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Purification of Acetylcholine-Receptor Enriched Membranes by Use of Affinity Partitioning

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Membrane fragments rich in acetylcholine receptor from the electric organ of *Torpedo californica* were purified by a combination of classical methods and affinity partitioning, a method analogous to affinity chromatography. Affinity partitioning is based upon the phase partition method where two water-rich liquid phases are formed upon the addition of sufficient quantities of water soluble polymers such as poly(ethylene oxide) and dextran. A phase system in which less than 2% of acetylcholinesterase, adenosine triphosphatase and α-toxin binding activities distribute into the poly(ethylene oxide) rich phase was chosen. By adding cholinergic derivatives of poly(ethylene oxide), selective changes in the distribution of membrane fragments rich in acetylcholine receptor into the poly(ethylene oxide) rich were achieved. Sodium dodecyl sulfate polyacrylamide gel electrophoresis as well as assay of cobra α-toxin binding, acetylcholinesterase and adenosinetriphosphatase activities indicate that substantial purification of membrane-bound acetylcholine receptor was achieved.

Purification of neurotransmitter receptors has been considered to be an important first step towards the characterization of their molecular properties. Thus far, the most successful example is the nicotinic receptor which, after solubilization, has been purified to a high degree by affinity chromatography[1–4]. Fortuitously, the binding properties of the nicotinic acetylcholine receptor remain intact upon solubilization in the presence of non-ionic detergents. Because of this, it has been possible to correlate binding of a number of cholinergic agents with their known pharmacological effects. However, in the case of the muscarinic receptor, the binding properties are substantially altered by the use of non-ionic detergents[5]. Also, it is possible that solubilization may uncouple the interactions of binding sites with other receptor functions, such as guanyl cyclase.

Many of these problems may be alleviated by the direct purification of membrane fragments rich in neurotransmitter receptor formed upon homogenization of receptor-rich tissue. Classical approaches to the purification of receptor enriched membranes have been based upon differences in physical properties between them and other membrane fractions[6,7]. Most procedures employ a combination of rate and density gradient centrifugation. If the source tissue is rich in a specific membrane component containing high den-

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sities of receptor, then substantial purification may be achieved[8,9]. When the source of tissue is as complex as mammalian brain, it is unreasonable to expect that the simple application of centrifugation will allow complete resolution of membrane fragments rich in a particular neurotransmitter receptor[10].

An attractive alternative to classical methods is the use of affinity chromatography to purify membrane fragments. Although this approach should, in principle, yield direct purification in a single step, there have been few reports of this approach in the purification of neurotransmitter receptor enriched membranes. Many neurotransmitters have ionic or hydrophobic character. Thus, potential difficulties may arise due to non-specific sorption propertities of neurotransmitter congeners employed in affinity chromatography.

Several important neurotransmitter antagonists such as the alkaloid, atropine[11], appear to bind both high and low affinity sites in rat brain. It is desirable to purify only those membrane fractions rich in high affinity binding sites. However, this may be difficult to achieve in practice because the attachment of affinity ligands to a solid matrix may produce a heterogeneous population with a complex distribution of binding properties. In fact, in several applications of affinity chromatography to the purification of soluble proteins with specific binding sites, the binding capacity of the affinity matrix is substantially less than the amount of ligand covalently attached to the affinity matrix[12,13]. This indicates that a substantial fraction of ligand molecules are sterically unavailable for soluble protein binding. Hence, it is difficult to ascertain the effective ligand concentration in the affinity matrices. In view of this, it is difficult to ascribe the binding of membrane fragments to affinity matrices as being due to either the interaction with high or low affinity receptor sites. Since the apparent low affinity binding sites may be due to non-specific interactions, binding to affinity columns may not be true affinity chromatography. In order to establish that true affinity chromatography is obtained, it is important to demonstrate that specific elution can be achieved with concentrations of competitors consistent with their known dissociation constants. In some applications of affinity chromatography to the purification of membrane fragments or intact cells containing specific binding sites, membrane fractions are eluted over a wide range of concentrations or may not be achieved even at extremely high levels of specific competitor[14,15]. Thus, it is difficult to interpret such results in terms of competitor binding to discrete sites.

In an attempt to overcome these difficulties we have developed an alternative method called affinity partitioning. Affinity partitioning is an extension of the phase partition method developed by Albertsson and co-workers[16,18]. In the phase partition method purification is achieved by extraction between phases formed by mixing aqueous solutions of two or more polymers. In the most commonly employed method, dextran of 500 000 molecular weight and poly(ethylene oxide) of 6 000 molecular weight are added to water or to an aqueous buffer solution. Two phases, both rich in water, are formed. Each of the phases is rich in one of the polymers; the top in poly(ethylene oxide) and the bottom is dextran rich. This and similar phase systems have proved useful in the purification of a number of enzymes and cell organelles. If components of the mixture to be purified differ in distribution, then they may be separated by several extractions or by use of countercurrent distri-

bution. Difference in distribution may be due to a variety of physical and chemical differences but primarily upon the net electrical charge[16]. This technique has, with phase systems containing isotonic buffer, been applied to the separation of intact cells which remain metabolically active after extraction in these systems[18]. In our affinity partitioning method, ligands are attached to one of the polymers used in the phase system, in this case, the linear polymer, poly(ethylene oxide). Since poly(ethylene oxide) contains two hydroxyl groups per molecule, it is possible to synthesize well defined reagents which should have homogeneous binding properties.

Upon adding poly(ethylene oxide) derivatives containing specific ligands to a phase system, we have shown that the partition coefficients of soluble proteins that bind the specific ligand are changed towards the poly(ethylene oxide) rich phase. These effects were antagonized by the addition of small molecular weight ligands that partition almost equally between the phases[19]. Furthermore, we were able to show that the quantitative behavior of such systems could be predicted on the basis of thermodynamic considerations.

As a first application of this method to membrane fragments containing specific receptors, we chose to study membranes from the electric organ of *Torpedo californica*. Since the post-synaptic membranes, which are rich in acetylcholine receptor, could be partially purified by sucrose density gradient centrifugation after high shear homogenization[8], it seemed likely that the receptor is present on membrane fragments distinct from those that are rich in acetylcholinesterase and Na^+, K^+ stimulated adenosinetriphosphatase. In addition, there have been reports that the density of acetylcholine receptor in this tissue is extremely high[20]. In view of these considerations, it was expected that this would be an ideal system for testing the usefulness of affinity partitioning for purifying membranes.

Derivatives of poly(ethylene oxide) containing ligands with cholinergic binding properties were synthesized from α, ω dibromoderivative of poly(ethylene oxide):

General Formula:

$$R\text{—}(CH_2\text{—}CH_2\text{—}O)_{156}\text{—}CH_2\text{—}CH_2\text{—}R$$

Derivative:	R:
Poly(EO)	—OH
MA-Poly(EO)	—N^+(H)(H)—H
TMA-Poly(EO)	—N^+(CH_3)(CH_3)—CH_3
PTMA-Poly(EO)	—NH—C_6H_4—N^+(CH_3)(CH_3)—CH_3

One of these derivatives, α, ω *bis*[trimethyl(4-aminophenyl)]poly(ethylene oxide) [PTMA-poly(EO)] is similar to a ligand which has been employed in affinity chromatography to purify solubilized acetylcholine receptor. In order to test for the possibility that any effects of these quaternary amino derivatives were simply due to electrostatic effects, we also synthesized an α, ω *bis* methylamino derivative of poly(ethylene oxide) [MA-poly(EO)], which, although positively charged at neutral pH, should have relatively low affinity for the acetylcholine receptor. At low ionic strength, we found all three charged poly(ethylene oxide) derivatives had a substantial effect on the partition of membranes rich in acetylcholine receptor. Upon increasing the ionic strength five-fold, we found that the effects of MA-poly(EO) were attenuated, whereas TMA-poly(EO) and PTMA-poly(EO) continued to have a potent effect on the partition of acetylcholine receptor rich membrane fragments as determined by the binding of ^{125}I-labelled *Naja naja siamensis* α-toxin (Fig. 1).

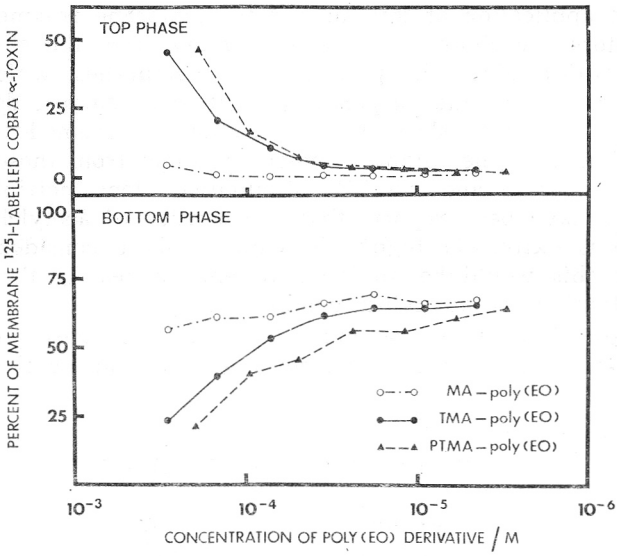

Fig. 1. Distribution of membrane bound ^{125}I-labelled α-toxin in presence of various concentrations of MA-poly(EO), TMA-poly(EO) and PTMA-poly(EO). The final phase system contained 4.32% (w/w) Dextran T-500, 3.59% (w/w poly(EO) 6 000, 4.75 mmol sodium phosphate (pH = 7.4), and 24.75 mmol NaCl per kg phase system. Unsubstituted poly(EO) was replaced with an equal amount of poly(EO) and derivative to give the indicated concentrations of terminal ligands. Substitution grades of PTMA-poly(EO), MA-poly(EO) and TMA-poly(EO) were calculated from elemental analysis to be 66%, 96% and 103%, respectively, where 100% substitution grade is two terminal molecules of ligand per linear poly(EO) molecule. We express the molarity of polymer-ligand as the ligand molarity, were it free in solution. Sufficient iodinated toxin to occupy approximately 7.3% of the α-toxin binding sites was added to a membrane fraction purified by sucrose density gradient centrifgation[8]. Phase system containing membrane bound ^{125}I-labelled α-toxin were mixed and placed on ice for 15 min and then centrifugated at 500 *g* for 15 min at 5.5 °C. Aliquots of top and bottom phases were colleted in a Nuclear Chicago gammacounter. (From Flanagan, Taylor and Barondes, *Nature*, in press).

Bisquaternary methonium compounds such as decamethonium and hexamethonium should be potent antagonists of the cholinergic derivatives of poly(EO) binding to the acetylcholine receptor. This was found to be the case. Additionally, the potency of decamethonium, hexamethonium and trimethonium

in blocking the effects of the addition of PTMA-poly(EO) paralleled the potency of these agents in reducing the initial rate of α-toxin binding (Table I).

TABLE I.

Effect of bisquaternary methonium compounds on toxin binding and affinity partitioning. Initial rate of ^{125}I-labelled *Naja naja siamensis* α-toxin binding was determined by a modification of the method of Schmidt and Raftery[22]. Reaction mixtures contained 4×10^{-8} M acetylcholine receptor and four-fold molar excess of ^{125}I-labelled *Naja naja siamensis* α-toxin (5—10 Ci/mmol) in 10 mM phosphate, pH = 7.4. The reaction rate approximated pseudo first order kinetics. During the incubation period in the absence of inhibitory ligand the reaction progressed to 30—40% of completion and initial rates were calculated assuming a first order approach to equilibrium. The concentrations of bisquaternary methonium compounds required to inhibit the affinity partitioning of membrane bound ^{125}I-labelled α-toxin in the top phase were determined using a system like that in Fig. 2 with 1.85×10^{-4} M PTMA-poly(EO). (From Flanagan, Taylor and Barondes, *Nature*, in press).

	Concentration/M that inhibits initial rate of toxin binding by 50%	Concentration/M that inhibits PTMA-poly(EO) effect by 50%
Decamethonium	3×10^{-8}	2×10^{-7}
Hexamethonium	1×10^{-6}	3×10^{-6}
Trimethonium	7×10^{-5}	$> 5 \times 10^{-4}$

As an additional control for the specificity of the affinity partitioning effect observed, it was found that the addition of an excess of α-toxin blocked the effect of the cholinergic poly(EO) derivatives; whereas, the addition of α-toxin in the absence of cholinergic derivatives of poly(EO) had little effect on the distribution of the acetylcholine receptor rich membrane fraction.

Upon subjecting a crude membrane pellet to a single extraction in the presence of PTMA-poly(EO), a seven-fold purification was achieved by collecting the membrane fraction partitioning into the poly(EO) rich phase. A 25-fold reduction in ATPase specific activity was observed (Table II) in this fraction, while a five-fold decrease in the specific activity of acetylcholinesterase was observed. Of the recovered fraction, 25% of the protein was acetylcholine receptor. This value was calculated on the basis of one toxin binding site per 100 000 molecular weight.

Further purification was achieved by a combination of sucrose density gradient centrifugation and affinity partitioning. Electron microscopy of the membrane fragments, negatively stained with neutral phosphotungstic acid, was performed by John Duguid, California Institute of Technology. Under these conditions, 60% of the membrane fragments were observed to contain the characteristic morphological structures previously observed with highly purified acetylcholine receptor[21].

Upon sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis of the highly purified membrane fraction, Commassie blue bands were observed at 40 000, 49 000, 60 000 and 67 000 molecular weight which are characteristic of the acetylcholine receptor purified from *Torpedo* species (Fig. 2). In addition, a protein band at approximately 105 000 molecular weight was observed. As

TABLE II.

Specific activity of fractions from single extraction in phase system containing PTMA-poly(EO). The phase system used in this extraction contained 4.32% (w/w) Dextran T-500, 3.59% (w/w) poly(EO) 6000, 1.35×10^{-4} M PTMA-poly(EO), 24.75 mmol NaCl, 4.75 mmol sodium phosphate, (pH = 7.4), per kg phase system. A particulate fraction was partially purified from the homogenate of electroplax by differential centrifugation (8). This fraction, containing 15.6 mg protein was resuspended in 2 ml of 1 mM phosphate buffer, pH = 7.4, and added to the phase system to give the above concentrations in 40 g of total system. The mixture was placed on ice, mixed by repeatedly inverting the tube, and then added to 60 ml syringe fitted with a valve. The syringe assembly was centrifuged at 1 000 rpm for 30 min at 5.5 °C in a GLC-1 centrifuge (Sorvall). The top phase was collected directly and bottom phase was collected through valve. The top and bottom phases were diluted with distilled water and centrifuged for 3 hr at 35 000 rpm in a Type 35 rotor (Beckman Instruments). Of the protein added to phase system 6.63 mg was recovered in bottom phase and 0.51 mg was recovered in top phase. No determination was made of the constituents in the interphase. (From Flanagan, Taylor and Marondes, *Nature,* in press).

	ATPase[a]	AChE[b]	Toxin binding[c]
Pellet from differential centrifugation	62.8	35	353
Bottom phase	26.4	20	201
Top phase	2.5	7	2,499

[a]μmol of ATP hydrolyzed per hr per mg-protein (23)
[b]μmol of acetylcholine hydrolyzed per min per mg-protein (24)
[c]pmol of ^{125}I-labelled *Naja naja siamensis* toxin bound per mg-protein (22)

a result of the contaminating membranes, it is difficult to determine whether or not this protein is a component of the acetylcholine receptor rich membranes. It should be noted in this regard that a 105 000 molecular weight protein was observed in a wide range of membrane fractions after sucrose density gradient centrifugation. This protein appears to be the major protein species in the crude membrane preparation.

In comparing affinity chromatography with affinity partitioning we should emphasize that with affinity partitioning it was simple to perform controls required to rule out the possibility of nonspecific electrostatic effects. These controls were performed by using various concentrations of polymer derivatives and antagonists. Similar controls would be more complicated when performed with affinity chromatography, requiring the synthesis of a number of affinity matrices containing various concentrations of ligands.

Similar strategies may be usefully employed in the purification of a number of neurotransmitter receptors. It is reasonable to expect that other post-synaptic membrane fragments may contain similar densities of receptor. On the other hand, it is difficult to predict whether this method will be useful for purification of other membranes, or intact cell which contain a substantially lower density of specific binding sites. Due to the low affinity of the ligands used in this study, it was impossible to use saturating concentrations of the poly(EO) derivatives without risking a possible change in the gross physical properties of the phase system. Consequently, it was impossible to

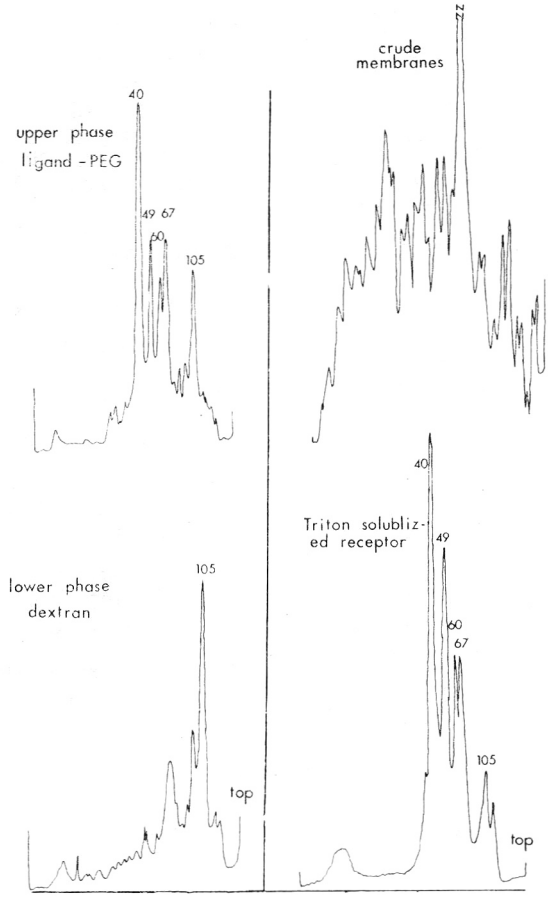

Fig. 2. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of purified membrane fractions and solubilized acetylcholine receptor. Crude membranes were prepared by differential centrifugation[18]. These membranes were further purified by sucrose density centrifugation and a single affinity partitioning extraction. The phase system composition was the same as that described in Table II. Acetylcholine receptor, solubilized in the presence of Triton-X-100, was purified by affinity chromatography[25]. Membranes and receptor purified in this manner were subjected to SDS polyacrylamide gel electrophoresis[26] and stained for protein with Commassie blue.

determine the effect of full occupancy of the acetylcholine receptor by poly (ethylene oxide) derivatives. Further experiments using ligands of higher affinity would be useful in this regard.

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DISCUSSION

E. Heilbronn:

Do you ever see any formation of synaptosomes or any fragments of presynaptic membranes sticking to the presynaptic ones in your *Torpedo* preparations?

S. D. Flanagan:

We have only used negative staining techniques in electron microscopy studies of our purified acetylcholine receptor rich membranes. Thus it is difficult to access the fraction of other membranes such as presynaptic fragments in our preparations. However, it should be pointed out that if there is a protein characteristic of synaptic cleft material, distinct from the acetylcholine receptor itself, little was observed in the fraction containing the highest specific activity of toxin binding.

E. Heilbronn:

Have you done any cross reactions between your membrane and antibodies to purified receptor from *Torpedo californica*?

S. D. Flanagan:

No, we have not attempted such studies.

M. E. Eldefrawi:

I noticed that you consistently used neurotoxin to determine receptor sites on your membrane, and as I mentioned before you may see toxin binding even though the acetylcholine binding site may be denatured. Have you looked at acetylcholine binding to purified membranes to see how subjecting them to the poly(ethylene oxide) affects acetylcholine binding?

S. D. Flanagan:

No, we have not tried to measure the binding of acetylcholine using, for example, the equilibrium dialysis methods that you employ. However, since the membrane

fraction rich in toxin binding activity are purified rapidly from the electric organ and are not exposed to detergents, it may be useful to perform such studies with our membrane preparation.

T. L. Rosenberry:

Do poly(ethylene oxide) polymers act as detergents to solubilize part of the receptor in your system?

S. D. Flanagan:

Membranes, purified by affinity partitioning extraction in this study, were collected from the phase solutions by centrifugation. This implies that the collected material remains membrane bound. Although poly(ethylene oxide) is a component of a number of non-ionic detergents such as Triton X-100 and Brij, these detergents contain hydrophobic groups such as aliphatic chains to form amphipathic molecules with solubilizing activity. The derivatives of poly(ethylene oxide) we used contain relatively hydrophilic ligands and would not be expected to be strong solubilizing agents.

Studies by Albertsson and Walter indicate that poly(ethylene oxide) is a gentle agent, and erythrocytes and other intact cells may be purified by polymer phase systems containing poly(ethylene oxide) and dextran. Erythrocytes are relatively sensitive to the effects of detergents, and the addition of such agents frequently results in hemolysis. Thus it is unlikely that poly(ethylene oxide) has substantial detergent activity.

E. Heilbronn:

We have recently been developing a method for the preparation of presynaptic membranes from brain using the old observation of ours that phospholipase A_2 breaks down synaptic membranes but leaves the synaptic junction reasonably intact. Do you think that a combination of this technique with yours might give nice results?

S. D. Flanagan:

I gather that treatment of synaptic complexes with phospholipase A_2 would render the synaptic membranes more accessible to the poly(ethylene oxide) derivatives used in affinity partitioning. If such is the case, then such pretreatment would be extremely useful in combination with affinity partitioning to yield a high degree of purification of cholinergic or other neurotransmitter synaptic complexes.

SAŽETAK

Čišćenje membrana obogaćenih acetilkolinskim receptorom metodom afinitetne raspodjele

S. D. Flanagan, P. Taylor i S. H. Barondes

Acetilkolinskim receptorom bogati fragmenti membrana električnog organa vrste *Torpedo californica,* čišćeni su kombinacijom klasičnih metoda i primjenom afinitetne raspodjele, metode analogne afinitetnoj kromatografiji. Afinitetna raspodjela zasniva se na metodi raspodjele slojeva, gdje se dodatkom polimera topljivih u vodi, kao što su polietilenoksid i dekstran, oblikuju dva vodom bogata sloja. Odabran je sistem u kojem se manje od 2% acetilkolinesteraze, adenozintrifosfataze i aktivnosti koje vežu α-toksin distribuira u polietilenoksidom bogati sloj. Dodatkom kolinergičnih derivata polietilenoksida, dobivene su selektivne promjene u raspodjeli fragmenata membrana bogatih acetilkolinskim receptorom. Elektroforeza na poliakrilamidnom gelu i pokusi vezanja α-toksina kobre, aktivnosti acetilkolinesteraze i adenozintrifosfataze upućuju na to, da je čišćenje acetilkolinskog receptora vezanog za membrane u znatnoj mjeri uspjelo.

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