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Immunological and Binding Studies on Chloroform—Methanol Extracts from Electroplax of *Torpedo marmorata* and from Rat Brain Cortex. Comparison of the Material Solubilized by Organic Solvents with the Receptor Proteins Solubilized by Detergents

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We have investigated a chloroform – methanol extracted material from electric organ of *Torpedo marmorata* with respect to binding of [^{3}H]-acetyl-α-neurotoxin and [^{3}H]-acetylcholine. No binding was observed which is in contrast to observations with detergent extracted receptor. Further, no crossreactivity could be demonstrated between the chloroform – methanol extracted proteolipid and antibodies against the detergent extracted receptor. Labelling in the membrane with [^{3}H]-acetyl-α-neurotoxin before extraction with chloroform – methanol gave a very low yield of extracted labelled compound. The protein yield is about 0.3 % of the total in the homogenate and only 0.04 % of the specifically bound neurotoxin.

Using the alkylating affinity label [^{3}H]-*N*-propylbenzilylcholine mustard (*1*) and also [^{3}H]-atropine, which both bind, at low concentrations, specifically to the muscarinic acetylcholine receptor, binding was studied to the chloroform – methanol extracted proteolipid from rat brain cortex. No specific binding could, however, be demonstrated. Prelabelling in the membrane with *1* and subsequent extraction with chloroform – methanol gave a protein yield of about 1 % and an extraction of 26 % of the specifically bound label.

Extraction and subsequent chromatography in chloroform – methanol of acetylcholine receptors have been described.[1–4] In these experiments organic solvent extracts from either smooth muscle, electric organ, skeletal muscle or housefly head were equilibrated with labelled cholinergic ligands such as [^{3}H]-atropine, [^{14}C]-acetylcholine, [^{14}C]-tubocurarine and [^{3}H]-α-neurotoxin prior to loading onto a Sephadex LH-20 column. The finding that labelled protein peaks could be eluted from Sephadex LH-20 columns was taken as indication that cholinergic proteolipids had been isolated. This interpretation has been criticized; Levinson and Keynes [5] showed that the co-occurrence of ligands and proteins in the eluate may be a consequence of co-chromatography. They also showed that acetylcholine containing peaks can be created using receptor-free and protein-free extracts, which suggests that the association of ligand with these peaks is nonspecific.

Ligand binding studies with the assumed cholinergic eluate from Sephadex LH-20 columns have been carried out using a biphasic partition method.[6] Fractions from Sephadex LH-20 columns, resolubilized in Ringer solution [5] did, however, not show any binding to cholinergic ligands. This would indicate that organic extraction does not solubilize cholinergic receptors in an active form.

Other workers have solubilized the nicotinic acetylcholine receptor (nAChR) from the electric organ of *Torpedo* [7–9] or *Electrophorus* [10–12] by means of non-ionic detergents. The binding properties of the isolated receptors to neurotoxins and nicotinic ligands have been described from several laboratories.[11,13,14] Antibodies against nAChR have been raised [9,15,16] and their ability to demonstrate the occurrence of nAChR in various isolation steps has been shown.[26]

An alkylating affinity label for muscarinic acetylcholine receptors (mAChR) has been introduced,[17] which made it possible to label the receptor *in situ*, thus allowing the study of mAChR extraction and chromatography with less risk of artifactual co-chromatography.

By using antibodies against nAChR, labelling of nAChR with α-neurotoxin prior to membrane extraction in the presence and absence of other cholinergic ligands and covalent affinity labelling of mAChR, we have studied the applicability of chloroform–methanol extraction for the preparation of cholinergic receptors.

EXPERIMENTAL

Materials. All chemicals used were of analytical grade. [^{3}H]-acetylcholine chloride (250 mCi/mmol) and [^{3}H]-atropine (500 mCi/mmol) were purchased from Radiochemical Centre Amersham, U.K. Acetyl-α-neurotoxin from *Naja naja siamensis* was prepared and subsequently [^{3}H]-labelled as previously described.[18] *1* (28 Ci/mmol; 2.9 nM) was activated according to Burgen *et al.*[17] This substance was a gift from Dr R. Squires, Ferrosan AB, Copenhagen. Antibodies against the nicotinic acetylcholine receptor were raised in rabbits as described by Heilbronn and Mattsson.[9]

Extraction of the nicotinic acetylcholine receptor (nAChR). An electroplax membrane homogenate (30 % w/v) from *Torpedo marmorata* in physiological solution at pH 7.3, containing 0.02 % sodium azide, was incubated with 10^{-5} M [^{3}H]-acetyl-α-neurotoxin (2.7 μg toxin/g electroplax) in the presence and absence of 10^{-2} M carbamylcholine or 10^{-3} M tubocurarine. Labelling with the toxin was carried out for 10 min at room temperature. The homogenate was then cooled to 4 °C and centrifuged at 100 000 *g* for 1 h. The resulting pellet was freeze-dried for about 20 h. In a preliminary experiment the pellet was washed with buffer to remove free toxin. The supernatant was shown to contain only about 1 % of the total radioactivity, and this step was subsequently excluded.

Extraction in hydrophobic media and all the following steps were carried out at 4 °C as described.[2] Prelabelled lyophilized electric tissue (0.2–1.0 g) was extracted with 10–20 ml of chloroform–methanol (2:1) for 30 min. The extract was centrifuged for 5 min at 3000 rpm. The supernatant was assayed for radioactivity and protein. Chloroform–methanol extracted unlabelled material was incubated with [^{3}H]-acetyl-α-neurotoxin for 15 min at 4 °C and subsequently treated according to La Torre.[2] In parallel experiments unlabelled material was extracted and the supernatant was loaded onto a Sephadex LH-20 column (1 × 20 cm). Fractions of 5 ml were collected and assayed for protein, binding of [^{3}H]-acetyl-α-neurotoxin and [^{3}H]-AcCh and crossreactivity towards nAChR antibodies.

Protein was determined in all extracts and in the eluted fractions after evaporation of the organic solvent under N_2-atmosphere and resolving the residue in 0.1 M phosphate buffer pH 7.4, or in Ringer solution with or in some experiments without 1 % Triton X-100 present. The protein was measured according to Kalckar[19] and Lowry.[20]

Binding studies of [^{3}H]-acetyl-α-neurotoxin were carried out using the Sartorius membrane filter assay[9] while binding of [^{3}H]-AcCh was measured by equilibrium dialysis (20 h, 25 °C) in Krebs-Ringer solution, pH 7.5, containing 1 % Triton X-100 in a multiple cell apparatus.[14] The AcCh concentrations varied between 10^{-6} M and 10^{-3} M. Rocket immunoelectrophoresis was carried out according to Weeke.[21]

Extraction of the muscarinic acetylcholine receptor (mAChR). Rat brain homogenate (10 % w/v) was incubated with the [^{3}H]-*N*-propylbenzilylcholine mustard in Ringer solution for 15 min at room temperature in the absence and presence of 10^{-5} M atropine. Labelling with *1* was stopped by addition of sodium thiosulfate (0.5 mM) to the incubation mixture, which was then centrifuged at 100 000 *g* for 1 h. The resulting pellet was washed with Ringer solution twice, until less than 0.5 % of the total radioactivity was found in the supernatant. The washed pellet was freeze-dried overnight prior to extraction with 2 × 10 ml chloroform–methanol (2:1) for 30 min at 4 °C. The extracts were pooled, aliquots were taken for protein and radioactivity assays and the rest was applied to a Sephadex LH-20 column prepared and eluted according to La Torre.[2] In another experiment rat brain homogenate (10 % w/v in sucrose) was freeze-dried without labelling with *1*. The freeze-dried material was extracted with 2 × 10 ml chloroform–methanol (2:1) and chromatographed on a Sephadex LH-20 column as earlier. The eluted protein fractions were subjected to equilibrium dialysis against 10^{-8} and 10^{-7} M [^{3}H]-atropine in Ringer solution for 36 h at 4 °C. Control of nonspecific binding was carried out in the presence of "cold" atropine 10^{-5} M.

RESULTS AND DISCUSSION

Binding of α-neurotoxin and AcCh to organic extract of electric organ from Torpedo marmorata. The chloroform–methanol extract from unlabelled *Torpedo marmorata* electric organ homogenate was incubated with [^{3}H]-acetyl-α-neurotoxin for 15 min at 4 °C. The extract was then loaded on a Sephadex LH-20 column. The elution pattern in Fig. 1 is the same as de-

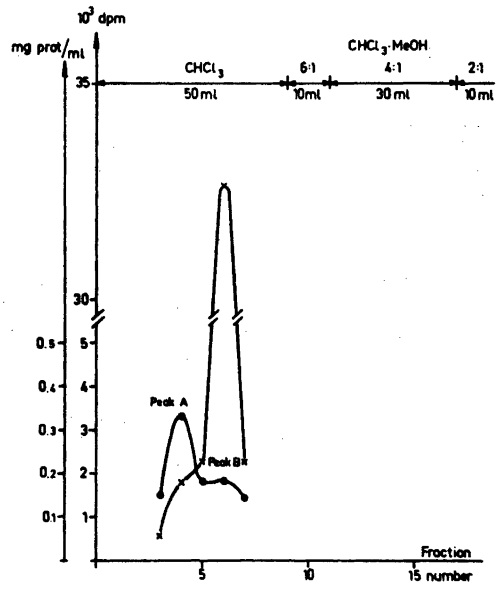

Fig. 1. Elution profile from Sephadex LH-20 column with toxin labelled chloroform–methanol extract from electric organ of *Torpedo marmorata*. ×, radioactivity; ●, protein concentration.

scribed earlier [2] and reproduced by us [24] and others.[3,5] The radioactivity is found in peak B.

In the presence of carbamylcholine chloride binding of [^{3}H[-acetyl-α-neurotoxin to the homogenate is inhibited to about 50 % (Table 1). About 50 % of the total binding may thus represent the specific toxin binding (0.1 nmol/mg of protein) which is in good agreement with

Table 1. The effect of chloroform–methanol extraction on the nicotinic receptor labelled by [^{3}H]-acetyl-α-neurotoxin. Specifically bound: 5.95 nmol (P^a) and 0.001 nmol (S^b).

	Protein		Binding of [^{3}H]-acetyl-α-neurotoxin		
	mg	%	nmol/mg	nmol	%
In the absence of carbamylcholine					
P	75	100	0.15	11.2	100
S	0.2	0.3	0.024	0.005	0.04
In the presence of 10^{-2} M carbamylcholine					
P	75	100	0.07	5.25	46
S	0.2	0.3	0.02	0.004	0.04

[a] Pellet after centrifugation of labelled homogenate. [b] Supernatant after centrifugation of chloroform-methanol extract.

results from other laboratories.[22,23] (Possible desensitation due to carbamylcholine is not discussed here.) As seen in Table 1 the protein yield after extraction with chloroform–methanol (2:1) was about 0.3 % of the total protein found in the original homogenate and only 0.04 % of the specifically bound neurotoxin. When the extract is chromatographed on Sephadex LH-20 an elution pattern similar to that shown in Fig. 1 is obtained, but the specific radioactivity of peak B is much lower than in the experiment with labelling the chloroform–methanol extract.

A reason for this discrepancy in the specific activities may be that the toxin-receptor com-

Table 2. Comparison between chloroform–methanol extracts and Triton X-100 solubilized nAChR.

Fraction	Protein mg/ml	mg (total)	AChE-activity μmol/min mg	[^{3}H]-acetyl-α-neurotoxin-binding nmol/mg	[^{3}H]-ACh-binding nmol/mg
Peak A					
In 0.1 M phosphate buffer containing 1 % Triton X-100	0.95	2.4	0	0	0
In 0.1 M phosphate buffer	3.24	9.7	–	0	–
Peak B					
In 0.1 M phosphate buffer containing 1 % Triton X-100	0.15	0.52	0	0	0
In 0.1 M phosphate buffer	0.28	0.84	–	0	–
Triton X-100 solubilized nAChR	6.8	102	0.15	0.03	0.04

Table 3.[a] Extraction of bound [^{3}H]-*N*-propylbenzilylcholine mustard by chloroform–methanol.

	In the absence of atropine		In the presence of 10^{-5} M atropine	
	Bound pmol	Protein mg	Bound pmol	Protein mg
Total *1* added	101	444	101	444
Bound *1*	61.4 ± 2.8	444	48.9 ± 4	444
Bound after washing the pellet twice	54.9 ± 3.1	340	40.1 ± 2.2	344
Extracted with 2 × 10 ml chloroform–methanol (2:1) from the freeze-dried pellet	9.5 ± 1.2	4.8	5.6 ± 1.7	5.3

[a] Data from three experiments.

plex is not as readily solubilized as the unlabelled receptor (*cf.* Fig. 1) or that the receptor and the toxin have been separated from each other during purification. It is also possible that the toxin binds to other components than nAChR.

In order to see if the high specific radioactivity of peak B (Fig. 1) is due to a receptor-toxin complex, the binding capacity of unlabelled chloroform–methanol extracted and chromatographed membrane material was examined. Unlabelled material corresponding to peaks A and B was assayed for protein, for binding of [^{3}H]-acetyl-α-neurotoxin and [^{3}H]-acetylcholine and for crossreactivity with antibodies against nAChR. Chloroform–methanol fractions were evaporated and resolved in 0.1 M phosphate buffer, containing 1 % Triton X-100 (see Experimentals). This treatment may have removed lipids from a cholinergic proteolipid present but seems justified as it is known that cholinergic protein isolated in aqueous solvent containing nonionic detergents binds cholinergic ligands and also that it reacts with antibodies. Table 2 shows that no binding of toxin or AcCh occurs to peak A or peak B. As previously described by us [24] rocket immunoelectrophoresis of material from the two different peaks without and with Triton X-100 phosphate buffer gave no crossreaction with antibodies against nAChR prepared as described.[9] Barrantes *et al.*[25] confirmed this lack of crossreactivity with anti-nAChR on detergent solubilized proteolipids from *Electrophorus*. It appears that no proteins could be isolated by chloroform–methanol, which either by toxin binding or immunological studies could be identified as active or recognizable nicotinic acetylcholine receptor molecules.

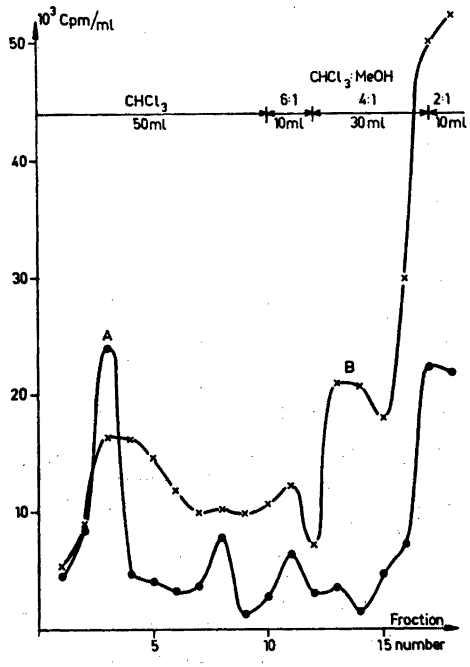

Fig. 2. Chromatography on Sephadex LH-20 column with chloroform–methanol extract from rat brain. The homogenate was labelled with [^{3}H]-*N*-propylbenzilylcholine mustard in the absence and presence of 10^{-5} M atropine before extraction. ×, radioactivity in the presence of 10^{-5} M atropine; ●, radioactivity with no atropine present.

Binding of atropine and 1 *to organic extract of rat brain.* Table 3 shows that a specific, by 10^{-5} M atropine inhibitable binding of *1* to rat brain homogenate occurs and that extraction of the freeze-dried pellet leads to release of 14–18 % of *1* bound in the absence and presence of atropine. The protein yield after ex-

traction with chloroform–methanol (2:1) was about 1 % of the total protein found in the original homogenate. Nonspecific binding of *1* represents a much higher portion of the bound label than the portion which is released by extraction. Thus it is difficult to conclude whether the released *1* represents molecules which were bound to specific or nonspecific sites. An additional problem is the slow release of *1* from binding sites. Fig. 2 shows the elution profile of a Sephadex LH-20 column loaded with the organic extracts of labelled rat brain homogenates. It can be seen that the elution profile is dependent on whether the labelling with *1* was carried out in the absence or presence of atropine. A "specific" labelling is observed in peak A. The chromatogram of the extract labelled in the presence of atropine shows a peak B of radioactivity in the chloroform–methanol (4:1) fraction as well. This peak is missing from the corresponding curve, suggesting that in the presence of atropine the label is bound to positions of different hydrophobicity than those to which the label is bound in the absence of atropine. The last raise of radioactivity in the elution profile probably represents the free ligand.

In yet another series of experiments labelling with *1* and examination of the atropine binding capacity was undertaken after extraction of freeze-dried rat brain homogenate with chloroform–methanol (2:1). No specific labelling by *1* or specific binding of atropine could be detected in the extract. It should be noted that chloroform–methanol "binds" [^{3}H]-atropine when assayed with equilibrium dialysis technique. However, after correction for this "binding" no specific binding of this ligand is observed. Thus, chloroform–methanol extraction does not yield muscarinic receptor molecules which are capable of binding antagonists. The very limited release of covalently labelled material is of limited value as no active component could be isolated by this extraction method.

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