ANALYSIS OF THE SOLUBILIZED NICOTINIC ACETYLCHOLINE RECEPTOR OF *DROSOPHILA MELANOGASTER*

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1. Introduction

Nicotinic acetylcholine receptors (AchR) from various vertebrate organisms have been intensively investigated because of their significance for the control of ion permeability within the process of synaptic transmission [1,2].

The presence of a nicotinic cholinergic system in the central nervous system of insects, although not yet conclusively demonstrated, is strongly supported by a series of findings: (i) Mutants for two metabolic enzymes of acetylcholine, choline acetyltransferase and acetylcholine esterase, produce neuroanatomical, electrophysiological and behavioural defects in *Drosophila melanogaster* [3]. (ii) α-Bungarotoxin (αBgt), a specific ligand for the nicotinic AChRs of vertebrates, binds to a component present in heads of *Drosophila melanogaster* [4–7] and *Musca domestica* [5], in brain tissue of the moth *Manduca sexta* [8] and in abdominal nerve cords of *Periplaneta americana* [9]. The binding fulfils the criteria of saturability, pharmacological specificity and tissue localization, as required for a nicotinic AChR [4–7]. (iii) Electrophysiological evidence corroborates the biochemical and pharmacological studies. αBgt blocks transmission at the central-nerve giant-fiber synapses in the terminal abdominal ganglion of *Periplaneta* [9]. This blockage is also exerted by isothiocyanate and nicotine at concentrations similar to those required for the inhibition of the [^{125}I]αBgt binding to extracts from *Periplaneta* and *Drosophila* [9]. These findings make it very plausible to assume the existence of cholinergic synapses in the central nervous system of insects and to regard the αBgt binding component as a putative nicotinic AChR.

The characterization of the AChR in *Drosophila* is of phylogenetic interest in the study of its evolutionary stability. Moreover, it offers a possibility to approach to the genetic dissection of the synaptic process of ion permeability control, since *Drosophila* enables the application of powerful genetic techniques to the isolation of mutants in this system.

A requisite for the analysis of AChR mutants is the biochemical characterization of the wild-type receptor. Furthermore, the demonstration of a biochemical similarity between the well-studied AChRs of vertebrates and that of *Drosophila* would prove it to be an appropriate model system.

We here report the solubilization of the putative AChR of *Drosophila* and some of its biochemical and pharmacological properties, using [^{125}I]αBgt as a specific, high-affinity ligand. From our results we conclude that *Drosophila* may be used as an appropriate model for studies of the function of the AChR.

2. Materials and methods

Heads of *Drosophila* (wild-type strain 'Oregon R') were harvested according to the technique of Harris et al. [10], and homogenized in 130 mM NaCl, 5 mM KCl, 3 mM EDTA, 20 mM Tris–HCl, pH 7.8 (buffered *Drosophila*-Ringer) at a concentration of 800 heads/ml. Homogenization was performed on ice in a Bühler blender homogenizer for 3 × 30 s at maximum speed. The homogenate was freed of cuticle particles by filtration through a nylon cloth (pore size 60 μm) and centrifuged for 5 min at

Abbreviations: AChR, acetylcholine receptor; αBgt, α-bungarotoxin; [^{125}I]αBgt, [^{125}I$_2$]diiodo-α-bungarotoxin

800 × *g*. The pellet was resuspended in homogenization buffer and centrifuged again. The collected supernatants were further centrifuged on a cushion of 35% sucrose in homogenization buffer at 30 000 × *g* for 25 min. Most of the αBgt binding activity was found in the interphase between sucrose solution and supernatant. The pellet of this centrifugation contained the eye screening pigment; it was found to contain no αBgt binding activity. The interphase was collected, diluted with homogenization buffer and centrifuged at 40 000 × *g* for 25 min. The pellet ('interphase pellet') was used for the following experiments.

The interphase pellet was resuspended to a final concentration of 5–10 mg protein/ml in 50 mM NaCl, 50 mM Tris, containing 1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml Pepstatin as protease inhibitors, and the pH adjusted with HCl to the value to be tested for solubilization efficiency. After addition of detergent to a final concentration of 1%, the mixture was incubated for 30 min at 25°C and afterwards centrifuged for 1 h at 100 000 × *g*. The following conditions were chosen as a standard solubilization procedure (see section 3): Incubation in 100 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 10 mM Tris–HCl, pH 8.7, at 25°C for 30 min followed by the above-mentioned centrifugation. The supernatant is referred to as 'soluble extract'.

[^{125}I]αBgt with a specific activity of 500-700 Ci/mmole was prepared according to the method of Vogel et al. [11]. It was further purified from radioactive contaminants on a 1 ml DEAE-cellulose column to minimize unspecific binding to the DEAE-cellulose filters used in the assay of solubilized material.

Binding assays were performed under saturating conditions (10 nM [^{125}I]αBgt, 0.1–1 nM AChR), unless otherwise stated, in a total volume of 50 μl in 50 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5, (incubation buffer) and, additionally, 1 mM nicotine to measure unspecific binding and 1% Triton X-100 when testing soluble receptor. After incubation at 25°C for 90 min, unless otherwise indicated, the samples were diluted with 0.2 ml of incubation buffer and filtered. [^{125}I]αBgt receptor complex was retained on Sartorius SM 11107 cellulose acetate filters (in the case of membranes) or on Whatman DE 81 DEAE-cellulose filters (in the case of solubilized material) and washed twice with 3 ml of incubation buffer. Filters were then dried and radioactivity determined in 0.5% PPO/toluene.

0.2 ml of soluble extract, supplemented with β-galactosidase (*E. coli*, grade IV), catalase (beef liver), cytochrome *c* (horse heart) and blue dextran, were filtered through a 30 ml Sepharose 6B column, which had been equilibrated with 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM Tris–HCl, pH 8.0 at 4°C, and fractions of 0.7 ml were collected. β-Galactosidase and catalase were assayed as described in [12] and [13], respectively. [^{125}I]αBgt binding activity was measured using the DEAE-cellulose filter technique.

0.2 ml of soluble extract were centrifuged on a 5–20% sucrose gradient in the same buffer plus marker proteins, as used for Sepharose 6B gel filtration for 3.5 h at 60 000 rev./min in a Beckmann SW 65 rotor at 20°C. Fractions of 140 μl were collected and aliquots assayed as described above.

Protein concentrations were measured as described by Lowry et al. [14], using bovine serum albumin as a standard. Prior to spectrophotometry, samples containing Triton X-100 were centrifuged in a Beckmann Microfuge B to eliminate the precipitate produced in the presence of the detergent.

3. Results and discussion

Synaptic membranes of *Drosophila* seem to be very resistant against extraction by detergents. Dudai reported [6] that Triton X-100, which yields quantitative solubilization of the AChR from vertebrates [15], is only slightly effective in the case of *Drosophila* AChR. It was shown [6] that the Triton 'solubilized' receptor, as judged by centrifugation at 100 000 × *g* for 1 h, sedimented at higher forces and longer times, and appeared as complexes of high molecular weight upon gel filtration. Taking centrifugation as a provisional criterion, we find (table 1) that, by increasing the pH, the solubilization of the *Drosophila* AChR achieved with Triton X-100 is improved. Moreover, extraction with deoxycholate turns out to be more efficient than that with Triton X-100, and also exhibiting the same pH dependency. A combination of both detergents does not increase the yield of soluble AChR. In the experiment shown in Table 1 the improvement of receptor solubilization runs in parallel with an increase of the specific receptor concentration in the soluble fraction. This result

Table 1
Solubilization efficiency with two detergents and various pH-values

Solubilization conditions	Receptor concentration (nM)	Protein content (mg/ml)	Specific receptor concentration (pmol/mg)	% Solubilization
'Interphase' (not treated)	5.9	4.25	1.39	100
1% Triton X-100 pH 7.5	0.6	1.85	0.32	10
1% Triton X-100 pH 8.1	0.9	1.90	0.49	16
1% Triton X-100 pH 9.0	1.5	1.95	0.77	25
1% Sodium deoxycholate pH 8.1	2.0	2.80	0.71	34
1% Sodium deoxycholate pH 9.0	2.9	2.45	1.18	49
1% Triton X-100 + 1% Sodium deoxycholate pH 9.0	2.9	2.50	1.16	49

For details of solubilization procedure and binding assay see section 2. [^{125}I]αBgt binding activity was compared in aliquots of the material not treated with detergents ('interphase') and the supernatants of the centrifugation at 100 000 × *g* for 1 h after various detergent treatments. '% solubilization' means the fraction of AChR concentration found in the supernatants as compared to the interphase

suggests that the AChR of *Drosophila* is more resistant to solubilization than other membrane proteins. A substantial fraction of the receptor extracted with 1% deoxycholate at pH 9.0 can be regarded as really solubilized, as shown by its inclusion in a Sepharose 6B column (fig.1A). Thus, we conclude, that experimental conditions bringing a reasonable fraction of the AChR into a soluble state have been established, allowing us to proceed with further steps towards its total purification.

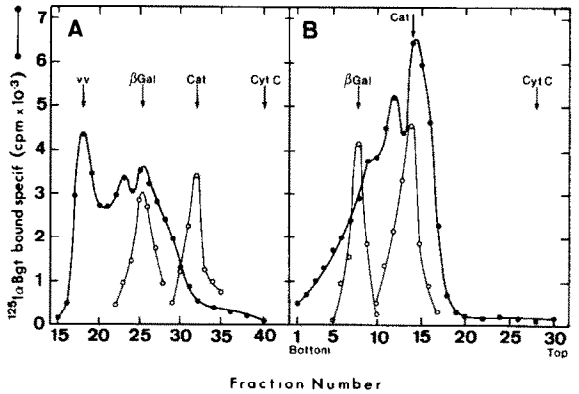

Fig.1. Estimation of the molecular weight of the AChR complex by (A) gel filtration on Sepharose 6B and (B) sucrose density gradient. For experimental details see section 2. vv, void volume; βGal, β-galactosidase; Cat, catalase; Cyt C, cytochrome *c*. •, [^{125}I]αBgt bound specifically to 50 μl aliquots of the fractions; ○, enzyme activity in aliquots of the fractions, represented in arbitrary units.

Equilibrium (K_D) and kinetic constants are difficult to compare between different reports, because the K_D is highly dependent on the content of cations such as Na^+, K^+, NH_4^+ or $Tris^+$ in the incubation mixture, probably due to a competitive binding of these ions to the αBgt binding site [16,17]. Therefore it is necessary to give a measured K_D as an apparent value and to specify exactly the conditions of the experiment. Table 2 shows kinetic and equilibrium constants, measured under identical conditions for the receptor in the membrane bound as well as in the soluble state. It turns out that solubilization does not alter the physicochemical properties of the AChR for the binding with [^{125}I]αBgt. Furthermore, K_Ds agree well with each other when determined by kinetic and equilibrium measurements. The K_D values are close to those reported for the binding of α-neurotoxins to the AChR of *Electrophorus* [18], rat diaphragm [21] and the putative AChR from rat brain [15,19,20], assuming a similar dependency of α-toxin binding upon small cation concentrations in these systems [17] as was found in *Drosophila* for αBgt binding [16]. The dissociation constant we found for the AChR-αBgt complex in *Drosophila* is about 10–50 times higher than those found in electric organs and in the vertebrate neuromuscular junction, but is in the same range as the dissociation constants

Table 2
Kinetic and equilibrium constants of the [^{125}I]αBgt receptor binding reaction

State of receptor	k_{+1} (on-rate) (M^{-1} s^{-1})	k_{-1} (off-rate) (s^{-1})	$K_D = \frac{k_{-1}}{k_{+1}}$ (M)	K_D from equilibrium measurements (M)
Membrane bound	$5.5 \cdot 10^5$	$7.7 \cdot 10^{-5}$	$1.4 \cdot 10^{-10}$	$1.6 (\pm 0.3) \cdot 10^{-10}$
Soluble state	$4.6 \cdot 10^5$	$8.3 \cdot 10^{-5}$	$1.8 \cdot 10^{-10}$	$2.3 (\pm 0.6) \cdot 10^{-10}$

On-rate kinetics were measured as described for the [^{125}I]αBgt binding assay (AChR concentration ~1 nM). The reactions were stopped by addition of 0.2 ml of 1 mM nicotine at the appropriate times. The reaction rate within the first 4 min was taken as the basis for calculation of the kinetic constant. Off-rate kinetics were started by addition of non-labelled αBgt to a final concentration of 1 μM after 45 min of preincubation of the material in 10 nM [^{125}I]αBgt. K_Ds were also derived from equilibrium measurements using Scatchard plot analysis with αBgt concentrations between 10 pM and 10 nM and an incubation time of 60 min

found in some instances for the putative AChR from rat brain [15,19,20]. In this latter case, values ranging from $5.8 \cdot 10^{-5}$ s^{-1} [19] to $3.1 \cdot 10^{-6}$ s^{-1} [20] have been reported, the discrepancies being attributed to variation in experimental conditions [20]. Similar considerations might also explain the different dissociation constants obtained for *Drosophila*. Whereas those reported here and by Dudai [6] are similar, Schmidt-Nielsen et al. [4] found the receptor-toxin complex to be practically irreversible. It seems as if the determination of these parameters were particularly sensitive to experimental modifications in the case of AChRs from central nervous systems.

In vertebrates various drugs are known to be either agonists or antagonists of the neurophysiological effect of acetylcholine on various types of AChRs. Table 3 summarizes the inhibitory effects of some of these drugs on the binding rate of [^{125}I]αBgt to membrane bound and soluble AChR. High affinities are found for all nicotinic ligands tested, suggesting that the αBgt binding site is a nicotinic AChR. The affinity of atropine for this putative AChR turns out to be higher than that for nicotinic AChRs in vertebrates, but still much lower than that of all nicotinic ligands tested in *Drosophila* excepting carbamylcholine, which is known to be a rather weak agonist. Furthermore Dudai reported a much higher affinity of atropine (K_I = 4 nM) to a putative muscarinic

Table 3
Inhibition by various cholinergic ligands of the rate of [^{125}I]αBgt binding reaction

Type of inhibitor	Inhibitor	IC_{50} (μM)	
		Membrane bound	Soluble state
Nicotinic agonists	Carbamylcholine	60	70
	Nicotine	1.0	0.8
Nicotinic antagonists	d-Turbocurarine	2.4	2.7
	Flaxedil	5.5	6.3
Muscarinic agonist	Muscarine	1500	1000
Muscarinic antagonist	Atropine	25	100

The reaction was started by addition of [^{125}I]αBgt (10 nM) after 30 min preincubation in the presence of various concentrations of inhibitor, and stopped after 3 min by the addition of 0.2 ml 1 mM nicotine. IC_{50} is the concentration of inhibitor necessary to reduce on-rate reaction to 50% of the value without inhibitor. 'Membrane bound' refers to the interphase pellet, 'soluble state' to material coeluting with β-galactosidase (see fig.1A) in gel filtration

AChR of *Drosophila* [22]. Thus the rather high affinity of atropine for the putative AChR described here does not justify to classify it as a muscarinic or mixed nicotinic/muscarinic one. In electric fishes the affinity for cholinergic antagonists remains stable upon solubilization, whereas the affinity for agonists increases in *Electrophorus* [23] and decreases in *Torpedo* [24–26]. These results have been interpreted assuming that the receptor is present under different states of affinity and that extraction by detergents may cause an interconversion between these states [24–26]. The fact that in *Drosophila* solubilization does not change the affinity either for agonists and antagonists (Table 3) might mean that the AChR does not exist under different states of affinity. However, the finding of negative cooperativity in the inhibition by acetylcholine of the αBgt binding to the membrane-bound receptor [7] suggests that such a heterogeneity of affinity states does exist.

Slightly different results for the affinity of the soluble receptor for antagonists have recently been reported by us [16], but crude extract was used in those studies instead of the more pure fraction (receptor co-eluting with β-galactosidase in gel filtration) used here. Acetylcholine and/or other unknown ligands of the AChR might reduce, due to competitive interaction, the apparent affinity for agonists when assaying the crude fraction.

Determination of the apparent molecular weight of the soluble native AChR provides very similar values to those reported for *Electrophorus* [13] and the putative AChR from rat brain [15]. Fig.1A shows that part of the deoxycholate extracted material (≤40% of total binding activity) eluted with the void volume in gel filtration. This can be interpreted as either high molecular weight membrane particles due to incomplete solubilization or to re-aggregation of soluble material. Apart from this high molecular weight fraction a considerable amount of the binding activity elutes together with β-galactosidase (420 000 MW) upon gel filtration and co-sediments with catalase (250 000 MW) in sucrose gradient centrifugation. These hydrodynamic properties are in exact agreement with those reported for the AChR from *Electrophorus* [13] and the putative AChR from rat brain [15]. Another fraction of higher molecular weight (fig.1A, fract. 23; fig.1B, fract. 12), which exhibits binding activity, is also found and may represent dimers of the receptor complex.

The results presented here indicate that the putative nicotinic AChR of *Drosophila* exhibits several biochemical properties similar to those of the AChR from vertebrates, suggesting a rather high evolutionary stability. Therefore we conclude that *Drosophila* may be used as an appropriate model for studies on the function of the AChR system. The first steps towards a genetic dissection of the system have been made with the recent description of genetic variants that affect AChR structure [27].

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