.3 mM dithiothreitol; after dilution of the latter, submaximal alkylation was performed with 1 μM bromo[^{3}H]ACh in the absence (■) and presence (□) of 5 μM α-BuTX. The labelled membranes were extracted for 3 hr at 4° with 2% Triton/25 mM phosphate buffer, pH 8; after centrifugation, AChR (incompletely labelled and hence able to bind α-toxin) in the extract was purified by absorption onto α-toxin-Sepharose resin, followed by washing and elution with 2% SDS/1% β-mercaptoethanol. In the case of solubilised AChR from cat denervated muscle (C), it was purified by selective absorption on α-toxin Sepharose followed by washing; the immobilised AChR was reduced, alkylated with (□) and without (■) the inclusion of α-BuTX and eluted as outlined above. All the samples were subjected to electrophoresis in SDS polyacrylamide gels under reducing conditions; the gels were cut to 2 mm (A, B) or 1 mm (C) slices and their radioactive contents determined by scintillation counting. Mobilities of the samples were calculated relative to bromophenol blue. The mobility of a series of standard proteins (bovine serum albumin, catalase, glutamate dehydrogenase, ovalbumin, lactate dehydrogenase and chymotrypsinogen A versus log of their molecular weights is shown for each gel (◆). Adapted from Shorr *et al.* [20].

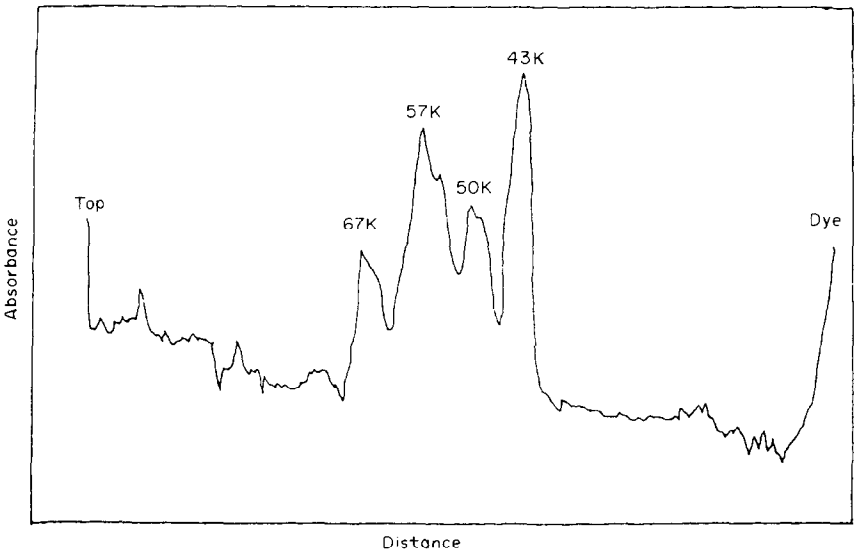

Fig. 5. Subunit composition of the AChR from chicken muscle. The purified receptor from chicken denervated muscle was subjected to electrophoresis in an SDS/polyacrylamide (1 mm thick) slab gel, using the procedures of Sumikawa *et al.* [35] except that 8.75% acrylamide was used. Staining was with Coomassie Brilliant Blue: a scan at 550 nm is shown. M_r values were obtained from a linear plot given by four protein standards run in parallel track. The 43,000-M_r (43 K α) polypeptide, when eluted and analysed by micro-sequencing, gave the sequence shown in Fig. 3. The 50,000 (β), 53,000 (γ) and 57,000 (δ) polypeptides have been tested similarly: they have given N-terminal sequences homologous with that of the 43,000 confirming that all are receptor subunits. The sequencing has shown that there are four homologous polypeptides in the receptor in all. The 67,000 band is not from a receptor subunit, being a contaminant obtained only when—as here—the receptor was eluted from the toxin-affinity column by SDS instead of a specific ligand, to obtain a full yield from [31].

been obtained for *Torpedo* AChR [39]; certainly, it would be interesting to establish in which subunit(s) these reside, but this could prove rather difficult. Peptide mapping of 2 subunits from chick [35] or rat [22] muscle AChR did not reveal strong structural similarities. As this was also found to be the case with all the *Torpedo* AChR subunits, peptide maps are an insensitive guide to such homologies; in contrast, antibodies raised against isolated, renatured preparations of individual subunits from *Torpedo* AChR could readily detect a high level of structural resemblances among *all* of the polypeptides [40].

D. MOLECULAR GENETICS OF AChR

Perspective. AChR has become the first receptor to reach the stage of gene cloning. Thus, it was reported in 1982 that the cDNA molecule encoding the α-subunit of the AChR from *Torpedo* electric organ had been produced and sequenced [41, 42] and those for the the other 3 subunits have been described in 1983 [43, 44]. Hence, the amino acid sequence of the entire oligomeric AChR protein has been obtained, transforming with startling rapidity our knowledge of this receptor. At the same time, the mRNAs coding for these polypeptides were recognised and translated to yield the functional protein, as described below. Here, again, the AChR is surely the prototype for the future analysis of other receptors. The amounts of receptors in general are so small and the difficulties in determining their structures by conventional protein-chemical methods are so great [31] that it will be essential to elucidate these structures at the DNA level. Moreover, a variety of questions on the requirements for different subunits, segments of subunits or even single amino acid residues, in the various functional and structural characteristics of the receptor are capable of being answered by manipulating mRNAs in cellular translation or DNAs by the techniques of molecular genetics. The recent advances in AChR molecular biology will, it seems safe to predict, prove a model for the future analysis of many other receptor molecules.

Recognition of the receptor mRNA. Torpedo electric organ is a good source of AChR mRNA but on translation of this in the conventional cell-free translation systems no receptor activity was obtained [45–47] even with microsomal supplementation. The reason for this is that the processing and membrane transport needed for the assembly and insertion of an integral membrane protein cannot be obtained there. It was found, however, that the *Xenopus* oocyte can correctly process the products of translation of AChR mRNA, after micro-injection thereof (Fig. 6). The binding of α-BuTX and of nicotinic ligands is then present in the product, which is 9S in size and inserted in the plasma membrane of the oocyte [31, 46, 48]. The 4 subunits of fish AChR, identical electrophoretically and in peptide mapping with those of the AChR purified from the source tissue, are produced in this system. It was also shown by a cell-free translation technique that the four polypeptides of the *Torpedo* AChR are produced by four separate genes [47]. A further question at once

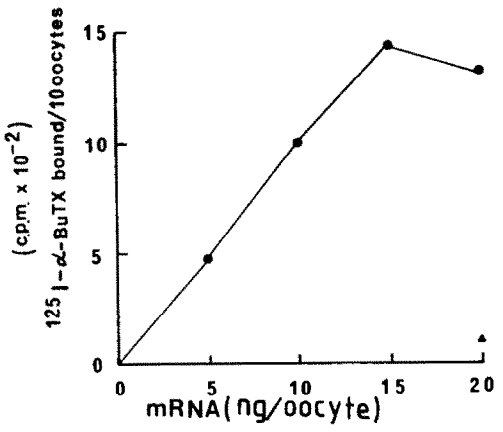

Fig. 6. α-BuTX binding activity in *Xenopus* oocytes after micro-injection with muscle AChR mRNA. Duplicate batches of 10 oocytes were microinjected with various amounts of poly (A)$^+$ mRNA that had been extracted from cat denervated muscle. These were then cultured at 21° in 150 μl modified Barths' medium for 48 hr, and homogenized in 200 μl of 50 mM phosphate buffer, pH 7.2/1% Triton X-100/1 mM EDTA/1 mM EGTA/0.1 mM phenylmethylsulphonyl fluoride. The ^{125}I-α-BuTX binding activity (●) in the resulting supernatants was measured. A control in which a large excess of unlabelled α-BuTX was added just before the assay gave very low values (▲). (Results of K. Sumikawa and E. A. Barnard.)

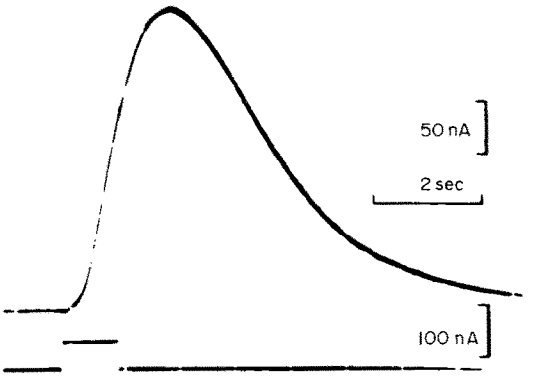

Fig. 7. *In vitro* translation of AChR mRNA. A single-cell test system for the production of receptor channels, formed in the *Xenopus* oocyte membrane 2 days after the micro-injection of 20 ng of a partly-purified poly (A)$^+$ mRNA fraction from *Torpedo marmorata* electric organ, is illustrated. This mRNA fraction was shown to have the ability to direct protein synthesis to give all the four subunits of the *Torpedo* AChR. ACh was applied (pulse, in lower line) by iontophoresis to the surface of the cell. A recording across the voltage-clamped (−70 mV) cell membrane, is shown. Atropine (2.5×10^{-7} M) is present to block the oocyte's own muscarinic receptors; the non-injected oocyte has no detectable nicotinic receptors of its own. In contrast to the response shown, no response at all is found (a) when mRNA coding for other proteins is injected and tested similarly; (b) when α-BuTX (1 μg/ml) treatment is given prior to the application of ACh; (c) when d-tubocurarine, at receptor-blocking concentrations, is applied with the ACh; (d) when the ACh is applied, instead, to the inside of the cell, showing that the correct orientation of the receptors has been produced. By change of the membrane potential it was also shown that the reversal potential is about −8 mV, as for the receptor in its original membrane. This and other evidence shows that the AChR channel characteristics are reproduced in the spontaneous insertion of the receptor subunits into this foreign cell membrane. The same results are obtained with mRNA from muscle. Taken from [48].

arises: do these mRNAs together produce the specific cation channel of the AChR? Production of a ligand-binding structure may not be sufficient for the production of the entire functional receptor system. This question was answered affirmatively by the demonstration that an ion channel having the predicted properties appears in the membrane of the oocyte, only after the micro-injection of the AChR mRNA fraction, the channel being opened by ACh applied by micro-iontophoresis to the surface of the oocyte (Fig. 7). These results, first obtained with *Torpedo marmorata* mRNA [48] have been repeated in an extension of that study [48, 49] with denervated muscle AChR mRNA. Comparable production of a receptor chloride channel in the oocyte has been elicited by micro-injection of mRNA for the γ-aminobutyrate receptor [50].

Cloning of receptor subunit cDNA. N-Terminal amino acid sequences, up to 54 residues long, were available from chemical studies of the *Torpedo* receptor subunits [37]. A short stretch of such sequence for the α-subunit was used to construct an equivalent DNA fragment, so that this could be used as a ^{32}P-labelled probe for hybridisation screening. By the methods of molecular genetics, starting with the *Torpedo* mRNA fraction identified (as in Fig. 6) as coding for the AChR, clones of *E. coli* containing inserts of the corresponding cDNAs were produced and screened by the above-mentioned AChR α-subunit ^{32}P-probe. By this means cDNA coding for the *Torpedo marmorata* α-subunit was obtained and sequenced [41]. This showed an initiation codon followed by codons for a typical signal peptide (of the type [47] which determines membrane insertion in general) of 24 mainly hydrophobic residues (Fig. 8). This is followed immediately by codons for the chemically-established [37] N-terminal sequence of the *Torpedo* AChR α-subunit, confirming the identity of the cDNA cloned. The rest of the subunit sequence is, of course, also then revealed (Fig. 8). This was the first receptor subunit to be cloned and sequenced. The α-subunit of *Torpedo californica* AChR was, likewise, sequenced by S. Numa and colleagues [42], that of *Torpedo marmorata* further by Devillers-Thiery *et al.* [51], the γ-subunit of *Torpedo californica* by Claudio *et al.* [43] and its other subunits by Noda *et al.* [44]. The numbers of amino acids present in each subunit, as determined from the cloning, are shown in Table 5. The true molecular weight of the protein is 267,751 (for *Torpedo californica*). With the approximately 4% carbohydrate present [52], this would give M_r about 278,000. That number should be compared with the values, discussed above, for M_r estimated in solution or in the membrane.

In a recent advance, S. Numa and colleagues have used the cDNA for the *Torpedo* AChR α-subunit to clone the cDNAs for the calf and human muscle

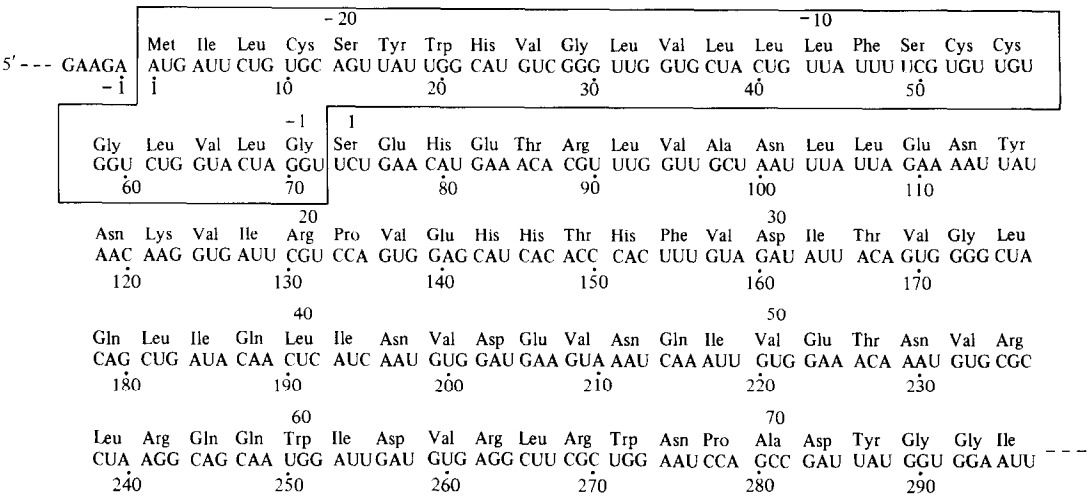

Fig. 8. The signal peptide and N-terminal region of the α-subunit of *Torpedo marmorata* AChR. The corresponding mRNA sequence is also shown. This was obtained from the cloning of the cDNA for this subunit [41]. Note that amino acids 1–54 are those known from protein sequencing [37]; this was how the α-subunit cDNA was identified. The sequence runs on beyond that shown—eventually all of the sequence to position 437 was obtained. After an untranslated region at the 5' end (only a part of which is shown), there is an initiation codon (AUG) and a 24-amino acid translated sequence which is not present in the α-subunit isolated from the protein. This is the signal peptide (shown in the box), necessary for the protein to be inserted and assembled in the membrane *in vivo*, and subsequently removed by an endopeptidase [47]. This composition, with a great preponderance of hydrophobic side-chains, is typical for signal peptides in membrane proteins in general. A similar but not identical signal peptide has been found in the β, γ and δ subunit precursors, proving that the subunits are encoded by separate genes. The same applies to the muscle AChR α-subunit [53].

AChR α-subunits [53]. Sequencing has shown that all of these α-subunits are of the same length (Table 5). Cloning for the other subunits of muscle AChR, and for other species, is in progress in several laboratories, and its full structure can be expected to be known in the near future.

Homologies in AChR structure. There is a startling similarity in the sequences of the α-subunits from electric organ, calf and human muscles (Fig. 3, Table 5). Obviously, this is a protein which has evolved to a stage where it accepts very little change, indicating strong functional constraints in its structure. The 4 subunit types within one species (*Torpedo californica*) are also strongly related, although much less homologous than the α-subunits in different species (Table 5). This has naturally suggested a common origin for all of the 4 subunits [44, 53]. This homology explains the immunochemical cross-reactivity of the 4 subunits [40].

Models of the AChR. Some approach to model building for the receptor molecule in the membrane can now be made. The amino acid sequence of each subunit can be subjected to the empirical secondary-structure-prediction procedure of Chou and Fasman [54], and the variation of the hydrophobicity in stretches along the sequence can be estimated by means of one of several available indices of side-chain hydrophobicity. α-Helical segments of suffi-

Table 5. Homologies between the subunits of AChR

Source	Subunit	Number of amino acids	Homology Identical	Conservative
*Torpedo**	α	437		
	β	469	19%	54%
	γ	489	(α, β, γ, δ)†	(α, β, γ, δ)†
	δ	501		
Calf muscle‡	α	437	97%	
Human muscle‡	α	437		
Torpedo	α	437	80%	

* From Noda *et al.* [42, 44], Sumikawa *et al.* [41], Claudio *et al.* [43] and Devillers-Thiery *et al.* [51], for both *Torpedo californica* and *Torpedo marmorata*.

† Positions identical, or identical strictly plus conservatively replaced, between all 4 *Torpedo* subunits, when aligned with gaps for maximum homology.

‡ Noda *et al.* [53].

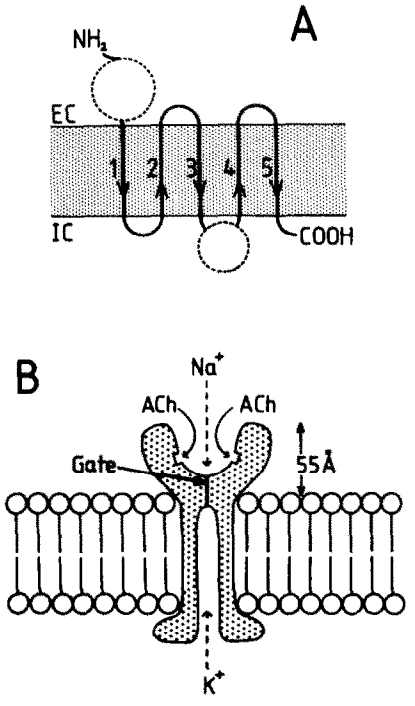

Fig. 9. (A) A model for the arrangement of one of the AChR subunits in the synaptic membrane. This is based on one interpretation of the secondary structure of the α-subunit of *Torpedo* AChR as predicted from the amino acid sequence determined [41, 42] by cDNA cloning. The prediction is by standard empirical methods [54] modified for membrane proteins [55, 56]. The structure is found thus to have a large non-helical N-terminal segment, which is placed on the extra-cellular (EC) side of the membrane because of the usual location of the N-termini of membrane proteins on that face and because of the location of the sole N-glycosylation site on the α-subunit in the sequence in that region. Five interrupted runs of α-helix each predominantly hydrophobic, are presumed to form 5 trans-membrane segments, leaving the C-terminus on the intracellular (IC) side, although the latter point is still uncertain. The ACh and α-toxin binding site is located in the N-terminal projection, above the plane of the membrane. The sequences of the β, γ and δ subunits are homologous to this one (Table 5), and similar analysis of these sequences shows that similar structures will form in the membrane from each subunit; hence, it is presumed that an equivalent intra-membrane region of each subunit contributes to the walls of the ion channel.

(B) A model for the arrangement of the receptor protein in the plane perpendicular to the membrane. The two ACh binding sites per AChR are shown above the extra-cellular face of the membrane, since α-BuTX binding has been observed at such a location in electron microscopic and X-ray diffraction studies of toxin-treated *Torpedo* membranes [58]. The projection on the extra-cellular face has been deduced from similar analyses (see text) and appears to comprise segments of all of the 5 subunits, probably in a ring in the order α, β, γ, δ [62]. It is presumed that these projections are contributed by the large N-terminal non-helical segment of each subunit. The model assumes that a gate of relatively small dimensions keeps a channel in the membrane closed, except when a structural change provoked by the binding of ACh at both of its sites is relayed to open this gate. The channel structure and its insulation are contributed by intra-membrane segments (see A) of all of the subunits.

cient length ($\geq$23 residues) and above-average hydrophobicity are then presumed to span the membrane. By these means it has been deduced that at least four trans-membrane helical segments are formed by each subunit [42, 43, 51]. However, there is uncertainty in the starting points and lengths of these helices, and different methods give different predictions here. Since the Chou–Fasman formulae have a globular protein base and under-predict membrane protein α-helices [55, 56], a version modified for such proteins gives rise to a different model, with 5 trans-membrane segments (Fig. 9A). The interpretation of primary structure is not precise enough to derive the topology with certainty, and chemical evidence will be required to decide if a model such as that of Fig. 9 (A) is correct. The N-terminus of the α-subunit is shown as located externally, because of evidence of this from partial proteolytic degradation [57] and the sole N-glycosylation at Asn-141 in the α-subunit [41], which occurs in the sequence before the predicted first helical segment (Fig. 9A).

All five subunits present in the AChR probably have similar topologies. Their actual chain lengths do not differ greatly (Table 5), the larger differences deduced from SDS-gel analyses in Section C being due to artefacts in that technique, and analysis as above leads to the same 5-segment model for each subunit. How these subunits form the receptor structure is hypothesised in Fig. 9(B). There will be 25 trans-membrane helices in all, sufficient to form a barrel structure for a central ion channel, with interlocking concentric layers. A central well is seen in negatively-stained electron micrographs of receptors in *Torpedo* membranes [16], presumed to be this channel [58]. X-Ray diffraction, image reconstruction and electron microscopy, all applied to receptor-rich membranes [59–61] have led to deductions of the form of the receptor as a cylinder with an external funnel, with the approximate shape and dimensions through and above the membrane shown in Fig. 9(B). The uppermost face can be decorated by α-BuTX and its antibody [58], leading to the postulated location of the ACh site as shown. As noted earlier, there are 2 per receptor molecule and they are both occupied by ACh for channel opening. The model places the high-affinity ACh binding site of the in α subunit in the N-terminal non-helical region of Fig. 9(A). The amino acid sequence shows that a disulphide bond, known to be adjacent to the ACh site (see Section C), *can* be present there.

A gating region is shown in the model as being on the extracellular side of the channel structure. This is based on the evidence that the binding of ACh leads to a conformational change in the structure which opens the channel. The hypothesised fixed channel (containing clathrate-frozen water) and a gate will bring the energy changes involved in this transition into the range of the binding energy available. The overall conformational change may nevertheless be quite considerable. The open channel must be about 6.5 Å across at its narrowest, by the limiting ionic radii of permeant cations. How the various segments of the 5 subunits contribute to the gating mechanism and to the channel structure, and how far the closing up of the protein structure proceeds,

in fact, are examples of the more precise structural questions which can now be addressed. We can look forward to the future identification of particular regions by immunological dissection and by directed mutagenesis, both based on the information provided by the tools of molecular genetics.

E. IMMUNOCHEMICAL STUDIES ON MUSCLE AChR

Polyclonal antibodies. These have been raised against muscle AChR from a number of species (Table 3). In most of such studies symptoms characteristic of experimental myasthenia were induced but, unfortunately, detailed properties of the antibodies were often not investigated. Recently, a collection of muscle AChR antibodies was characterised [33] with respect to their ability (a) to inhibit α-BuTX binding to solubilised AChR of denervated muscle; (b) to decrease the rate of blockade by α-BuTX of neuromuscular transmission in mouse diaphragm; (c) to reduce the amplitude of miniature endplate potentials in the latter tissue. Of 6 different antibody preparations, only one (made against cat denervated muscle AChR) produced any reduction of the synaptic potentials; although it decreased α-toxin binding to solubilised homologous AChR, inhibition of the interaction of AChR with carbamylcholine could not be detected by radioimmunoassay. Some of the other antibodies that were without effects on these potentials reduced α-toxin binding *in situ* to membrane-bound AChR of innervated muscle and also, solubilised muscle extracts [see (a) and (b) above]. It was deduced that the majority of the antibody species appeared to be directed against determinants that were not located at the actual ACh recognition site *per se* or the ion-channel; binding of the relatively large α-toxin molecule was probably inhibited by antibody interaction with loci in the vicinity of the ACh site. However, the heterogeneity of polyclonal antibodies makes it difficult to draw definite conclusions from such studies; similar difficulties are experienced in experimentation with antisera from different human myasthenics, as their specificities vary greatly [29].

Monoclonal antibodies. To help overcome the aforementioned problems and to obtain a series of probes that might selectively perturb the structure/function of AChR, the hybridoma technique was used to prepare monoclonal antibodies against a homogeneous preparation (see above) of AChR from chick denervated muscle [5]. The characteristics of four of the resultant monoclonals which were of the IgG_1, sub-class are detailed in Table 6. Analysis of their interaction with homologous AChR-^{125}I-α-BuTX complex by sucrose-gradient centrifugation showed that two (2A3 and 7B2) appeared to crosslink the antigen intra-molecularly, as the immune complex gave a single size peak on the gradient ($s_{20,w} \sim 12$S) regardless of the antibody concentrations used. In contrast, monoclonal 7B3 exhibited inter-crosslinking of AChR oligomers (Table 6) that resulted in creation of a broad peak at a high molecular weight position. One of each of these two antibody types induced experimental myasthenia in mice when the hybridomas were grown in the peritoneal cavity. It is well established that anti-AChR antibodies can increase the rate of AChR degradation by a process that involves crosslinking of AChR molecules [4]; this is responsible, to a major extent, for the decreased muscle AChR content in human and experimental myasthenia. As monoclonal 7B3 is a more potent myasthenogen than 7B2 (Table 6), it is likely that its ability to crosslink AChR, and presumably accelerate its degradation, contributes to this. In the case of monoclonals 2A3 and 7B2, intramolecular crosslinking of AChR may result from a more avid interaction with two copies of the same

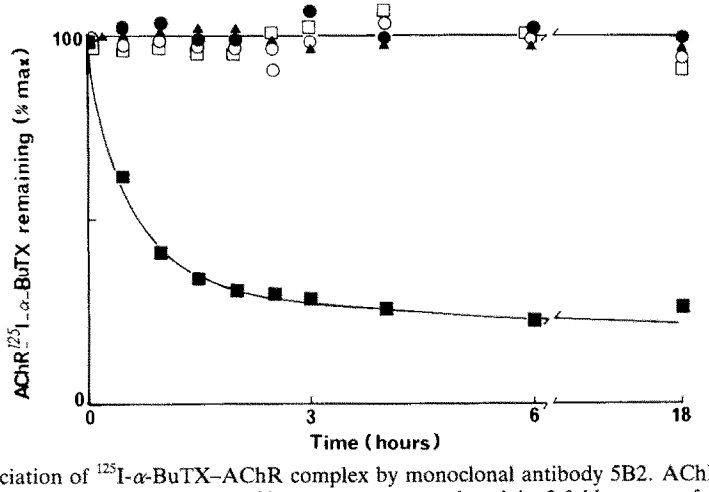

Fig. 10. Dissociation of ^{125}I-α-BuTX–AChR complex by monoclonal antibody 5B2. AChR from chick denervated muscle in crude Triton X-100 extract was complexed in 2-fold excess of ^{125}I-α-BuTX; 2.5 pmoles were then diluted in a final volume of 2 ml of the same extraction buffer, containing 100 μl of ascites fluid (●) 2A3; (■) 5B2; (○) 7B2; (□) 7B3 or normal mouse serum (▲). At intervals, aliquots were placed on DE-81 filter and washed in 25 mM sodium phosphate buffer containing 0.2% Triton X-100, for a standard length of time (2 × 6 min). Specific counts were calculated by pre-complexing AChR in excess α-BuTX before addition of ^{125}I-α-BuTX; these values of non-specific binding have been subtracted. When excess α-BuTX was included at zero time, no change in the apparent rate of dissociation was observed. Taken from Mehraban *et al.* [5].

Table 6. Properties of monoclonal antibodies to AChR from chick muscle

Clone	Myasthenia in donor	Myasthenogenicity of ascites in Balb/c mice (%)	Immunoglobulin sub-class	True (μM) and apparent [nM] titres	(%) Inhibition of ^{125}I-α-BuTX binding to AChR 18h 4°C	Immune complexes apparently involved	Reactivity with BTBC purified from chick brain—% of apparent titre towards homologous antigen	
							Optic lobe	Cortex
2A3	+ve	0	G1	19.0 [175]	0	Interaction with one oligomer	0	0
7B2	+ve	20	G1	60 [200]	0	Interaction with one oligomer	11	11
7B3	+ve	60		0.05[2]	60	Cross-linking of oligomers	15	12
5B2	+ve	0	G1	9.0 [83]	60	—	0	0

Titres were determined against homologous AChR (Triton X-100 extract of muscle) complexed to ^{125}I-α-BuTX; they were measured by double precipitation with anti-mouse immunoglobulin and are expressed as amount of ^{125}I-α-BuTX binding sites precipitated by a given volume of ascites fluid. Purified preparations of BTBC were used after being complexed to ^{125}I-α-BuTX. Apparent titres were obtained from the initial slope of immuno-precipitation curves whereas true titres were taken as the amount of complex precipitated when an aliquot of ascites (usually 0.02 μl) was saturated with the antigen. Inhibition of ^{125}I-α-BuTX binding was measured by incubating AChR in crude Triton X-100 extract (~1 pmole) with ascites fluid (40 μl) under the conditions shown, followed by assay of the mixture for AChR activity using the DE-81 disc assay. Percentage inhibition of ^{125}I-α-BuTX binding activity by monoclonal antibody is expressed relative to that obtained with normal mouse serum; the activity seen in the presence of ineffective antibody was identical to the latter. Non-specific binding was measured likewise in a sample to which excess α-BuTX was added before ^{125}I-α-BuTX. Immunoglobulin sub-classes were determined by Ouchterlony double diffusion in agarose gel against sub-class specific antisera; for preparation 7B3 unequivocal determination was difficult because of the low immunoglobulin content of ascites fluid. Mice suffering from experimental autoimmune myasthenia gravis had characteristic postures and complete paralysis of hind limbs accompanied by loss of weight. The size of immune complexes formed with AChR-toxin complex were analyzed by sucrose gradient centrifugation, using a wide range of antibody concentrations. Modified from Mehraban *et al.* [5].

determinant in the one oligomer. It has been proposed that monoclonal antibodies against *Torpedo* AChR, exhibiting a similar pattern of reactivity, bind to a common determinant located in the two α-subunits of each oligomer [63]. The myasthenogenicity of antibody 7B2 could result from complement-mediated [4] destruction of post-synaptic membrane.

With the aim of finding functional perturbants for AChR, the ability of these antibodies to affect α-BuTX binding to homologous AChR was examined. Two monoclonals (7B3 and 5B2) produced appreciable inhibition (Table 6); as the antibodies were screened with AChR complexed to ^{125}I-α-BuTX, it is assumed that they bind to loci outside the toxin site and that the inhibition observed results from steric hindrance or an antibody-induced conformational change. In fact, 5B2 (but not any others) reacted with preformed AChR-^{125}I-α-BuTX complex and induced a rapid dissociation of toxin (Fig. 10), presumably by altering the conformation of AChR to a state with low affinity for α-BuTX. Other useful features of these monoclonals include the ability of 7B3 to show greater reactivity with AChR of chick innervated rather than denervated or embryonic muscle [5]. A variety of analytical techniques failed to distinguish AChR from these sources [21, 35] but some minor differences in pI [22] and immunoreactivity with polyclonal anti-AChR [33, 64] antisera have been detected in synaptic and extrasynaptic AChR of rat muscle; other dissimilarities in the latter [7, 14] such as turnover rate (Table 1) and open-time of AChR channel may result from differences in the membrane environment. Lastly, the preferential reaction of monoclonal 5B2 with proteolysed AChR from chick innervated or denervated muscle indicates the subtle specificity of such antibodies which, therefore, have many potential uses. In particular, the availability of antibodies which perturb the ion-channel of AChR or affect desensitisation could prove to be useful tools; already, monoclonals against *Torpedo* AChR have been identified that differentiate two distinct ACh binding sites in the molecule [28].

F. α-BuTX BINDING PROTEIN FROM THE CENTRAL NERVOUS SYSTEM

For neurobiologists the AChR from brain is of great interest, but progress on its study has been limited because of (a) the very low AChR content in brain tissue, (b) lack of a convenient preparation for electrophysiological and hence pharmacological studies and (c) the inability of α-BuTX to inhibit neurotransmission therein [65, 66]. Electrophysiological evidence has been obtained, however, that α-BuTX can block nicotinic transmission in the optic tectum of goldfish, lizard, turtle and pigeon [65]; also, saturable binding of α-BuTX to preparations from chick optic lobe, which is antagonised by nicotinic ligands, has been demonstrated [67, 68]. On the basis of these important observations, an α-BuTX binding component (BTBC) was purified from optic lobe of day-old chicks [69]. This involved extraction of the membranes with Lubrol PX in buffer containing a cocktail of anti-proteases, followed by affinity chromatography on α-BuTX Sepharose resin, with elution by carbamylcholine. Any contaminating brain actin was removed by adsorption onto DNase-Sepharose prior to a final purification step on lentil lectin gel, as described for muscle AChR. The specific activity of the resultant protein was ~3 nmoles of toxin bound/mg protein and its glycoprotein nature was shown by absorption to plant lectins (Table 4). Purity was ascertained by isoelectric focusing of the ^{125}I-iodinated material, when a single peak of radioactivity (pI = 5.4) was obtained; this was completely shifted in the presence of α-BuTX, indicating that all the radioactivity present comprised a biologically-active binding protein. Also, the preparation was homogeneous with respect to size as a single peak of radioactivity was seen on sucrose density gradient centrifugation.

The most important question to answer was whether this purified BTBC represented the true AChR. Certain biochemical properties of the glycoprotein were similar to those of chick muscle AChR (Table 4) indicating it might be a functional receptor. It exhibited ability to bind nicotinic agonists and antagonists with affinities that are typical of central but not peripheral AChR. Moreover, it can be affinity-labelled selectively with bromo[^{3}H]ACh following reduction with dithiothreitol [69], suggesting that, as in the case of peripheral AChR, a disulphide bond exists in the ligand recognition site which can be reduced and alkylated. However, the size of the polypeptide labelled in this manner differs from that in chick muscle AChR though this may not reflect a difference in the protein size but another variation in structure (e.g. carbohydrate moieties) that can alter mobility on SDS gels. Also, the dissociation rate for bound α-BuTX is very much faster [69]. Finally, a glycoprotein showing very similar properties (Table 4) can be purified likewise from chick brain cortex [69]; yet, nicotinic synapses in this brain region (at least in other species) are not thought to be affected by α-BuTX. A series of anti-AChR (fish electric organ) antibodies, including some monoclonals made against muscle AChR, were reported not to cross-react with BTBC of rat brain [70], thus, additional criteria are required to prove the identity of this isolated protein from chick optic lobe and cortex.

Cross-reactivity between the BTBC purified from chick optic lobe and muscle AChR has been obtained using polyclonal antibodies against cat muscle AChR [69]; unfortunately, the extent of this was low (Table 4). Likewise, one polyclonal antiserum against rat muscle AChR shows some definite reactivity with BTBC of rat brain [71]. More convincing evidence for a central AChR with structural similarity to its peripheral counterpart has recently been produced using two monoclonal antibodies (7B2 and 7B3) made with chick muscle AChR. Both of these preparations were found to have a significant titre with the optic lobe protein (Table 6). This was shown by the immuno-precipitation of the pure protein complexed to ^{125}I-α-BuTX and, also, after direct labelling with ^{125}I (Fig. 11). It is noteworthy that the preparation from chick brain cortex showed identical reactivity to the latter with both the polyclonal [69] and monoclonal (Fig. 11; Table 6) antibodies. Another common feature is the formation of immune complexes

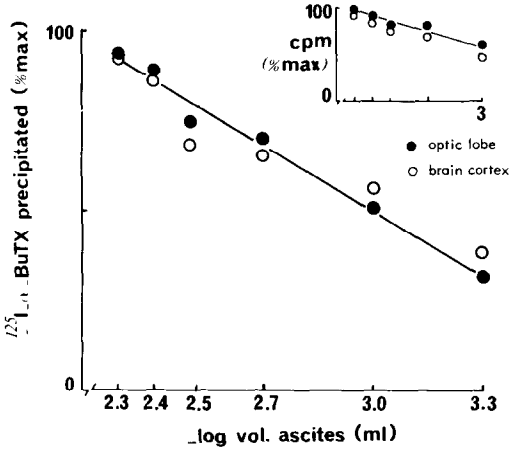

Fig. 11. Immunoprecipitation of chick brain BTBC by monoclonal antibody 7B2. Purified BTBC (1 pmole; 8 nM final conc.), complexed to mono-^{125}I-iodo-α-BuTX present in 3-fold excess, was incubated overnight at 4° with ascites fluid containing antibody 7B2. Immune complexes were precipitated by reaction with protein A-bearing cells of *Staphylococcus aureus* for 1 hr at 23°, washed and their radioactive contents determined. The amounts of radioactivity precipitated non-specifically were determined similarly by adding an equivalent quantity of non-immune serum; these have been subtracted from the data plotted. With the concentrations used in this experiment, the maximum quantity of complexed BTBC from optic lobe (●) and brain cortex (○) precipitated were 0.1 pmole. However, when much higher antibody concentrations were used, in density-gradient centrifugation experiments, immune complexes of practically all the optic lobe BTBC present could be formed as indicated by a shift to higher molecular weight values. *Insert:* BTBC, purified from optic lobe (●) and brain cortex (○), were ^{125}I-iodinated; after free ^{125}I was removed, it was used in a similar immunoprecipitation experiment as detailed above. From Mehraban *et al.* [5].

with the optic lobe protein of a single molecular size, indicative of intra-molecular cross-linking as observed with muscle AChR; this suggests the presence of two copies of a common determinant in each oligomer of these peripheral and central proteins. Such antibodies are now allowing this central receptor to be purified quickly with minimal proteolysis by immunoaffinity chromatography (Unpublished results); unlike the conventional procedure, this method has yielded material with a multiple-subunit pattern analogous to muscle AChR though the apparent sizes of some of the polypeptides (e.g. that which binds bromo[^{3}H]ACh) are larger (Table 4). Monitoring of the effects of these cross-reactive antibodies by neurophysiological and behavioural methods should help decipher the functional importance of this central AChR in different areas of the central nervous system, while immunocytochemical studies could establish its precise location therein.

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