The Isolation of an Acetylcholine- and Decamethonium-Binding Protein from Housefly Heads

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Protein fractions thought to contain an acetylcholine-receptor molecule have been isolated from a variety of nervous tissues. The experimental approach used by Miledi & Potter (1971) and Miledi et al. (1971) has been to label receptor molecules in the electric organs of fish or vertebrate muscle preparations with radioiodinated \( \alpha \)-bungarotoxin in order to facilitate their isolation by fractionation techniques involving the use of detergent solutions. De Robertis and his co-workers (De Robertis & Fiszer de Plazas, 1970; Gomez et al., 1970; De Robertis et al., 1970; Lunt et al., 1971) have used chloroform–methanol mixtures to extract from vertebrate brain and muscle tissue and from the electroplax of the electric eel hydrophobic protein–lipid complexes (proteolipids) that show high-affinity binding for cholinergic ligands and exhibit many of the properties expected of an acetylcholine-receptor molecule. Both groups have demonstrated that acetylcholine-binding activity is not due to the presence of acetylcholine esterase in their preparations. It is possible that the fractions isolated by these two radically different experimental approaches contain the same acetylcholine-binding component.

We now describe experiments showing that a proteolipid fraction with similar properties to those of the 'acetylcholine-receptor proteolipids' described by De Robertis and his co-workers can be isolated from insect nervous tissue. The presence of an acetylcholine-binding component in aqueous homogenates of housefly heads has been reported (Eldefrawi & O'Brien, 1970).

Methods

Housefly heads were obtained by manually shaking frozen flies (Musca domestica) for 2 min and then using sieves to separate the heads from the remainder of the insect. The heads (usually 10 g), as a 10% (w/v) suspension in water at 4°C, were disrupted by mechanical homogenization (ten passes at 1150 rev./min in a Teflon–glass homogenizer), and the resultant brei was filtered through nylon mesh (159 \( \mu \)m aperture). The residue was washed once and the combined filtrates were centrifuged for 10 min at 20000g. The supernatant, which contained 52% of the total acetylcholine esterase activity, was freeze-dried, and this material (approx. 900 mg dry wt.) was then extracted by stirring with 35 ml of chloroform–methanol (2:1, v/v) for 14–16 h at room temperature. Particulate material was removed by filtration through a no. 3 sintered-glass funnel, and the filtrate, after the addition of 15 ml of chloroform, was evaporated to a volume of 9 ml. This extraction procedure is essentially that described by De Robertis & Fiszer de Plazas (1970).

To test for acetylcholine- or decamethonium-binding activity, radioactive ligand was incubated with the concentrated extracts for 1 h at room temperature. The incubation mixtures were then loaded on to a Sephadex LH-20 column (18 cm \( \times \) 1.5 cm) that had been prepared and equilibrated with chloroform by the procedures described by Soto et al. (1969). The sample was eluted with the following sequence of solvents: 50 ml of chloroform, 30 ml of chloroform–methanol (15:1, v/v) and 50 ml of chloroform–methanol (2:1, v/v). Fractions (3–6 ml) were collected and analysed for protein, phosphorus and radioactivity by using the methods described by Cattell et al. (1971).

All solvents were redistilled before use. Dimethyl-tubocurarine hydrochloride was obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). [\( ^{14} \mathrm{C} \)]Acetylcholine chloride (250 mC/mmol), [\( ^{1} \mathrm{H} \)]acetylcholine chloride (13.7 mC/mmol) and [\( ^{3} \mathrm{H} \)]decamethonium chloride (329 mC/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) and used without further purification.

Results

In four experiments an average of 0.5% of the total protein present in the 20000g supernatant fraction obtained from fly heads was extracted by chloroform–methanol.

Fig. 1 shows the elution profile obtained when the proteolipid fraction (2.5 mg of protein) was incubated in a 4 ml volume of chloroform–methanol (2:1, v/v) containing 1.1 \( \mu \)M-[\( ^{14} \mathrm{C} \)]acetylcholine chloride for 1 h and then chromatographed on a Sephadex LH-20 column. The bulk of the protein present in the extracts was resolved into three main peaks, of which two were eluted with chloroform and one with chloroform–methanol (2:1, v/v). A minor protein component was eluted with chloroform–methanol (15:1, v/v). Of the [\( ^{3} \mathrm{H} \)]acetylcholine chloride applied to the column 90% was recovered with the
Fig. 1. Fractionation on Sephadex LH-20 of housefly head proteolipids (2.5 mg of protein) that had been incubated with 1.1 μM-[Ac-3H]acetylcholine chloride

Experimental details are given in the text. ---, Protein; ·······, radioactivity; ----, phosphorus.

Fig. 2. Fractionation on Sephadex LH-20 of housefly head proteolipids (1.76 mg of protein) that had been incubated with 3.45 μM-[Me-3H]decamethonium chloride

Experimental details are given in the text. ---, Protein; ·······, radioactivity; ----, phosphorus.

second protein peak eluted with chloroform. The phosphorus-containing material was separated into two peaks, one, which had a shoulder, being eluted with chloroform, the other with chloroform–methanol (2:1, v/v). The recoveries of protein, radioactivity and phosphorus from the column were 91, 100 and 84% respectively.

To test that the [Ac-3H]acetylcholine chloride
eluted from the column was bound to a component of the proteolipid and not merely $[Ac-3^H]$acetylcholine chloride co-chromatographing with the protein and lipid, 4ml of 1.1 $\mu m$-[1-$^{14}C$]acetylcholine chloride was chromatographed through the column with the same elution procedure. Only 4.3% of the radioactivity was recovered from the column, with 0.5% being eluted with chloroform alone. The remaining radioactivity appeared to be irreversibly bound to the Sephadex LH-20.

Similar experiments were done to determine whether decamethonium would bind to the proteolipid. Fig. 2 shows an elution trace of a proteolipid fraction (1.76mg of protein) that had been incubated with 3.45 $\mu m$-[Me-$^{3}H$]decamethonium chloride. An elution profile similar to that obtained with $[Ac-3^H]$-acetylcholine chloride was obtained, the $[Me-^{3}H]$-decamethonium chloride being eluted with the same protein peak. The recoveries of protein, radioactivity and phosphorus from the column were 106.3, 80.6 and 121% respectively. In a control experiment, with the same concentration of free $[Me-^{3}H]$decamethonium chloride, 2.9% of the radioactivity applied to the column was eluted with chloroform and a further 2.9% with chloroform–methanol (2:1, v/v). These results are similar to those reported by De Robertis et al. (1970) for the binding of cholinergic ligands to proteolipid isolated from electroplax.

These workers also showed that the binding of cholinergic ligands to electroplax proteolipid was strongly inhibited by dimethyltubocurarine. Therefore it was decided to test the effect of this compound on acetylcholine binding to the proteolipid from fly head. Fly head proteolipid was preincubated with dimethyltubocurarine (5.8 or 99 $\mu m$) for 1h at room temperature before the addition of 1.1 $\mu m$-$[Ac-3^H]$-acetylcholine chloride. After 1h the test solutions and controls that had not been preincubated with dimethyltubocurarine were chromatographed on Sephadex LH-20 columns. It was found that, even at the higher concentration, dimethyltubocurarine had no effect on the binding of acetylcholine to the proteolipid fraction and that elution profiles similar to that shown in Fig. 1 were obtained with both dimethyltubocurarine-treated and control preparations.

**Discussion**

The specificity of the interaction between the two cholinergic ligands used in this study and a single proteolipid fraction is emphasized by the finding that the ligands were associated with only one of the four proteolipid peaks separated from the chloroform–methanol extracts by chromatography on Sephadex LH-20. It is possible that the ligands could be binding to the lipid components of the proteolipid. However, this is unlikely, since in some experiments the phosphorus-containing material (presumably phospholipid) was almost completely separated from the protein and associated radioactivity by Sephadex LH-20 chromatography. In addition, all of the protein and radioactivity can be separated from the bulk of the lipid material by ether precipitation. Therefore it is highly probable that the cholinergic ligands bind to the protein component of the proteolipid.

The acetylcholine-binding proteolipid isolated from fly head tissue has similar properties to the 'acetylcholine-receptor proteolipids' isolated from electroplax (De Robertis et al., 1970) and muscle tissues (Lunt et al., 1971). Thus all of these proteolipids are soluble in chloroform–methanol, contain both protein and lipid components, are eluted by chloroform alone from Sephadex LH-20 and show a marked and quantitatively similar ability to bind cholinergic ligands. In the experiment in which the proteolipid fractions were incubated with 1.1 $\mu m$-$[Ac-3^H]$acetylcholine chloride before Sephadex LH-20 chromatography, 10.2nmol of $[Ac-3^H]$acetylcholine chloride/mg of protein was recovered in the proteolipid peak. In a very similar experiment De Robertis et al. (1970) found that 13.4nmol of $[1-^{14}C]$-acetylcholine chloride/mg of protein was associated with the fractionated proteolipid from electroplax. One major difference between the insect and vertebrate proteolipids is the finding that dimethyltubocurarine does not inhibit acetylcholine chloride binding in the former case. However, this complements the results of other investigators, who showed that dimethyltubocurarine has a mode of action on the insect nervous system markedly different from its action on that of vertebrates (Flattum et al., 1967).

Our results are therefore consistent with the suggestion that the acetylcholine- and decamethonium-binding proteolipid isolated from fly heads could be an acetylcholine receptor.


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