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SOLUBILIZATION AND PROPERTIES OF THE SOLUBLE AXONAL CHOLINERGIC BINDING MACROMOLECULE*

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SUMMARY

Lysolecithin has been used to solubilize the axon plasma membrane preparation from lobster walking legs. This was accomplished with complete recovery of activity of the axonal cholinergic binding macromolecule and retention of the basic properties of the membrane-bound macromolecule. Sedimentation of the soluble protein through a sucrose gradient containing [^{3}H]nicotine enabled the separation of the axonal cholinergic binding macromolecule from acetylcholinesterase and demonstrated the apparent dissociation of the axonal cholinergic binding macromolecule in low ionic strength solutions.

INTRODUCTION

The existence of an axonal cholinergic binding macromolecule in an axon plasma membrane preparation from the walking leg nerves of the lobster *Homarus americanus* was previously described[1]. The loss of [^{3}H]nicotine binding activity by treatment with phospholipase A was taken as partial evidence that this macromolecule is a phospholipoprotein[1]. The possibility that this inactivation by phospholipase A was caused by the production of lysolecithin was examined. However, it was observed that low concentrations of lysolecithin solubilized the axon plasma membrane preparation while maintaining all of the binding activity. This phenomena was studied in more detail and the results are presented here along with a characterization of the soluble axonal cholinergic binding macromolecule.

MATERIALS AND METHODS

The axon plasma membrane fraction was isolated by differential centrifugation from a hypotonic homogenate of the main sensory–motor nerve bundle from the walking legs of 1.5-lb lobsters, *Homarus americanus*. Details on the preparation and its characteristics have previously been described[2]. Axonal cholinergic binding activity was measured as binding of [^{3}H]nicotine (specific activity 355 Ci/mole) by equilibrium

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dialysis. Nicotine is the reversible cholinergic ligand with the highest affinity toward the axonal cholinergic binding macromolecule[1].

The washed axon plasma membrane fraction was suspended in 0.05 M Tris, pH 7.8. Lysolecithin was added to 0.7-ml aliquots of this suspension and the sample was kept on ice for 45 min with occasional shaking. It was then centrifuged in an SW 50L rotor with small tube adaptors at $100000 \times g$ for 1 h at 4 °C. The supernatant was removed with a Pasteur pipet and the pellet was resuspended in 0.7 ml 0.05 Tris, pH 7.8. Both fractions were assayed for protein[3], acetylcholinesterase[4], and axonal cholinergic binding macromolecule[1].

RESULTS AND DISCUSSION

The axon plasma membrane preparation was treated according to the solubilization procedure with different initial concentrations of membrane proteins and of lysolecithin so that a wide range of ratios of lysolecithin/protein (mg/mg) was obtained and so that at least two points with different protein concentrations were obtained for each ratio. The results of these experiments are summarized in Fig. 1, where the percent of the total activity or protein found in the supernatant varied with the ratio of lysolecithin/protein. The remainder of the activities was found in the pellet. The total recovery of protein and axonal cholinergic binding macromolecule was essentially 100%, while there was an enhancement of total acetylcholinesterase. This enhancement varied with different membrane preparations from 1.3–2.0-fold at a ratio of lysolecithin/protein of 3. It is observed that lysolecithin produces no differential solubilization of axonal cholinergic binding macromolecule or acetylcholinesterase. Instead, it apparently disrupts the entire membrane structure releasing all of the protein to an identical extent. In addition, it is the ratio of lysolecithin/protein that appears to determine the extent of solubilization, with maximal release occurring at ratios greater than 3. In this manner lysolecithin interacts with the axon plasma membrane preparation in a way similar to which other detergents solubilize membrane-bound enzymes[5–8]. It is interesting that this ratio of detergent/protein of 3 has been

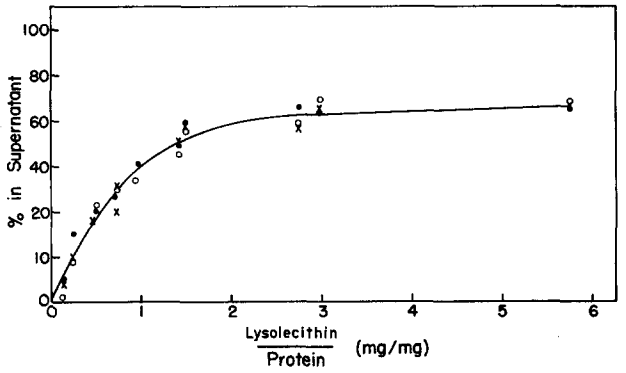

Fig. 1. Solubilization of protein (○—○), acetylcholinesterase (×—×), and axonal cholinergic binding macromolecule (●—●) by different ratios of lysolecithin/protein. The initial protein concentration was varied from 0.5–2.0 mg/ml and the lysolecithin concentration was varied from 0.2–3 mg/ml. The amount of activity in the supernatant is represented as the percent of the total recovered activity, the remainder being in the pellet.

previously observed to be optimal with several different detergents and membranes[5–7]. Perhaps this is indicative of the nature of the interaction between detergents and membranes.

The ability of other detergents to solubilize the axonal cholinergic binding macromolecule was tested. Triton X-100, sodium dodecyl sulfate, cholate, and deoxycholate all caused loss of axonal cholinergic binding macromolecule activity at concentrations which removed it from the pellet so that no more than 10% of activity was found in the supernatant. These detergents successfully dissolved the membrane protein and in most cases maintained the activity of the acetylcholinesterase. Only Lubrol solubilized active axonal cholinergic binding macromolecule but not in as good a yield as did lysolecithin.

Some of the binding properties of the soluble axonal cholinergic binding macromolecule were compared with those of the membrane-bound macromolecule. The binding of [^{3}H]nicotine was measured over the concentration range of $1 \cdot 10^{-8}$–$2 \cdot 10^{-6}$ M. From these data a complete binding curve was generated in which only a single class of binding site was found with a $K_D = 1.2 \cdot 10^{-6} \pm 0.1 \cdot 10^{-6}$ M. Nicotine bound to the maximal extent of 240 ± 30 pmoles/mg protein. For the membrane-bound form we have reported $K_D = 4.2 \cdot 10^{-7} \pm 0.4 \cdot 10^{-7}$ M and a maximum binding of 245 ± 40 pmoles/mg protein[1]. The binding of nicotine to the soluble axonal cholinergic binding macromolecule was inhibited by cholinergic agonists and antagonists in a similar way to the membrane-bound macromolecule[1]: curare, atropine, acetylcholine, and procaine at 10^{-5} M each inhibited the binding of 10^{-7} M nicotine to the extent of 85, 60, 20 and 60%, respectively. It was previously shown that acetylcholine and procaine were competitive inhibitors of nicotine binding to the membrane-bound axonal cholinergic binding macromolecule[1].

The membrane protein solubilized by lysolecithin remains in solution after the lysolecithin has been removed by dialysis. This property is particularly useful for further purification of the soluble axonal cholinergic binding macromolecule. Fractionation of the solubilized axon plasma membrane preparation was accomplished by sedimentation in a sucrose gradient. In order to detect axonal cholinergic binding macromolecule activity all the solutions used to make the gradient and to solubilize the membrane contained 10^{-7} M [^{3}H]nicotine. Therefore, wherever axonal cholinergic binding macromolecule activity was present a peak of radioactivity was observed because of the binding, while in the fractions containing the solution of the applied sample there was a decrease in radioactivity. This approach is analogous to the gel filtration experiments run in the presence of ligand and used to determine binding constants of soluble enzymes[9]. It is also simpler than taking each fraction from the gradient and assaying for binding by equilibrium dialysis and perhaps most important it enables the detection of small amounts of reversible binding of ligands so that the use of essentially irreversible toxins and affinity labels is not required.

In a typical experiment 2.5 mg solubilized protein in 0.3 ml of a 2% lysolecithin, 0.05 M Tris, pH 7.8, solution was layered on top of a 5-ml 5–20% sucrose gradient containing 0.2% lysolecithin throughout. The tubes were centrifuged in an SW-50L rotor at 35000 rev./min (100000 × g) for 16 h at 4 °C. Fractions of 10 drops (about 0.16 ml) were collected from the bottom of the punctured tube, and 0.1 ml aliquots were counted for radioactivity and 5 μl were used for acetylcholinesterase assay. Some results of these experiments are shown in Fig. 2. It was observed that when the sucrose

gradient contained lobster Ringer's (457 mM NaCl, 15 mM KCl, 25 mM $CaCl_2 \cdot 2H_2O$, 4 mM $MgCl_2 \cdot 6H_2O$, 4 mM $MgSO_4 \cdot 3H_2O$, 10 mM Tris, pH 7.8) a single, symmetrical peak of axonal cholinergic binding macromolecule activity was obtained with a

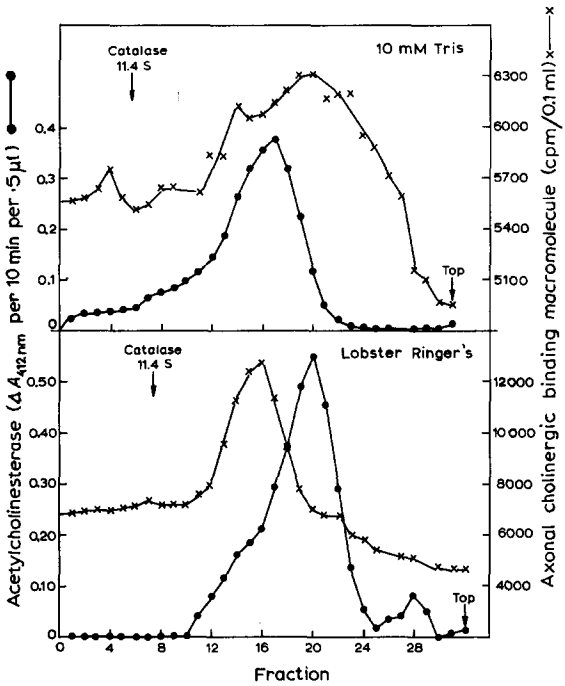

Fig. 2. Distribution of axonal cholinergic binding macromolecule (× — ×) and acetylcholinesterase (○—○) after sedimentation in a 5–20% sucrose gradient. The centrifugation was done at 100000 × *g* for 16 h at 4 °C. The run in the lower graph was done with lobster Ringer's present in all solutions while that in the upper graph had only 10 mM Tris. [^{3}H]Nicotine at 10^{-7} M and 0.2% lysolecithin were present throughout the gradient.

sedimentation coefficient of 7.8 S, and an unsymmetrical peak of acetylcholinesterase activity with a sedimentation coefficient of 5.8 S. When the sedimentation was done in the absence of salts, with only 10 mM Tris, pH 7.8, present, the nicotine binding was reduced and the axonal cholinergic binding macromolecule activity was in two broad peaks with sedimentation coefficients of 7.9 S and 5.2 S. The acetylcholinesterase was again in an unsymmetrical peak but with a sedimentation coefficient of 6 S. The sedimentation coefficients calculated are all relative to the standard of 11.4 S for catalase[10], which is assumed to have the same value in the 0.2% lysolecithin as in water. Although the changes in the sedimentation coefficient of axonal cholinergic binding macromolecule may be interpreted to reflect a dissociation phenomenon it is also possible that a salt dependent conformational change may significantly alter the shape of some of the axonal cholinergic binding macromolecule such that the rate of sedimentation is correspondingly changed. In order to explain the effects of salts on the binding of nicotine, it was postulated that a conformational change was induced in axonal cholinergic binding macromolecule upon binding of some inorganic

cations[11]. Such ion-induced conformational changes in axonal macromolecules are an integral part of several theories of axonal conduction[12,13].

These sedimentation experiments clearly demonstrate the physical separation of the axonal cholinergic binding macromolecule and acetylcholinesterase. Previous evidence for these two macromolecules not being identical relied on relatively indirect results such as different affinities for ligands and different stabilities[1].

Judging from the value of sedimentation coefficient, the lobster axonal acetylcholinesterase appears to be smaller than acetylcholinesterases from other sources which have been similarly characterized[14,15]. However, this lobster axonal enzyme has other properties which differ from those of other acetylcholinesterases; namely its resistance to inactivation by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and the ability of the local anaesthetic procaine to be a competitive inhibitor with respect to acetylcholine[1,16]. Similarly the axonal cholinergic binding macromolecule is apparently smaller than the acetylcholine receptor (9.5 S) isolated from *Torpedo* electroplax[17]. These apparent size differences do not necessarily imply polypeptides of different molecular weights. The major determinant in the rate of sedimentation may be the amount of phospholipid bound to the soluble protein. This is particularly true for the axonal cholinergic binding macromolecule since the phospholipid has been shown to be an absolute requirement for binding of nicotine[1]. A change in the amount of phospholipid bound to the axonal cholinergic binding macromolecule may also be responsible for its apparent dissociation in the presence of low ionic strength solutions.

In conclusion, lysolecithin which has earlier been reported to solubilize neural tissue[18,19] was here used to solubilize the axon plasma membrane preparation from lobster walking leg nerves. This was accomplished with nearly complete recovery of axonal cholinergic binding macromolecule activity and retention of the basic properties of the membrane-bound material. Sedimentation of the solubilized protein through a sucrose gradient has enabled the separation of the axonal cholinergic binding macromolecule from acetylcholinesterase and the demonstration of the apparent dissociation of the axonal cholinergic binding macromolecule in low ionic strength solutions.

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