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Neuronal acetylcholine receptors in *Drosophila*: the ARD protein is a component of a high-affinity α-bungarotoxin binding complex

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The *ard* gene of *Drosophila melanogaster* encodes a structural homologue of vertebrate nicotinic acetylcholine receptors (AChR) and is expressed exclusively in nervous tissue. To study the nature of the ARD protein, antibodies were raised against fusion constructs containing two regions of this polypeptide. One segment is putatively extracellular (amino acids 65-212), the other domain is exposed to the cytoplasm (amino acids 305-444). The ARD antisera obtained served to investigate the physical relationship between the ARD protein and α-bungarotoxin (α-Btx) binding sites occurring in *Drosophila*. Two different high-affinity binding sites for [^{125}I]α-Btx, a highly potent antagonist of vertebrate muscle AChR, were detected in fly head membranes. Equilibrium binding and kinetic studies revealed K_d values of ~0.1 nM (site 1) and ~4 nM (site 2). The estimated maximal binding (B_{max}) was ~240 and 1080 fmol/mg protein respectively. Both sites exhibited a nicotinic-cholinergic pharmacology. Immunoprecipitation experiments with the ARD antisera indicated that the ARD protein is associated with the [^{125}I]α-Btx binding site 1 only. These data support the previously postulated hypothesis that the ARD protein is part of an α-Btx binding neuronal AChR of *Drosophila*. Furthermore, they indicate heterogeneity in nicotinic-cholinergic binding sites in the insect nervous system.

Key words: neuronal acetylcholine receptor/α-bungarotoxin binding protein/*Drosophila melanogaster*

Introduction

Acetylcholine (ACh) is a major excitatory neurotransmitter at many synapses of both vertebrates and invertebrates. Two different receptor types respond to this signal molecule: muscarinic receptors which are coupled to G proteins and act via second messenger systems, and nicotinic acetylcholine receptors (AChR) which form an integral ion channel for signal transduction. In vertebrates, nicotinic receptors are found in both muscle and neurons of the peripheral and central nervous system (CNS). While muscular AChR action is readily blocked by the snake venom protein α-bungarotoxin (α-Btx) at very low concentrations, neuronal nicotinic receptors can be pharmacologically divided into several subtypes: AChRs which are antagonized by α-Btx, those which are not affected by the toxin, and α-Btx binding sites which may not represent functional AChRs (for review see Clarke, 1987). A similar heterogeneity is also reflected at the levels of vertebrate AChR proteins (Schneider *et al.*, 1985; Whiting and Lindstrom, 1987) and complementary and genomic DNAs (Boulter *et al.*, 1986; Goldman *et al.*, 1987; Nef *et al.*, 1988).

In insects, heterogeneity of nicotinic AChRs appears to be less pronounced. Here, transmission at neuromuscular junctions appears to involve transmitters other than ACh, while in the CNS elements of cholinergic synapses, e.g. the ACh synthesizing and degrading enzymes, choline acetyltransferase and acetylcholinesterase are present in high amounts (for review see Breer and Sattelle, 1987). Also, high levels of α-Btx binding to the neuropile region of the *Drosophila* CNS have been demonstrated (Dudai and Amsterdam, 1977; Schmidt-Nielsen *et al.*, 1977; Rudloff, 1978). Moreover, iontophoretically applied α-Btx can block synaptic transmission in cockroach ganglia (David and Sattelle, 1984). Using α-Btx as an affinity ligand, Breer *et al.* (1985) have isolated a homo-oligomeric 250-300 kd protein from the migratory locust consisting of several 65-kd subunits. The purified protein has been reconstituted in planar lipid bilayers and shown to form functional ACh-gated channels (Hanke and Breer, 1986).

Probes of vertebrate AChRs have been successfully used to isolate genomic and cDNAs encoding two different receptor-like polypeptides of *Drosophila*: the α-like subunit (ALS) which resembles ligand binding subunits of vertebrate AChRs (Bossy *et al.*, 1988), and the ARD protein which shows structural features of a non-ligand binding subunit (Hermans-Borgmeyer *et al.*, 1986; Wadsworth *et al.*, 1988). By *in situ* hybridization we have found that the *ard* gene is expressed in neural tissue (I.Hermans-Borgmeyer *et al.*, submitted). The time course of expression reflects the major periods of neuronal differentiation of the fly, i.e. late embryogenesis and pupal life, suggesting that the ARD protein may be a subunit of a neuronal AChR. This hypothesis is strongly supported by the data presented in this paper. We show that antisera raised against two different regions of the ARD protein recognize one of two high-affinity α-Btx binding sites solubilized from *Drosophila* head membranes.

Results

Production of antisera against ARD fusion proteins

Fusion constructs were designed to express both putative extracellular and cytoplasmic regions of the ARD protein in bacteria; antisera were subsequently raised against the chimeric proteins. As schematically illustrated in Figure 1, two different vector systems were employed to produce the following parallel constructs: amino acids 65-212 of the ARD protein fused to β-galactosidase (LAC65-212) and MS2 polymerase (MS65-212); amino acids 305-444 fused to β-galactosidase (LAC305-444); and amino acids 295-486 fused to MS2 polymerase (MS295-486). After partial

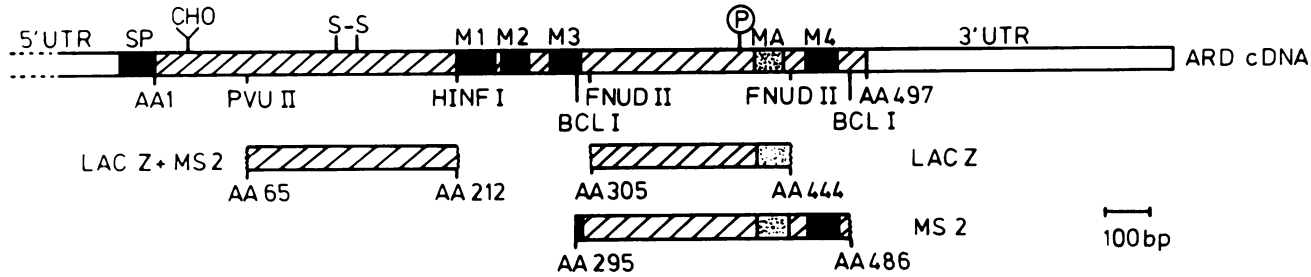

Fig. 1. Physical map of ARD cDNA and schematic representation of fusion constructs. Structural features of the ARD mRNA and the encoded ARD protein (hatched) are indicated: UTR = untranslated regions; SP = signal peptide; CHO = potential N-glycosylation site; S-S = disulphide bridge conserved in all AChR subunits; M1–M4 = hydrophobic membrane-spanning regions; MA = predicted amphipathic α-helix; P = potential cAMP-dependent phosphorylation site (for a detailed description see Hermans-Borgmeyer *et al.*, 1986; Gundelfinger *et al.*, 1986). Only restriction cleavage sites used to create fusion constructs are indicated. The first and last amino acids (AA) encoded by these fragments are given. LAC Z: respective fragment was cloned into the pEX3 expression vector (Stanley and Luzio, 1984) to yield ARD–β-galactosidase fusion proteins; MS2: fragments were subcloned into the pEX34c vector to express ARD-MS2 polymerase chimeric proteins (Strebel *et al.*, 1986). For details of construction see Materials and methods.

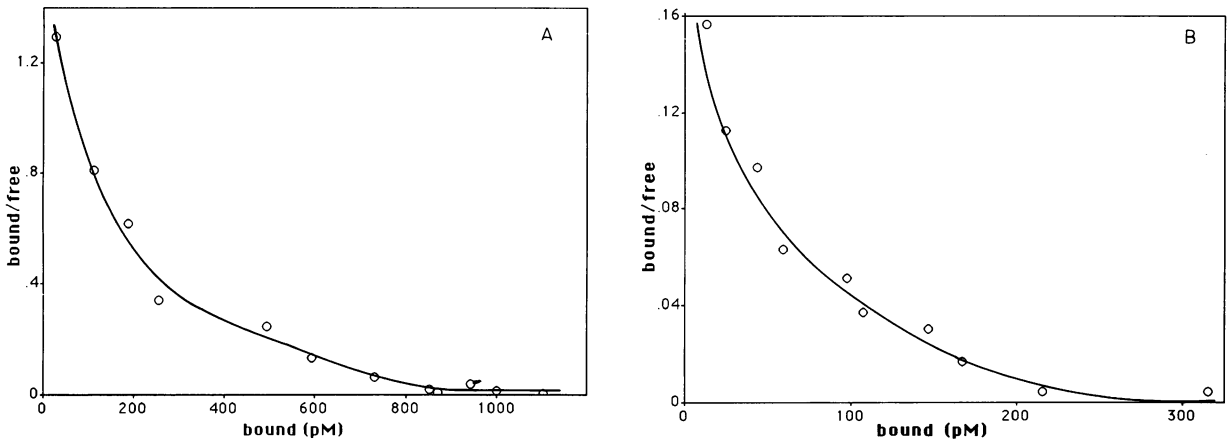

Fig. 2. Scatchard plots of [^{125}I]α-Btx binding to *Drosophila* head membranes (**A**) and detergent extracts (**B**). Two-site analysis of the Scatchard curves for specific [^{125}I]α-Btx binding was performed using a Gauss–Newton non-linear regression curve-fitting procedure (Meeker *et al.*, 1986). For binding conditions see Materials and methods. The resulting B_{max} and K_d values are summarized in Table I.

Table I. Summary of [^{125}I]α-Btx binding to *Drosophila* membranes and detergent extracts

	High-affinity site 1		High-affinity site 2	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Head membranes	0.15 ± 0.07	240 ± 93	4.3 ± 1.7	1080 ± 97
Head membranes (kinetic data)[a]	0.06 ± 0.02	–	4.5 ± 0.9	–
Detergent extracts	0.12 ± 0.09	80 ± 64	3.7 ± 1.2	800 ± 64
Embryo membranes	0.4 ± 0.26	5 ± 3.9	7.4 ± 4.4	68 ± 18
Supernatant after immunoprecipitation with AS8	0.37 ± 0.12	nd[b]	3.9 ± 0.9	nd
Supernatant after immunoprecipitation with AS21	ND[c]	ND	2.7 ± 0.4	nd
Supernatant after immunoprecipitation with AS6	ND	ND	7.0 ± 1.2	nd

[a]The data are calculated from those shown in Figure 3A and B. All other binding values presented in this table are calculated from equilibrium binding experiments.
[b]nd, not determined. B_{max} values were not determined after immunoprecipitation because of the addition of antisera and Pansorbin treatment.
[c]ND, not detectable.

purification, rabbit antisera were raised against MS65-212 and LAC305-444. The antisera were tested for recognition of the respective parallel constructs in Western blots. Antibodies were purified from positive sera by affinity chromatography on the respective parallel protein construct immobilized on Affi-Gel. Purified antisera AS21 and AS22 obtained against MS65-212, and AS6 against LAC305-444, were employed to study the physical relationship between the ARD protein and high-affinity α-Btx binding sites in the *Drosophila* CNS.

Characterization of α-Btx binding sites

The existence of α-Btx binding sites in *Drosophila* embryos (Salvaterra *et al.*, 1987) and adult flies (reviewed by Breer and Satelle, 1987) has been reported previously. We have analysed these α-Btx binding components of membranes

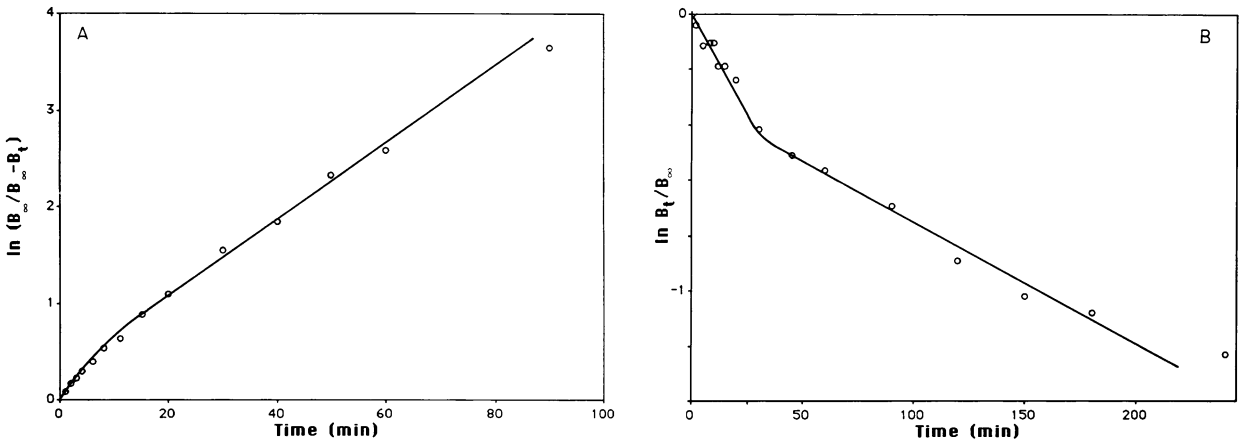

Fig. 3. Association (**A**) and dissociation (**B**) kinetics of [^{125}I]α-Btx binding to *Drosophila* head membranes. For the determination of association constants, ln $(B_\infty/B_\infty - B_t)$ is plotted as a function of time (Limbird, 1986). Estimated association rate constants are 4.6×10^7 M^{-1} min^{-1} and 8.0×10^6 M^{-1} min^{-1} for the fast and slow components respectively. Dissociation kinetics in the presence of unlabelled α-Btx are plotted as ln B_t/B_∞ as a function of time. Estimated dissociation rate constants are 3.0×10^{-3} min^{-1} and 3.6×10^{-2} min^{-1} for the fast and slow components respectively. (B_∞: amount of [^{125}I]α-Btx bound at equilibrium; B_t: [^{125}I]α-Btx bound at each time point.)

from fly heads before and after solubilization. [^{125}I]α-Btx binding was linear up to 0.5 mg protein (not shown). Scatchard analysis of [^{125}I]α-Btx binding (concentration range 0.05−200 nM) to head membranes revealed at least two different high-affinity binding sites (Figure 2A). The calculated K_d values were 1.5×10^{-10} M for the site of higher (site 1) and 4.3×10^{-9} M for the site of lower affinity (site 2). The corresponding B_{max} estimates were 240 and 1080 fmol/mg protein respectively (Table I). A very low-affinity site with a K_d of $>10^{-7}$ M and a concentration more than two orders of magnitude higher than that of the other two sites was not considered further.

Optimal solubilization of head membrane proteins was achieved at pH 8.5, using a mixture of sodium deoxycholate and Triton X-100 as detergents (data not shown). The efficiency of solubilization was in the range of 20−30% for wild-type flies and ~50% when membranes of the mutant strain 'white' were used. After solubilization, K_d values for α-Btx binding sites 1 and 2 were essentially unchanged (Figure 2B and Table I).

As levels of *ard* transcripts are high not only in heads of young flies, but also during late embryogenesis (Hermans-Borgmeyer *et al.*, 1986, and submitted), we have investigated [^{125}I]α-Btx binding to membranes of embryos of this developmental stage (13−19 h). Scatchard analysis revealed two binding sites with K_d values similar to those found in fly head membranes (Table I). However, their B_{max} values were 10- to 50-fold lower compared to adult heads.

Kinetic studies further confirmed the existence of two binding sites for [^{125}I]α-Btx. Equilibrium of binding was reached after 90 min of incubation (not shown). Transformed rate data for both association with and dissociation of [^{125}I]α-Btx from head membranes exhibited a biphasic behaviour (Figure 3), with association constants of 4.6×10^7 M^{-1} min^{-1} and 8.0×10^6 M^{-1} min^{-1}, and dissociation constants of 3.0×10^{-3} min^{-1} and 3.6×10^{-2} min^{-1}. The resulting kinetic K_d were calculated to be 6×10^{-11} M and 4.5×10^{-9} M for sites 1 and 2 respectively (Table I).

To test whether both α-Btx binding sites are of the nicotinic-cholinergic subtype, binding inhibition experiments

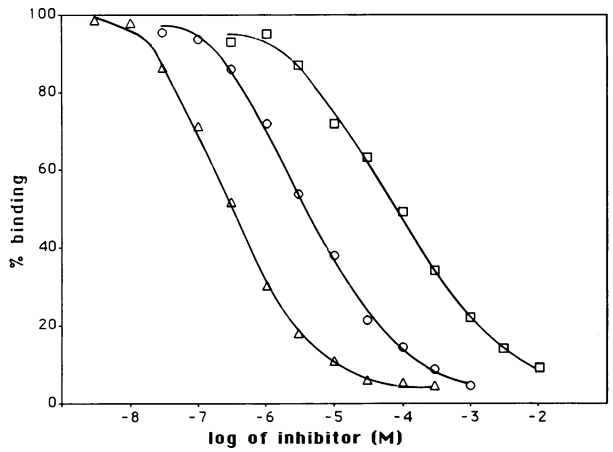

Fig. 4. Inhibition of [^{125}I]α-Btx binding to *Drosophila* head membranes by nicotine (△), *d*-tubocurarine (○) and carbamylcholine (□). [^{125}I]α-Btx binding was performed in the presence of various amounts of competitor as described in Materials and methods, except that the concentration of [^{125}I]α-Btx used here was 10 nM.

were performed using the agonists nicotine and carbamylcholine and the antagonist *d*-tubocurarine. All three ligands inhibited the binding of [^{125}I]α-Btx to fly head membranes (Figure 4). The IC_{50} values determined at a [^{125}I]α-Btx concentration of 10 nM were 3.5×10^{-7} M for nicotine, 3.8×10^{-6} M for *d*-tubocurarine and 8.0×10^{-5} M for carbamylcholine. The shape of all three inhibition curves is characterized by a shallow gradient (Limbird, 1986), again demonstrating the existence of more than one class of [^{125}I]α-Btx binding sites in *Drosophila*.

Immunoprecipitation of [^{125}I]α-Btx binding sites with ARD antisera

Purified ARD antisera AS21, AS22 and AS6 were tested for recognition of [^{125}I]α-Btx binding components present in detergent extracts of *Drosophila* head membranes. Increasing amounts of antisera were used for immunoprecipitation after toxin binding to the extracts. Control experiments showed that none of the sera competed with α-Btx binding, a prerequisite for successful immunoprecipitation of the

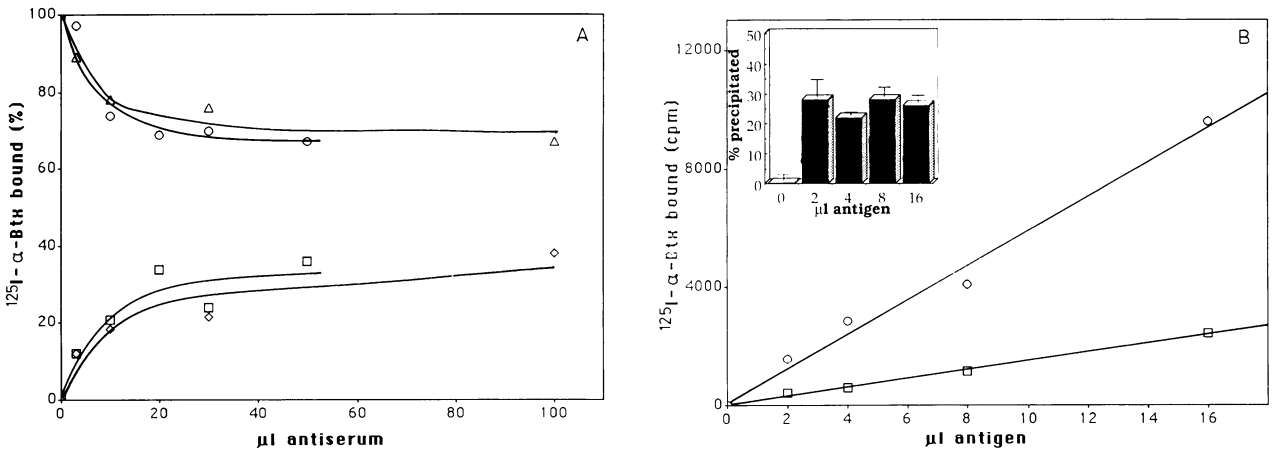

Fig. 5. Immunoprecipitation of [^{125}I]α-Btx binding sites by ARD antisera. (**A**) Titration of antisera. Aliquots of [^{125}I]α-Btx-labelled detergent extract of *Drosophila* heads were incubated with the indicated amounts of antisera and processed as described in Materials and methods. Both [^{125}I]α-Btx binding sites remaining in the supernatant after immunoprecipitation with AS6 (○) and AS22 (△), and those recovered from the immunoprecipitates of AS6 (□) and AS22 (◇) are plotted. (**B**) Determination of antigen concentration. Varying amounts of detergent extracts from *Drosophila* head membranes were incubated with 5 nM [^{125}I]α-Btx. After 2 h 20 µl of AS22 were added to each tube and the samples processed and analysed for [^{125}I]α-Btx binding sites in the supernatant (○) and pellet (□). Inset diagram indicates the percentage of immunoprecipitated [^{125}I]α-Btx binding sites at each point of measurement. Bars indicate standard deviations.

toxin−receptor complex (not shown). Maximally 30% of the solubilized [^{125}I]α-Btx binding sites were precipitable with both AS6 and AS22 (Figure 5A) as well as with AS21 (not shown). The remaining ~70% of binding sites were consistently found in the supernatant. Titration of AS22 with increasing amounts of antigen confirmed that only between 20 and 30% of the [^{125}I]α-Btx binding sites in the extracts were recognized by the antibodies (Figure 5B). These results suggested that ARD antisera may bind exclusively to high-affinity [^{125}I]α-Btx binding site 1 which represents ~25% of total toxin binding components. This hypothesis was confirmed by Scatchard analyses of [^{125}I]α-Btx binding to solubilized head membranes after immunoprecipitation with various antisera. While pretreatment with a control antiserum (AS8), which does not precipitate toxin binding sites, did not affect characteristics of the two high-affinity [^{125}I]α-Btx binding sites, both AS6 and AS21 eliminated binding site 1 from the detergent extracts (Figure 6). K_d values of the remaining [^{125}I]α-Btx binding component resembled that of the previously defined high-affinity toxin binding site 2 (Table I).

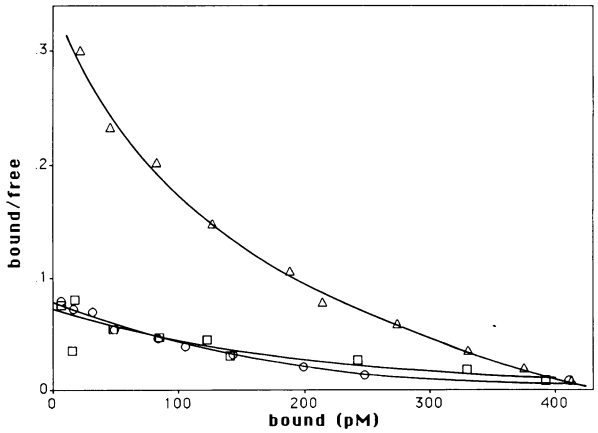

Fig. 6. Scatchard plots of [^{125}I]α-Btx binding to solubilized head membranes after incubation with ARD antisera. Detergent extracts from head membranes were incubated with an excess of various antisera. Two hours later Pansorbin was added for another hour. After centrifugation Scatchard analysis of [^{125}I]α-Btx binding to the supernatants of immunoprecipitation was performed as described in Materials and methods. [^{125}I]α-Btx binding behaviour after treatment with AS8 (△), an antiserum which does not immunoprecipitate [^{125}I]α-Btx binding sites, is shown as control. Two binding sites are seen with K_d values of 3.7×10^{-10} M and 3.9×10^{-9} M. Immunoprecipitation with AS21 (□) as well as with AS6 (○) eliminates the high-affinity binding site 1. The remaining sites show K_d values of 2.7×10^{-9} M and 7×10^{-9} M respectively.

Discussion

Equilibrium binding, kinetic, pharmacological and immunological data presented here clearly demonstrate the occurrence of at least two different classes of high-affinity binding sites for [^{125}I]α-Btx in *Drosophila* head and embryonic membranes. Toxin binding at both sites is displaceable by nicotinic-cholinergic ligands, such as nicotine, *d*-tubocurarine or carbamylcholine. Similar findings have been described for rat hypothalamus where two different toxin binding sites with K_d values in the range of $10^{-11}-10^{-9}$ M are found (Meeker *et al.*, 1986). A low-affinity site found in *Drosophila* is considered to represent 'non-specific' binding and was therefore not investigated in further detail. A similar low-affinity acceptor site has also been observed in vertebrates, e.g. in rat hypothalamus (Meeker *et al.*, 1986) and chick retina (Betz, 1981).

Previous studies of α-Btx binding to *Drosophila* membranes are rather controversial. Some authors have described the existence of a single binding component with K_d values ranging from 2×10^{-9} M to 1.6×10^{-10} M (Schmidt-Nielsen *et al.*, 1977; Dudai, 1978; Jimenez and Rudloff, 1980) while others have suggested the existence of multiple binding sites (Bartels, 1984; Nagursky, 1985). Whether α-Btx binding interferes with the physiological ACh response in *Drosophila*, as it does at the vertebrate motor endplate, is presently unclear. No inhibition of nicotinic-cholinergic transmission by α-Btx has been observed in grasshopper dorsal unpaired median neurons (Goodman and Spitzer, 1979). On the other hand, α-Btx at

concentrations of 10^{-7} to 10^{-8} M can block ACh responses in certain cockroach neurons (Lees *et al.*, 1983; David and Satelle, 1984). Clear evidence for the identity of α-Btx binding sites and a neuronal AChR in the migratory locust has been provided by reconstitution experiments with affinity-purified AChR preparations (Hanke and Breer, 1986). Thus, both α-Btx-sensitive and insensitive AChRs may exist in the insect CNS. The presence of two classes of high-affinity α-Btx binding sites of nicotinic-cholinergic pharmacology demonstrated here strongly argues for AChR heterogeneity in *Drosophila*. Furthermore, the exclusive immunoprecipitation of class 1 high-affinity α-Btx binding sites by ARD antisera proves that two physically distinct toxin binding proteins do exist in the fly. Binding site heterogeneity within a single receptor species, as documented in detail for *Electrophorus electricus* (Maelicke *et al.*, 1977) and *Torpedo californica* (Dowding and Hall, 1987), cannot explain our binding data.

Although the AChR nature of the high-affinity α-Btx binding component in *Drosophila* remains to be established, both the homology of the predicted ARD protein with known AChR subunits and its immunologically demonstrated association with α-Btx binding site 1 strongly indicate that the *ard* gene encodes a subunit of a neuronal cholinergic receptor. However, on the basis of the present data we cannot decide whether the ARD protein itself or an associated subunit of the receptor complex is the α-Btx binding component. Several arguments are in favour of the latter possibility. (i) The ARD protein lacks two consecutive cysteine residues (Cys192/Cys193) known to be associated with the toxin binding site of the α-subunit of muscle AChRs (Neumann *et al.*, 1986). (ii) The fusion proteins MS65-212 and LAC65-212 which should enclose the potential binding domain fail to bind α-Btx (P.Schloß, unpublished data), whereas a fusion protein covering the homologous region of the murine α-subunit of the muscle receptor has been shown to bind the toxin (Barkas *et al.*, 1987). Also, ARD antisera do not interfere with toxin binding to *Drosophila* membranes. It has to be noted that the negative results stated in (ii) support but do not prove that the ARD protein is not a ligand binding polypeptide.

In conclusion the receptor complex embodying the ARD protein may not be a homo-oligomeric complex as claimed for the neuronal nicotinic AChR of the locust (Breer *et al.*, 1985), but may resemble vertebrate neuronal AChRs for which a hetero-oligomeric quarternary structure with two types of subunits has been proposed (Whiting *et al.*, 1987). Whether the ARD protein assembles with the recently described α-like subunit ALS (Bossy *et al.*, 1988) to form a functional receptor in *Drosophila* remains to be clarified.

Materials and methods

Materials

Nicotine, carbamylcholine, *d*-tubocurarine and α-Btx were purchased from Sigma, Pansorbin from Calbiochem and [^{125}I]α-Btx from Amersham (specific activity ~240 Ci/mmol). The expression vector pEX34 (Strebel *et al.*, 1986) was kindly provided by Dr E.Beck, ZMBH. The expression vector pEX3 (Stanley and Luzio, 1984) and the bacterial host (*Escherichia coli* strain Pop 2136) were a gift from Dr K.Stanley, EMBL.

Cloning and expression of ARD fusion proteins

A *Hin*fI fragment of the ARD2 cDNA clone (Hermans-Borgmeyer *et al.*, 1986) encoding amino acids −23 to 212 of the ARD precursor was blunt ended by a fill-in reaction and subcloned into the *Sma*I site of pUC18 plasmid, resulting in clone '*Hin*f11'. The *Pvu*II/*Sal*I fragment of *Hin*f11 was ligated into the *Sma*I/*Sal*I sites of expression vector pEX3 to produce the fusion protein LAC65-212. The *Pst*I site overlapping the *Pvu*II site of *Hin*f11 was used to isolate a *Pst*I fragment encoding also amino acids 65−212. It was cloned into the *Pst*I site of the expression vector pEX34c, and the resulting fusion construct was termed MS65-212. The fusion protein LAC305-444 was constructed by cloning the *Fnu*DII fragment of the ARD1 cDNA corresponding to amino acids 305−444 of the mature ARD protein (Hermans-Borgmeyer *et al.*, 1986) into the *Sma*I site of pEX3. The *Bcl*I fragment of the ARD1 cDNA cloned into the *Bam*HI site of pEX34c resulted in fusion protein MS295-486 (see Figure 1). Expression of the different fusion proteins was performed as described by Zabeau and Stanley (1982).

For purification of fusion proteins, 1 l of induced bacterial culture was centrifuged for 10 min at 4000 *g*. The pellet was frozen at −70°C, thawed on ice and resuspended in 40 ml buffer 1 [50 mM Tris−HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF), 1 μM pepstatin and 1 μM leupeptin]. Lysozyme was added for 30 min at room temperature (2 mg/ml) before the solution was brought to a final concentration of 1% (w/v) Triton X-100. After three 10-s cycles of sonication the lysate was centrifuged for 30 min at 28 000 *g* through a cushion of 40% (w/v) sucrose in buffer 1. The pellet was resuspended in 2 ml phosphate-buffered saline (PBS) before adding 20 ml buffer 2 (8 M urea, 0.5 M Tris−HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 30 mM β-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin). The solution was dialysed for 2 h against 2 l of buffer 3 (50 mM Tris−HCl, pH 8.0, 0.5 M NaCl, 10% w/v glycerol) and then centrifuged at 20 000 *g* for 30 min. The soluble form of enriched fusion protein in the supernatant had a concentration of ~1 mg/ml. Protein concentrations were determined as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Immunization procedure

About 200 μg of enriched fusion protein were mixed with 1 ml RAS adjuvant (SEBAK) to form an emulsion and injected intradermally at multiple sites into a rabbit. Booster injections were given at 3-week intervals, and blood was taken 7 and 9 days after the boost. For purification of the antisera ~10 mg enriched parallel construct was coupled to Affi-Gel 15 (Biorad) according to the manufacturer's manual. Antisera were bound for 2 h at room temperature, and specific IgGs eluted with 0.1 M glycine−HCl, pH 2.5.

Preparation of membranes and detergent extract

Drosophila heads were harvested as described (Schmidt-Nielsen *et al.*, 1977) and ground in liquid nitrogen with pestle and mortar. One gram head material per 10 ml buffer A (10 mM Tris−HCl, pH 7.5, 280 mM sucrose, 0.01% w/v NaN_3, 100 μg/ml PMSF) was homogenized on ice in a Potter homogenizer. Nuclei and debris were removed by centrifugation at 1000 *g* for 10 min at 4°C, and membranes were collected by centrifugation at 20 000 *g* for 30 min at 4°C. After washing in 5 mM Tris−HCl, pH 7.5, and centrifugation as above, membranes were resuspended in PBS, frozen on liquid nitrogen and stored in aliquots at −70°C.

Solubilization of membrane proteins was achieved by suspending the membranes in 0.2 M NaCl, 1.8% (w/v) Triton X-100, 0.6% (w/v) sodium deoxycholate, 10% (v/v) glycerol, 10 mM Tris−HCl, pH 8.5, and protease inhibitors as described by Betz and Pfeiffer (1984). After 30 min at room temperature, the solution was passed twice through a 27-gauge cannula, centrifuged for 1 h at 48 000 *g*, frozen on liquid nitrogen and stored at −70°C. Preparation of embryo membranes was performed as described for head membranes, but without previous grinding in liquid nitrogen.

[^{125}I]α-Btx binding

Toxin binding to membranes and solubilized receptors was monitored as described (Betz, 1981), except that GF/C filters (Whatman) presoaked in 0.3% (v/v) polyethylenimine were used instead of DEAE−cellulose filters. If not stated otherwise, the concentration of [^{125}I]α-Btx was 5 nM. For association rate determinations binding was measured up to 2 h. Equilibrium was reached after ~90 min (data not shown). Dissociation rates were determined by adding 5 μM unlabelled α-Btx after 2 h of binding ('chase experiment'). All binding data are the mean of triplicate determinations.

Immunoprecipitation

Antisera were titrated as follows: solubilized *Drosophila* head membranes were incubated with 5 nM [^{125}I]α-Btx in the absence, or presence, of 5 μM unlabelled α-Btx for 2 h at room temperature. Aliquots (200 μl) were then incubated with 100 μl buffer S (10 mM Tris−HCl, pH 8.0, 10 mM NaCl, 0.3% w/v Triton X-100) containing increasing amounts of antisera. After another hour, 50 μl of Pansorbin (washed twice in buffer S) were added to bind the IgGs of the antisera. One hour later the incubations were diluted with 600 μl buffer S and centrifuged at 7000 *g* for 2 min. Aliquots of the

supernatants were used for the determination of remaining [^{125}I]α-Btx binding sites. The immunoprecipitates were washed once with 1 ml buffer S and counted in a gamma-counter.

For determination of antigen concentration, increasing amounts of solubilized *Drosophila* head membranes were incubated with 5 nM [^{125}I]α-Btx in the absence, or presence, of 5 μM cold α-Btx. After 2 h 20 μl of antiserum AS21 were added, followed by 50 μl of Pansorbin, centrifugation and determination of bound radioactivities in supernatant and immunoprecipitates as described above.

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