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Solubilization of cholinergic binding fractions from lobster axon membrane fragments

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During the past few years, our laboratory has been investigating the cholinergic binding properties of axonal membrane fragments from the walking leg nerves of the lobster, *Homarus americanus* [1, 2], a preparation first introduced by Denburg and O'Brien [3]. These studies have identified membrane-associated binding sites capable of interacting with [^{125}I]-α-bungarotoxin (BgTx), consistent with the requisites for a nicotinic cholinergic receptor, as well as sites capable of saturable, high-affinity binding of [^{3}H]quinuclidinyl benzilate (QNB), a highly specific muscarinic ligand. Purification and characterization of these axonal receptors require solubilization of the proteins, while retaining the native, functional form, into an environment suitable for affinity gel chromatography and other separation techniques. The present experiments were designed to survey the efficacy of a wide range of possible solubilizing conditions including salt extraction, ionic and nonionic detergents, sonication, and extraction with organic solvents [4].

MATERIALS AND METHODS

Axonal membrane fragments were prepared by the procedure of Denburg [5] and resuspended to a final protein concentration of 1–2 mg/ml in 10 mM Tris buffer, pH 7.8. The protein was incubated with a 3-fold weight excess or 1% concentration of a potential solubilizing agent for 1 hr at 5°. All studies employed initially the same criterion for solubilization, that is, protein not deposited upon centrifuging at 100,000 *g* for 60 min. Agents that solubilized a significant fraction of membrane proteins usually also caused a visually evident clearing of the microsomal suspension. It should be noted that solubilization does not exclude the possible presence of membrane micelles in the supernatant fraction.

The final supernatant fraction of solubilized proteins was dialyzed overnight against 500 ml Tris buffer at 5° to remove as much excess detergent as possible prior to determining the binding capacity of these proteins for cholinergic ligands. The binding assays utilized in this study have been described in detail elsewhere [2]. Earlier studies on the binding of cholinergic ligands to axon plasma membrane fragments yielded apparent dissociation constants (by Scatchard analysis) of 3.2×10^{-7} M for [^{3}H]nicotine [1], 1.1×10^{-7} M for [^{125}I]-α-BgTx [2], and 1.12×10^{-9} M for [^{3}H]QNB [2]. Binding to solubilized protein, therefore, was assayed at a single ligand concentration approximately twice the $K_{d_{app}}$, and binding was expressed (Table 1) in values relative to the binding capacity of untreated membrane proteins at the same ligand concentration, i.e. as per cent of nonsolubilized control.

For sonication, the membrane fragment suspension was kept at 5° in a salt–ice bath and exposed for 15–90 sec periods to sonication with a Branson probe-type sonifier.

The procedure of Sigrist *et al.* [6] was used to extract lipoproteins from the membrane fragments, with n-butanol as organic solvent in a single phase system and ether precipitation of the lipoprotein fraction. Liposomes were prepared by injecting the butanol extract into aqueous buffer solution (5 mM Tris, pH 7.8), as described by Batzri and Korn [7].

[^{3}H]Nicotine (sp. act. 250 mCi/m-mole) and [^{3}H]quinuclidinyl benzilate (sp. act. 16.4 Ci/m-mole) were purchased from Amersham Searle. [^{125}I]-α-bungarotoxin was a generous gift of Dr. Hai-Won Chang, Columbia University. Brij 36T was purchased from Emulsion Engineering, Elk Grove Village, IL. Lubrol WX was purchased several years ago from the Sigma Chemical Co. St. Louis, MO. Renex 30 was a gift of Imperial Chemical Industries, Wilmington, DE.

RESULTS AND DISCUSSION

The results of this study are summarized in Table 1. It is evident there is a wide variation of efficacy of solubilizing techniques with respect to the relative binding of nicotinic (nicotine and BgTx) and muscarinic (QNB) ligands. This supports the hypothesis of different binding sites, for these

Table 1. Solubilization of axon plasma membrane proteins—Relative binding capacity for cholinergic ligands*

Procedure	%Yield	Binding (%Nonsolubilized control) [^{3}H]NIC (1 μM)	[^{125}I]-α-BgTx (0.2 μM)	[^{3}H]QNB (2 nM)
Salt extraction				
2 M NaCl	24	59	89	85
Ionic detergents (1%)				
Sodium lauryl sulfate	100	0	0	ND+
Digitonin	54	100	49	70
Lysolecithin	90	100	83	188
Nonionic detergents (1%)				
Brij 36T	86	14	9	60
Lubrol WX	77	26	64	61
Triton X-100	83	33	57	60
Tween 80	6	292	311	721
Renex 30	48	52	26	161
Sonication (5°, 15–90 sec)	100	0	0	0
Butanol extraction	10	ND	0	0
Liposomes	10	ND	20	100

* Abbreviations: NIC, = nicotine; BgTx = bungarotoxin; and QNB, = quinuclidinyl benzilate.

+ No data.

ligands in axonal membranes [2]. It may, in fact, be possible to utilize the nonionic detergent to separate the two types of binding proteins. Brij 36T, a reagent used by Simon *et al.* [8] to solubilize the brain opiate receptor, and Renex 30, a compound similar to Triton X-100 but lacking the oxidizing contaminants of Triton [9], seem particularly promising for this purpose.

Although the temperature was carefully maintained at 5° and the duration of sonication was very brief, this technique failed to yield any functional membrane proteins in the 100,000 *g* supernatant fractions. Because it was observed in other studies that high energy sonication caused oxidation and degradation of membrane phospholipids [10, 11], these results may provide evidence for an essential role of phospholipids in the cholinergic binding properties of axonal membranes. Aronstam *et al.* [12] have shown that addition of phospholipids to rat brain muscarinic receptors markedly enhanced the binding of QNB, while phospholipases A and C inhibited QNB binding. Phospholipids have been shown to be important for BgTx binding activity in the post-synaptic cholinergic receptor of *Torpedo californica* [13] and to enhance stereospecific opiate binding to a rat brain synaptic membrane fraction [14]. In both of these systems, ligand interaction was reduced by phospholipase A_2. As the axon plasma membrane of lobster leg nerve has a high lipid content (66–75 per cent by weight) with a large predominance of cholesterol and phospholipids [15], compared to 30–33 per cent lipid content in *Torpedo* post-synaptic membranes [16, 17], studies have been undertaken to investigate the interaction of phospholipids with axonal cholinergic binding proteins. These experiments have shown that BgTx binding can be blocked by treatment with either phospholipase A_2 or phospholipase C and that the addition of phosphatidyl serine partially restores BgTx binding activity (J. K. Marquis and H. G. Mautner, unpublished observations).

The plant glycoside, digitonin, has been used successfully to solubilize adrenergic binding sites from frog erythrocyte membranes where several nonionic detergents, including Triton X-100 and Lubrol WX, were ineffective [18]. Digitonin, however, was not as effective as lysolecithin in solubilizing axon membrane cholinergic binding proteins.

Tween 80 (Polyoxyethylene sorbitan, monooleate) produced a striking one-step purification and possible activation of the nicotinic and, even more markedly, of the muscarinic binding proteins. Further studies are in progress to characterize these fractions.

In view of recent studies by Christopher *et al.* [19] that demonstrate structural alterations and loss of [^{3}H]decamethonium binding activity in the electroplax cholinergic receptor on attempting to purify it after extraction with organic solvents, it is not surprising to find that no cholinergic binding activity is retained in the butanol-extracted axonal membrane proteins. Chloroform–methanol extracted material from the electric organ of *Torpedo marmorata* also exhibited no specific binding for either nicotinic or muscarinic ligands and demonstrated no crossreactivity with antibodies against the detergent extracted receptor [20].

Solubilization of membrane proteins with retention of biological activity is one of the major obstacles in isolating and purifying these macromolecules. The solubilized species obtained from axon membrane fragments are clearly hydrophobic, 'intrinsic' membrane proteins [21] which can be removed from the membrane by emulsifying agents such as the detergents employed here that effectively incorporate the membrane proteins into an artificial, hydrophobic, lipid environment. It is encouraging to find, however, that a small but active fraction of the membrane proteins can be extracted into an aqueous environment using 2 M NaCl. Carson *et al.* [22] reported solubilization with 2 M NaCl of an atropine-binding material from ox cerebral cortex which has properties similar to those found for the muscarinic receptor. Kalderon and Silman [23] have purified and characterized a water-soluble acetylcholine receptor obtained by tryptic digestion, without use of detergents, from electrogenic tissue of *T. californica*. Although the yield is low in the present studies with axonal membrane proteins, the water-soluble species should lend itself to a wide variety of purification techniques.

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