ISOLATION BY AFFINITY CHROMATOGRAPHY OF A CHOLINERGIC PROTEOLIPID FROM NUCLEUS CAUDATUS.

H. Saraceno and E. De Robertis

Instituto de Biología Celular, Facultad de Medicina
1121 Buenos Aires, Argentina.

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SUMMARY

A cholinergic proteolipid fraction (i.e. a hydrophobic lipoprotein) was separated from the n. caudatus of the cow, using affinity chromatography with the lipophilic gel Sephadex LH-20 and p-phenyltrimethylamonium as the active group. High affinity binding studies showed that only the specific fraction, desorbed after an acetylcholine (or acid) pulse, and corresponding to 0,72% of the proteolipids, is the one that binds the cholinergic ligands. The binding of (^{3}H)atropine and (^{14}C)d-tubocurarine demonstrated that there are 814 picomoles/g fresh tissue of muscarinic sites and only 76 picomoles/g of nicotinic sites. The specific radioactivity for (^{3}H)atropine is 10,000 nmoles/g protein, suggesting a high degree of purification of the specific cholinergic proteolipid.

INTRODUCTION

High affinity binding for cholinergic drugs of nicotinic and muscarinic type was found to be localized in nerve-ending membranes of the rat cerebral cortex (1,2) and to be specially concentrated in the junctional complexes, which contain the post-synaptic membranes (3). Such binding was attributed to a proteolipid fraction (i.e. a hydrophobic lipoprotein) which could be extracted from the membranes with chloroform methanol (2:1) and further purified by Sephadex LH-20 chromatography and elution with solvents of increasing polarity (4,5). More recently Barrantes et al. (6) used affinity chromatography in organic solvents to separate a nicotinic proteolipid from skeletal muscle. The same column was also used to purify a muscarinic binding proteolipid previously isolated from smooth muscle (7).

In the present work the cholinergic affinity column was applied to the nucleus caudatus of the cow. It will be shown that this tissue contains a cholinergic proteolipid fraction of predominantly muscarinic characteristics.

METHODS

Cow brains were obtained as fresh as possible from the slaughter house and after packing on ice, were brought to the laboratory where the caudate nuclei were immediately dissected. The material was homogeneized at 10% concentration in distilled water, lyophilized and kept dry until use. For the extraction 500 to 1000 mg of the above material, corresponding to 2.5 to 5 g fresh tissue, was homogenized in 10-15 ml of chloroform-methanol (2:1) with an Ultra Turrax homogenizer (Karl Kolb, Frankfurt). After standing for 30 min the extract was filtered through Whatman paper N°2 and the residue was discarded. To the total lipid extract (TLE), half volume of chloroform was added and then it was concentrated under vacuum to a 2.5 - 3ml volume. The affinity chromatography system consisted of a dextran support, the lipophilic Sephadex LH-20, to which a spacer arm of 3,3 iminobispropylamine was covalently linked. The active group was p-phenyltrimethylammonium, a quaternary ammonium compound, linked to the free end of the spacer arm (Fig. 1). The column of 12 x 1.1 cm was equilibrated overnight with chloroform. The extract was loaded and the elution was carried out with chloroform and mixtures of chloroform-methanol of increasing polarity up to 1:1 (v/v). Fractions of 2-4 ml were separated using an LKB Uvicord fraction collector. The elution separated first all the non-specific proteolipids and when no further UV absorbing material was detected at 280 nm a pulse of 10 ml was applied, either of 10^{-3}M acetylcholine or of chloroform-methanol (1:1) acidified with 0.1N HCl. The elution was continued with the non-acidified solvent mixture until the specific cholinergic protein was desorbed (Fig. 1). Both with the acetylcholine or the acid pulse exactly the same results, regarding the amount and position of the specific proteolipid peak, were obtained. The latter procedure was, however, preferred because it was then easier to carry out the binding studies. If the peak is desorbed with acetylcholine the excess ligand should be eliminated by rechromatography in Sephadex LH-20; on the other hand, the presence of the acid milieu does not interfere with the cholinergic binding.

Binding studies were carried out in the non-specific as well in the specific peaks obtained from the affinity column (Fig. 1). After incubation with (^{14}C)acetylcholine, (^{14}C)d-tubocurarine or (^{3}H)atropine, aliquots of these peaks (50-100µg), were submitted to rechromatography on small columns of Sephadex LH-20 (16cm x 0.8cm) which were equilibrated overnight in chloroform. This method permits the separation of the bound from the free ligand (Figs. 2 and 3). The eluant, containing the rechromatographed proteolipid, was separated and collected for protein determination and radioactivity. For this purpose the fractions were evaporated to dryness at 60°C and counted in toluene containing 0.4% (w/v) PPO in a packard Tricarb or a Nuclear Chicago liquid scintillation spectrometer. The protein was determined by the method of Lowry as modified by Hess and Lewin[8] and the lipid P by the method of Chen et al.[9] For every ligand control experiments with free drug were carried out.

The radioactive drugs used were (acetyl-1-^{14}C)-choline iodide (3.84 mCi/mmol), d-tubocurarine-d-(methyl ^{14}C) ether iodide (112 mCi/mmol) and (G-^{3}H) atropine (318 mCi/mmol) all from Amersham.

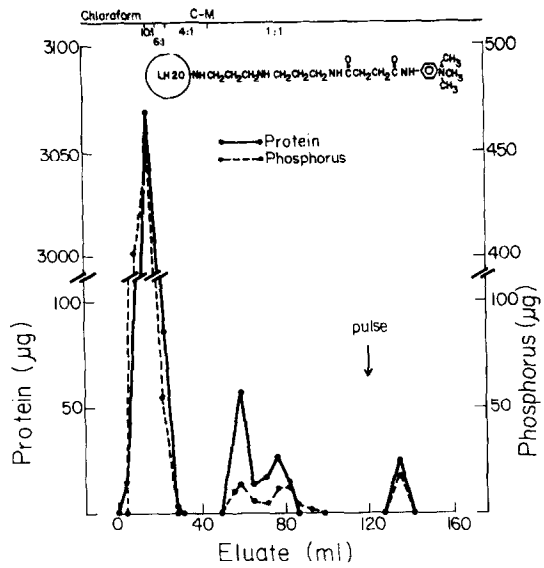

Fig.1 Separation of proteolipid protein and lipid P by affinity chromatography from the total lipid extract of 500 mg of lyophilized n. caudatus of the cow.
The inset shows the chemical structure of the specific affinity group (see the description in the text).

RESULTS AND DISCUSSION

Fig. 1 shows a typical affinity chromatogram made with the TLE of 500 mg of lyophilized tissue containing 35,9 mg of proteolipid protein. Fraction 1, eluting between 5 and 30 ml, contains 97.03% of the protein and 89.9% of the lipid P. Fraction 2, sometimes showing as in Fig. 1 two peaks, is eluted between 50 and 85 ml of chloroform-methanol (1:1) and contains 2.2% of the recovered protein and 7.3% of the lipid P. The specific fraction 3, eluted in acidified chloroform-methanol (1:1), between 125 and 135 ml, contains only 0.72% of the proteolipid protein and 2.7% of lipid P. The total recovery of the column for protein was 76.8% and for lipid P 85.4%.

Binding studies carried out on fractions 1 and 2 with (^{14}C) acetylcholine, (^{14}C)d-tubocurarine and (^{3}H)atropine showed negative results indicating that these proteolipid fractions were not cholinergic. On

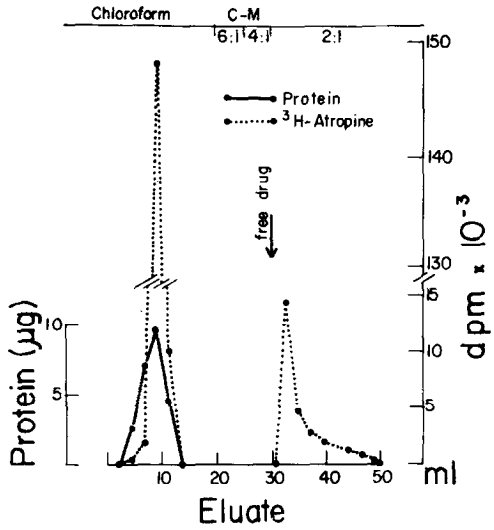

Fig. 2 Rechromatography of the specific peak (fraction 3 from Fig. 1) with 10^{-7}M (^{3}H)atropine on a Sephadex LH-20 column. Observe the coincidence between the protein peak and the bound radioactivity. The elution of the free drug is also indicated.

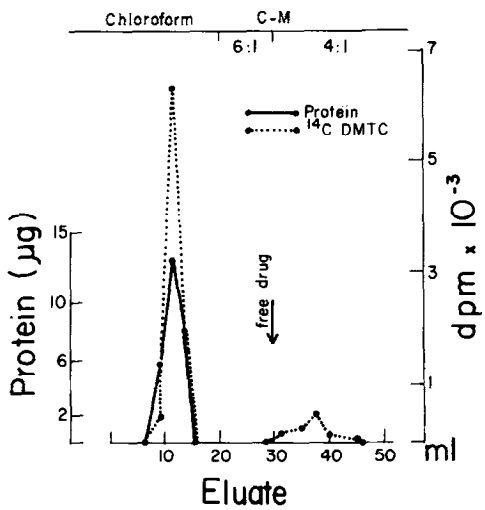

Fig. 3 Similar to Fig. 2 but showing the binding of 10^{-7}M (^{14}C) d-tubocurarine.

the other hand, when fraction 3 obtained after desorption (either by the pulse of acetylcholine or of acidified chloroform-methanol) was rechromatographed on small Sephadex LH-20 columns, it did show high

affinity binding for these cholinergic drugs (Figs.2 and 3). The protein appeared in a sharp peak between 3 and 15 ml of chloroform, in coincidence with a peak of radioactivity. In each case the separation between bound and free ligand was achieved, as could also be ascertained by the corresponding controls with free ligand.

Table I: Binding of cholinergic ligands to the specific protein peak separated by affinity chromatography from the n. caudatus of the cow.

Ligand	Concentration M	nmoles / mg protein	pmoles / g.fresh tissue
(^{14}C)acetylcholine	10^{-6}	10.41 ± 1.72	794
(^{3}H)atropine	10^{-7}	10.6 ± 1.73	814
(^{14}C)d-tubocurarine	10^{-7}	1.0 ± 0.17	76

The above results are the mean ± SD of three independent experiments. To carry out the binding studies aliquots of fraction 3 (see Fig. 1) were incubated with the ligand for 30 min and then rechromatographed on small Sephadex LH-20 columns as illustrated in Figs. 2 and 3.

Table 1 shows that while there is about the same amount of binding for (^{14}C)acetylcholine and (^{3}H)atropine per mg protein and per g fresh tissue, the binding of (^{14}C)d-tubocurarine is about 1/10th lower.

In agreement with microphysiological studies (10,11), binding experiments carried out on the total homogenate or on subcellular membranes of the n.caudatus have demonstrated that the cholinergic receptors are mainly of muscarinic nature (12-14). The results presented here agree with the above finding in that the specific

proteolipid fraction, separated by the affinity column, shows a predominance of muscarinic binding sites. Using (^{3}H)atropine and (^{14}C)d-tubocurarine (both at 10^{-7}M), as markers respectively for muscarinic and nicotinic binding, we found 814 picomoles/g fresh tissue of muscarinic sites and only 76 picomoles/g of nicotinic sites. In other words, the n.caudatus of the cow may contain about 90% of muscarinic and 10% of nicotinic receptors.

Using propylbenzilylcholine mustard, Hiley and Burgen (14) found 0.475 nmoles of binding sites/g protein in the total homogenate of the n.caudatus of the dog and Yamamura et al (13), assayed about 1 nmole of binding sites/g protein in the n.caudatus of the monkey. Since in our isolated proteolipid the specific radicactivity for (^{3}H)atropine is of about 10,000 nmoles/g protein, in comparison with the above results on total homogenate, we have reached a 10,000 to 20,000 fold purification.

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