PURIFICATION BY AFFINITY CHROMATOGRAPHY OF NICOTINIC AND MUSCARINIC HYDROPHOBIC PROTEINS SEPARATED BY SEPHADEX LH20.

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SUMMARY

Hydrophobic protein fractions, having nicotinic or muscarinic binding properties were separated respectively from skeletal muscle and smooth intestinal muscle using the lipophilic gel Sephadex LH20. Further purification of the specific fractions was carried out by a cholinergic affinity column, using elution with organic solvents, followed by a pulse of 10^{-3}M acetylcholine . The results obtained confirm the validity of the Sephadex LH20 technique for the isolation and binding of cholinergic proteins proposed by De Robertis et al (4) and discard the criticisms raised by Levinson and Keynes (7).

INTRODUCTION

Special hydrophobic protein fractions were isolated from Torpedo and Electrophorus electroplax (1) and skeletal muscle (2) and were shown to have nicotinic binding properties. Furthermore a cholinergic protein fraction of muscarinic type was isolated from intestinal smooth muscle (3). The standard procedure has been to extract the tissue with organic solvents and, after incubation with the specific ligand, to load it on a column made with the lipophilic gel Sephadex LH20 (4). This is followed by elution with chloroform and mixtures of chloroform-methanol of increasing polarity. In every case several protein fractions were separated, of which, a single specific peak bound the cholinergic ligand. The free drug was retained by the column or was eluted in the most polar regions of the chromatogram. For binding studies, another method used in our laboratory has been a biphasic partition system in which the ligand is placed in the aqueous phase and the two phases are stirred until binding equilibrium is reached (5).

In spite of all the published evidences for various receptor proteins (6)

and disregarding the results obtained by partition, Levinson and Keynes (7) have criticized the binding method using Sephadex LH20, suggesting that the association of the ligand with the protein was artifactual. In the present paper, in addition of showing that hydrophobic cholinergic proteins, having nicotinic and muscarinic binding properties, can be purified by affinity chromatography, the value of the Sephadex LH20 method (4) is fully confirmed.

EXPERIMENTAL

Lyophilised rat diaphragms were extracted with chloroform-methanol (2:1) (2). The circular layer of bovine intestine was dissected (3) and extracted without lyophilisation; the total lipid extract (TLE) was washed with theoretical upper phase (8). The extracts were submitted to column chromatography on a Sephadex LH20 column and the protein fractions, that bind the cholinergic drugs, were separated (Figs. 1A and 2A).

The affinity chromatography system consisted of Sephadex LH20, a spacer arm -3,3'-iminobispropylamine-, and a quaternary ammonium compound p-phenyltrimethylammonium of recognized cholinergic character(9) covalently linked to the free end of the spacer arm (10). The column of 17 x 1.7 cm was equilibrated with chloroform and the material (i.e. the previously isolated fractions) was loaded onto the column in 2.5 ml samples. Elution of the non-specific proteins was carried out by the use of chloroform and mixtures of chloroform-methanol of increasing polarity. When no further UV absorbing material was detected at 280 nm, a pulse of 10^{-3}M acetylcholine in 5 ml of chloroform-methanol (4:1) was applied. The elution was continued with the same solvent mixture until the specific cholinergic protein fraction was eluted (Figs.1B and 2B). A detailed description of the technique of cholinergic affinity chromatography in organic solvents is being published elsewhere (11).

RESULTS AND DISCUSSION

Fig. 1A shows the typical pattern previously described for skeletal muscle (2). The second protein peak (shaded area) is the specific one that binds

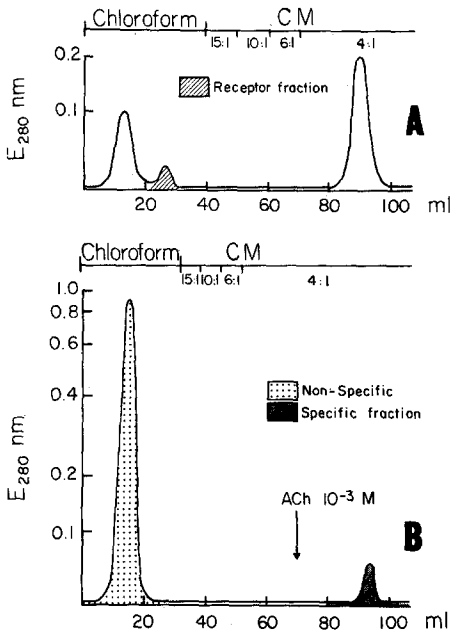

Fig. 1 Two-step separation of the cholinergic binding protein from rat diaphragm (A) Conventional chromatography on Sephadex LH20 of a TLE corresponding to 200 mg of lyophilised tissue. (B) Affinity chromatography in which the specific peak of (A) (i.e. shaded area) corresponding to 1 g of lyophilised tissue was used. This was equivalent to 32 hemidiaphragms.

The specific cholinergic binding protein (i.e. receptor peak) is eluted after the 10^{-3}M acetylcholine pulse (see the description in the text).

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various nicotinic drugs, including $[^{3}H]\alpha$-bungarotoxin (12). It contains an average of 200µg protein/g fresh tissue. Fig. 1B shows the result of affinity chromatography. The large non-specific peak contains most of the protein loaded onto the column. The specific protein (i.e. receptor peak), eluted after the acetylcholine pulse, contains 13 µg/g fresh tissue. Since the recovery is 95%, this amount corresponds to a 15.4 fold enrichement over the preceding step. If we consider that the total protein content in rat diaphragm is about 200 mg/g fresh tissue the total enrichement of this protein fraction is in the range of 15.000 fold. Upon rechromatography on Sephadex LH20, in the presence of 10^{-6}M $[^{14}C]$ acetylcholine iodide (Amersham 2.43 Ci/mole), the non-specific fraction did not show binding, indicating that the

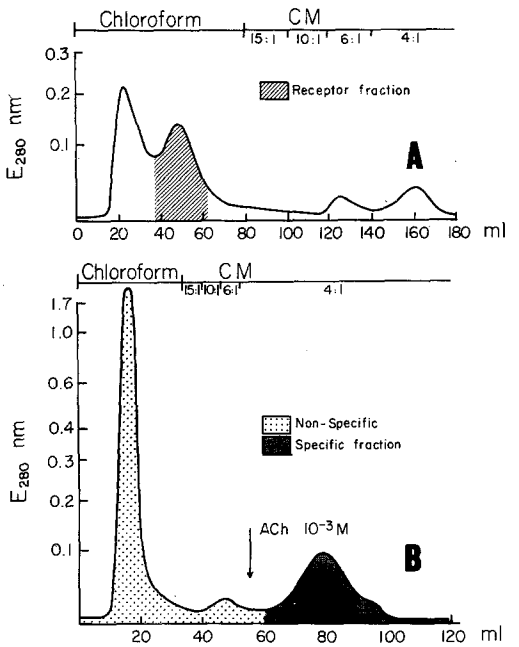

Fig. 2 Two-step separation of the muscarinic cholinergic binding protein fraction from the circular layer of the bovine intestine. (A) Conventional chromatography on Sephadex LH20 carried out with a TLE of 16g fresh tissue.
B) Affinity chromatography done with four specific peaks of (A). The cholinergic fraction (i.e. receptor peak) is eluted after the 10^{-3}M acetylcholine pulse (see the description in the text).

specific cholinergic binding protein had been completely removed by the affinity column. On the other hand, after elimination of the excess of acetyl choline from the receptor peak by Sephadex LH20 chromatography, the binding of [^{14}C] acetylcholine to this fraction was demonstrated by a new rechromatography on Sephadex LH20; Fig. 3 shows the single sharp peak of protein that appears in coincidence with the radioactive ligand in the chloroform. In this experiment as well as in the control, without protein, the free [^{14}C] acetylcholine is eluted in the more polar solvents. Similar results were obtained using as nicotinic ligand 8µg of [^{3}H]α-bungarotoxin (2Ci/mmole) kindly provided by Prof. E.A. Barnard. The binding of [^{3}H]α-bungarotoxin was also demonstrated by the partition method of Weber et al.(5). It should

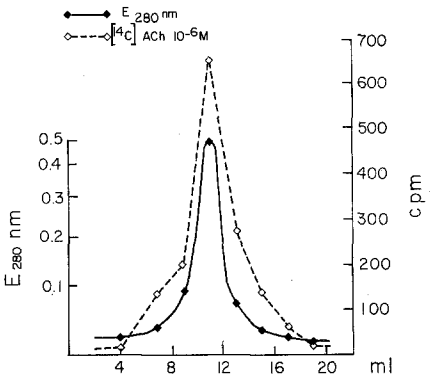

Fig. 3 Rechromatography on Sephadex LH20 of the receptor peak from rat diaphragm (see Fig. 1B), after incubation for 30 min with 10^{-6}M $[^{14}C]$ acetylcholine. The specific radioactivity reached in this experiment was 5 nmoles/mg protein.

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be emphasized that $[^{3}H]\alpha$-bungarotoxin has its partition coeficient 100% favorable to water.

In the case of the TLE of smooth intestinal muscle two peaks of protein were obtained by elution with chloroform using conventional chromatography (Fig.2A), instead of the three peaks previously found in the lyophilised tissue (3). Here the second peak (shaded area) is the specific one that binds $[^{14}C]$ acetylcholine and $[^{3}H]$ atropine (Amersham, 434 Ci/mole). This protein fraction contains 95 µg protein/g fresh tissue. After affinity chromatography the non-specific protein is eluted in two peaks (Fig.2A) and the specific fraction, eluted by the acetylcholine pulse, represents only 23% of the protein applied to the column. In this receptor peak 22 µg protein/g fresh tissue were estimated, which corresponds to a 4.3 fold purification over the preceding step and a total enrichment of the specific protein of about 4,500 fold (i.e. considering the total protein content to be about 100 mg/g protein) Upon rechromatography on Sephadex LH20 the non-specific fraction showed no binding for cholinergic drugs, while in the receptor peak the binding of $[^{14}C]$acetylcholine and of $[^{3}H]$atropine was demonstrated (Fig.4). Ochoa and

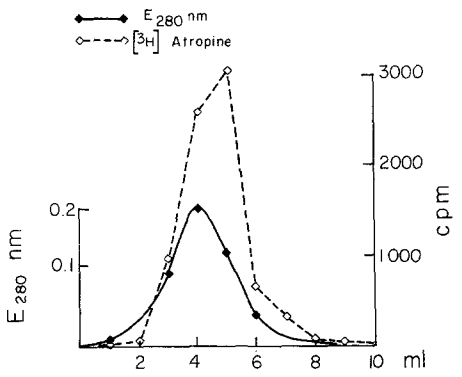

Fig. 4 Rechromatography on Sephadex LH20 of the muscarinic binding protein from intestinal smooth muscle (see Fig 2B), after 30 min. incubation with 1.07×10^{-8}M [^{3}H] atropine. The specific radioactivity reached at this low drug concentration was 0.25 nmoles/mg protein.

De Robertis (3) has previously shown that the muscarinic protein from smooth muscle does not bind [^{14}C] α-tubocurarine.

Present results demonstrate that the cholinergic affinity column, synthesized by Barrantes (10) is capable of separating both nicotinic and muscarinic proteins in organic solvents and is able to improve on the purification obtained by conventional chromatography in Sephadex LH20. Furthermore since acetylcholinesterase is not soluble in organic solvents (13), this technique only separates the cholinergic receptor protein fraction.

The use of a cholinergic affinity column, which works on the same general principles as those of affinity chromatography in aqueous solvents (11), confirms the value of the conventional technique based on Sephadex LH20 for the separation of receptor proteins and the study of the binding with specific ligands (4). Barrantes and Weber (11) have shown that the affinity column is able to remove entirely the cholinergic protein from the TLE of Electrophorus electroplax. In a work in progress, Saraceno and De Robertis (14) have observed that the cholinergic protein fraction from the TLE of the cerebral cortex is also completely removed by the affinity column and it is

only released by the acetylcholine pulse. These observations demonstrate that the cholinergic protein fractions were present in the TLE of Electrophorus and cerebral cortex. Even more conclusive is the finding shown here that the specific fractions isolated by Sephadex LH20 chromatography from skeletal and smooth intestinal muscle could be further purified by the affinity column.

The active fractions obtained by Sephadex LH20 chromatography (Figs.1A and 2A) were further separated into non-specific and receptor fractions; the latter being eluted after the acetylcholine pulse (Figs. 1B and 2B).

The use of the Sephadex LH20 technique under the same technical conditions, permitted to show that the non-specific fractions did not bind the cholinergic ligands, while the receptor fractions were able to bind the corresponding specific ligand, i.e. either nicotinic or muscarinic drugs. Furthermore, in these fractions the binding was carried out at drug concentrations small enough (10^{-8}M to 10^{-6}M) to minimize any kind of non-specific binding.

The methodologic approach used, in which two different methods of chromatography, produce additive results regarding the purification of the active protein, permit to discard completely the criticisms raised by Levinson and Keynes (7). We suppose that the drastic changes introduced by the authors in the Sephadex LH20 technique of De Robertis et al (4) were probably the cause of their artifacts. A complete analysis of the Sephadex LH20 technique, that confirms our findings, has been carried out by J.F. Donnellan, K.J. Cattel and coworkers, at the Shell laboratory in the United Kingdom, in their study of the hydrophobic cholinergic receptor protein of the fly head (Personal communication). Thus the value of this method for the separation of cholinergic receptor proteins and for the study of the binding with specific ligands is fully confirmed.

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