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Isolation of a Housefly Head Protein Fraction that Exhibits High Affinity Binding of Cholinergic Ligands

Philip J. Jewess, Barry S. Clarke, and John F. Donnellan

Woodstock Laboratory, Sittingbourne Research Centre, Shell Research Limited, Sittingbourne ME9 8AG, Kent, U.K.

The purification is described of a protein fraction, isolated from the central nervous system of housefly heads, that exhibits high affinity for cholinergic ligands. The purified material was found to bind with high affinity acetylcholine, nicotinic ligands such as nicotine and decamethonium as well as atropine, dexetimide and pilocarpine which are of a muscarinic nature. With all the ligands there appeared to be only a single site for binding with measured dissociation constants varying from 6.2×10^{-8} M (dexetimide) to 5.4×10^{-6} M (pilocarpine). The concentration of binding sites was in the range of 381 nmol g^{-1} of protein (atropine) to 560 nmol g^{-1} of protein (pilocarpine).

INTRODUCTION

Neurophysiological studies have indicated that cholinergic synapses are involved in the transmission of nerve impulses in the CNS of insects[1,2]. Although the extent of cholinergic innervation has not been quantified, the levels of AChE* throughout the nervous system suggest that the major mode of transmission is mediated by acetylcholine[3]. The electrophysiological information has been derived mainly from the study of the 6th abdominal ganglion of the cockroach *(Periplaneta americana)* and has shown that the insect AChR responds to both nicotinic and muscarinic cholinomimetics. This has led to the postulate that there is a single population of receptors that is less specific and perhaps less evolved in its binding characteristics than the more widely studied nicotinic and muscarinic vertebrate cholinergic receptors.

Biochemical studies with insect central nervous tissue also support the premise of a less specific type of receptor as housefly head extracts have been isolated that appear to bind with high affinity in a reversible manner both nicotinic and muscarinic types of cholinergic ligands[4,5,6]. The snake toxin, α-bungarotoxin, does not appear to interact with the insect cholinergic receptor either *in situ* or in direct binding studies[5] and has precluded the use of this nicotinic antagonist in the purification and characterization of the insect cholinergic receptor.

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* Abbreviations used: AChE — Acetylcholinesterase (E.C.3.1.1.7), AChR — Acetylcholine receptor, CAT — Choline acetyltransferase (E.C.2.3.1.6), PAGE — Polyacrylamide gel electrophoresis.

We describe here a method for the purification of a solubilised preparation of the flyhead AChR. This leads to the isolation of a protein fraction that shows a substantial enrichment in its capacity to bind reversibily a spectrum of cholinergic ligands with high affinity.

METHODS

Preparation of flyhead extracts

Flyheads were obtained by mechanically shaking frozen flies (*Musca domestica;* 2—3 days old) and using sieves to separate the heads from the debris. The heads were then homogenised in liquid nitrogen and the powdered material stored at —20 °C until required. The yield is approximately 100 g per 120 000 flyheads. An aqueous suspension was homogenised (10 passes at 1150 rev/min) using a Teflon-glass homogenizer and filtered through nylon net (159 μm aperture) to remove gross particulate material. The filtrate was used as the starting material for the purification procedure.

Assays

The binding of radiolabelled cholinergic ligands to flyhead extracts was assayed by equilibrium dialysis or more commonly by the ultrafiltration technique described by Clarke and Donnellan.[5] In the purification studies described below AChR activity was delineated by the capacity of extracts to bind decamethonium after incubation with 1 μM (Me—^{3}H) decamethonium for 10 min at 25 °C after the AChE had been inhibited by a pre-incubation step with 0.1 μM-paraoxon. CAT activity was measured essentially as described by Mannevik and Sörbo[7]. AChE activity was measured by the colorimetric procedure of Ellman *et al.*[8] The protein content of flyhead extracts was determined by the modified Folin-Ciocalteau method described by Cattell *et al.*[9]

Radiochemicals

Acetyl (*n*-Me—^{3}H) choline chloride (120 mCi/mmol), (1—^{14}C) acetylcoenzyme A (58 mCi/mmol), (Me—^{3}H)decamethonium chloride (418 mCi/mmol), (G—^{3}H)atropine (263 mCi/mmol) and (G—^{3}H)nicotine bitartrate (250 mCi/mmol), were obtained from the Radiochemical Centre, Amersham, U.K. (G—^{3}H)Pilocarpine (6.95 Ci/mmol) was from New England Nuclear, Boston, USA. (^{3}H)Dexetimide hydrochloride (600 mCi/mmol) was a generous gift from Dr W. Soudijn of Janssen Pharmaceutica, Belgium.

Isoelectric focussing

Gradients were constructed in a LKB 8101 column with 1.5% phosphoric acid as the lower electrode (anode) solution, a 47% to 0% sucrose gradient and an upper electrode (cathode) solution of 2% ethanolamine. The protein sample was incorporated in the gradient along with 2% LKB ampholites in the pH range of 3.5 to 10. Focussing took place over 60 h at 600 V and 2.2 mA. Gradient fractions were then removed and dialysed to remove the ampholites.

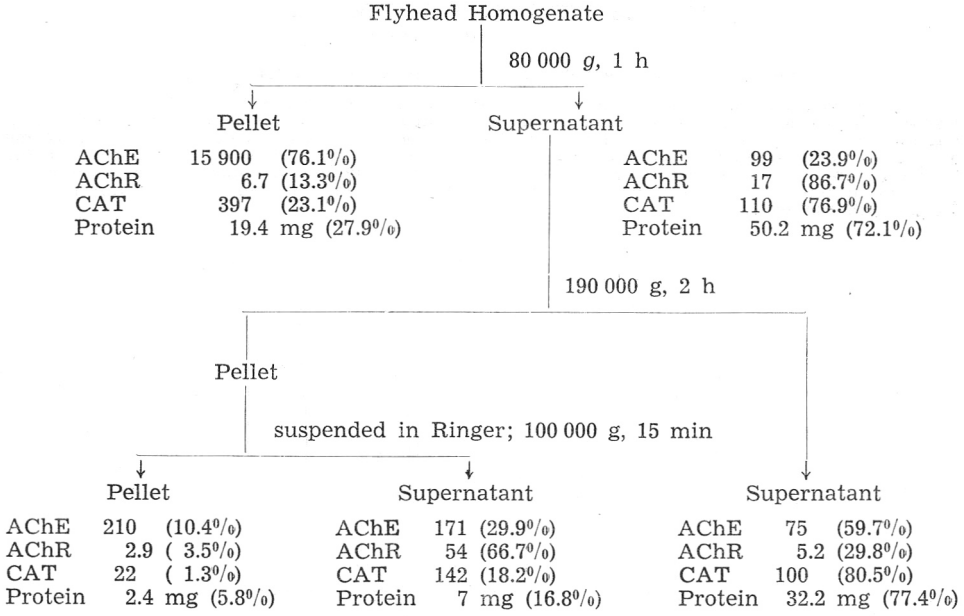
RESULTS

Eldefrawi *et al.*[4] and Donnellan *et al.*[6] have shown that the flyhead AChR is localised in a 80 000 *g* supernatant fraction obtained from aqueous homogenates of flyheads. Further centrifugation of this 'soluble' fraction at 190 000 *g* for 2 h results in the sedimentation of a membrane fraction enriched in the ability to bind a spectrum of cholinergic ligands[5,10]. This is shown in Table I which details the distribution and specific activities of AChE, AChR, CAT and protein observed in the fractions obtained by the differential centrifugation of a flyhead homogenate. The bulk of the AChE is located in the 80 000 *g* pellet while the CAT activity is predominantly in the 190 000 *g* supernatant.

TABLE I.

Differential centrifugation and solubilization of the flyhead AChR

A flyhead homogenate (3.6 g heads/360 ml water) was subjected to the centrifugation scheme shown above. AChE, nmoles acetylthiocholine hydrolysed min^{-1} mg^{-1} protein; AChR, nmoles bound decamethonium g^{-1} protein; CAT, nmoles acetylcholine synthesised 20 min^{-1} mg^{-1} protein. Figures in parenthesis show the % distribution of activity/binding capacity between pellet and supernatant (s).



Homogenization of the membrane fraction sedimented at 190 000 *g* in the Ringer solution (120 mM-NaCl; 5 mM-KCl; 10 mM-$MgCl_2$; 2 mM-$CaCl_2$ and 2 mM-tris, pH = 7.7) used in the binding assay leads to the apparent solubilization of the decamethonium-binding material as shown when the suspension is centrifuged for 15 min at 100 000 *g*. This leads to a three-fold purification in the decamethonium-binding capacity of the solubilised 100 000 *g* supernatant compared to the original 80 000 *g* supernatant.

A more significant purification can be obtained if the solubilised receptor extract is chromatographed through a Sepharose 6B column (5 × 80 cm, void volume 200 ml) equilibrated in Ringer. This results in the decamethonium-binding material being localised in a protein peak eluted between 400 and 700 ml. After concentration by ultrafiltration the pooled fractions are chromatographed through another Sepharose 6B column as shown in Fig. 1. It can be seen that the decamethonium-binding capacity of the 'receptor' peak can be increased up to 450 nmol g^{-1} protein. Using this procedure 100 g of flyheads from 120 000 flies yields approximately 50 mg of the Sepharose 6B extract enriched in decamethonium-binding capacity.

Isoelectric focussing of the Sepharose 6B extract showed that the decamethonium-binding material has a pI in the range 4.7 to 4.9 (Fig. 2). Similar values have been reported for the electroplax nicotinic receptor[11]. The anionic

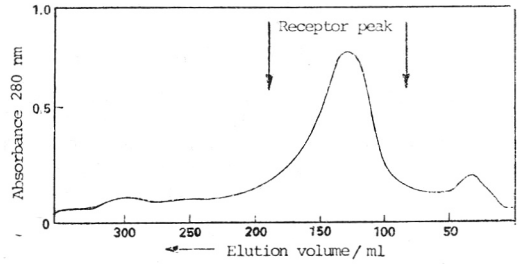


Fig. 1 — Chromatography on sepharose 6B of solubilised AChR.

120 g flyheads was processed through the procedure described in Table I and the 100 000 g supernatant chromatographed on a Sepharose 6B column (5 × 80 cm). The decamethonium-binding material, eluted between 400 and 700 ml, was concentrated to 15 ml (80 mg of protein) and applied to another Sepharose 6B column (2.6 × 98 cm). The elution profile in shown above. The receptor peak (69.4 mg of protein) bound 391 nmol of decamethonium per g of protein — AChE activity, 20 nmol min^{-1} mg^{-1} protein. Columns were equilibrated and eluted with Ringer solution.

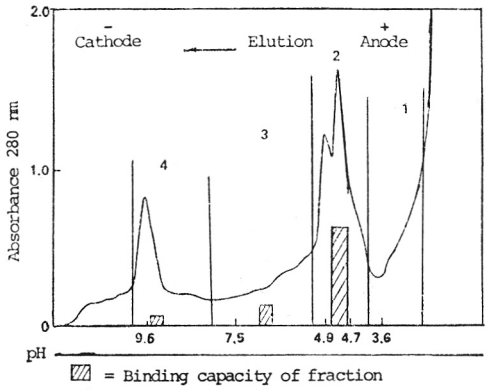


Fig. 2 — Isoelectric focussing of sepharose 6B AChR fraction.

Sepharose 6B extract (3 mg of protein) was focussed under the conditions described in the Methods Section. The 4 indicated fractions were dialysed to remove ampholites and analysed for protein, AChE and their capacity to bind decamethonium.

component in the Sepharose 6B extract can be separated by ion-exchange chromatography on DE-52 cellulose (Whatman Biochemicals Ltd., Maidstone, U. K.) as shown in Fig. 3. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of the receptor peak eluted from DE-52 cellulose shows the presence of two bands (82 000 and 90 000 daltons) on staining with Coomassie Blue. The native receptor has an apparent molecular weight of 350 000 daltons as judged by its elution position on a calibrated Sepharose 6B column.

Binding characteristics of the purified receptor

The ability of the purified Sepharose 6B extract to bind cholinergic ligands has been investigated by determination of bound ligand after incubation with concentration ranges (0.01 to 20 μM) of radiolabelled ligands. The apparent dissociation constant and concentration of ligand-binding sites were then obtained from double reciprocal plots of bound versus free ligand as analysed

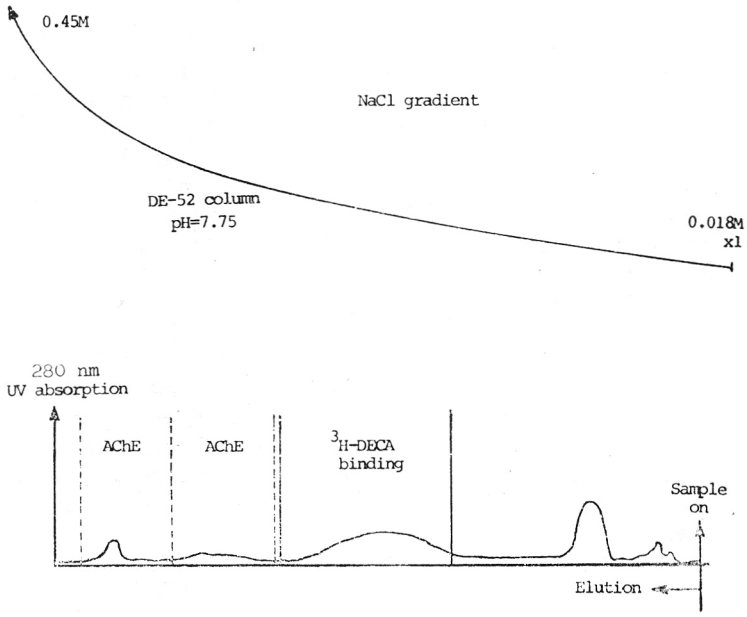


Fig. 3 — Ion-exchange chromatography on DE-52 cellulose.

A 4 ml aliquot (2.2 mg of protein) of the Sepharose 6B receptor fraction (see Fig. 1) was adsorbed onto a 1.9 × 9 cm column of DE-52 cellulose equilibrated in 0.1 strength Ringer. Elution at 1.8 ml/min with a 400 ml gradient of 0.1 to 2.5 strength Ringer gave the profile shown above. The AChR peak contained no detectable AChE activity and bound 580 nmol of decamethonium g^{-1} protein.

by the method of Wilkinson[12]. Table II illustrates the relatively broad ligand binding spectrum of the insect cholinergic receptor which binds nicotinic and muscarinic ligands with similar affinities. Of the ligands investigated dexetimide, a mammalian muscarinic antagonist, shows the highest affinity. Electrophysiological studies on the cockroach 6th abdominal ganglion preparation have shown that dexetimide is a potent antagonist of cholinergic transmission in the insect CNS (R. J. Dowson, personal communication).

TABLE II.

Binding studies with the flyhead cholinergic receptor

Ligand	Dissociation constant $K/\mu M$	Concentration of binding sites nmol g^{-1} protein	
Acetylcholine	5.027 ± 1.253	435 ± 59	(2)
Decamethonium	0.476 ± 0.09	575 ± 37	(3)
Atropine	5.223 ± 0.621	381 ± 25	(2)
(+)-Dexetimide	0.062 ± 0.009	579 ± 9	(2)
Pilocarpine	5.425 ± 0.719	560 ± 40	(2)
Nicotine	4.445 ± 0.484	508 ± 29	(2)

The binding of tritiated ligands was measured by the ultrafiltration method using the Sepharose 6B extract (Fig. 1) in the presence of 100 nM-paraoxon. Figures in parentheses indicate the number of separate binding runs — each with 8 to 10 ligand concentrations.

DISCUSSION

The solubilization by salt treatment of the AChR from flyhead membranes bears some resemblance to the procedures described for the solubilization of a nicotinic AChR from the optic ganglia of squid[13]. Differential centrifugation, solubilization (Table I) and chromatographic steps (Figs. 1 and 3) have permitted the isolation of a protein fraction that has the properties of the insect AChR in binding in a reversible manner a spectrum of cholinergic ligands (Table II). The purified receptor is essentially devoid of AChE and CAT activities which have the potential to interfere in the binding assay used to monitor ligand interactions with the AChR.

The insect AChR has an isoelectric point of 4.8 (Fig. 2) and appears from permeation chromatography to have a molecular weight of around 360 000 daltons. PAGE studies indicate a sub-unit composition of 82 000 and 90 000 daltons. Binding studies show a single site of binding for the ligands investigated (Table II). Earlier studies with an 80 000 g supernatant flyhead fraction had shown an apparent two site binding profile for both acetylcholine and decamethonium[6] and this may have been due to binding of these quaternary ammonium ligands to sites other than on the AChR in this relatively crude extract.

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SAŽETAK

Izolacija proteinske frakcije iz glave muhe koja pokazuje visok afinitet za vezanje kolinergičnih liganada

P. J. Jewess, B. S. Clarke i J. F. Donnellan

Opisano je čišćenje jedne proteinske frakcije visokog afiniteta za kolinergične ligande, izdvojene iz centralnog nervnog sistema glave muhe. Čišćena tvar vezuje s visokim afinitetom acetilkolin i nikotinske ligande kao što su nikotin i dekametonij, a također atropin, deksetimid i pilokarpin, koji su muskarinske prirode. Čini se da postoji samo jedno mjesto vezanja za sve ligande. Izmjerene konstante disocijacije kreću se od 6.2×10^{-8} M (deksetimid) do 5.4×10^{-6} M (pilokarpin). Gustoća mjestâ vezanja bila je od 381 nmol g^{-1} protein (atropin) do 560 nmol g^{-1} of protein (pilokarpin).

LABORATORIJ WOODSTOCK, SHELL,
ISTRAŽIVAČKI CENTAR SITTINGBOURNE,
KENT, ME9 8AG, ENGLESKA