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THE BIOCHEMISTRY OF AN ACETYLCHOLINE RECEPTOR

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The acetylcholine receptor from Torpedo californica electroplax has been studied at three levels of molecular organization: receptor-rich membrane fragments, solubilized and purified receptor, and reconstituted receptor in phospholipid vesicles. The binding of cholinergic ligands to the membrane-bound and the solubilized material is not cooperative, and the number of ligand sites is less than the number of toxin sites. In addition, the purified macromolecule contains the molecular features necessary for ion-translocation during postsynaptic depolarization, since a chemically excitable membrane can be formed from purified receptor and Torpedo phospholipids.

INTRODUCTION

Fundamental understanding of the mechanism of action of a physiological neuroreceptor, such as that for acetylcholine, is possible only if such a system can be studied at various physical levels or different levels of molecular organization. Electrophysiological studies have been conducted over many years for such receptors at the cellular level. Biochemical characterization at the membrane level, the isolated molecular level, and the reconstituted level have become possible in recent years based on (a) the discovery of alpha-bungarotoxin (1) and related neurotoxins from cobra species and (b) the extremely high content of acetylcholine receptors AcChR in organisms such as Torpedo species and

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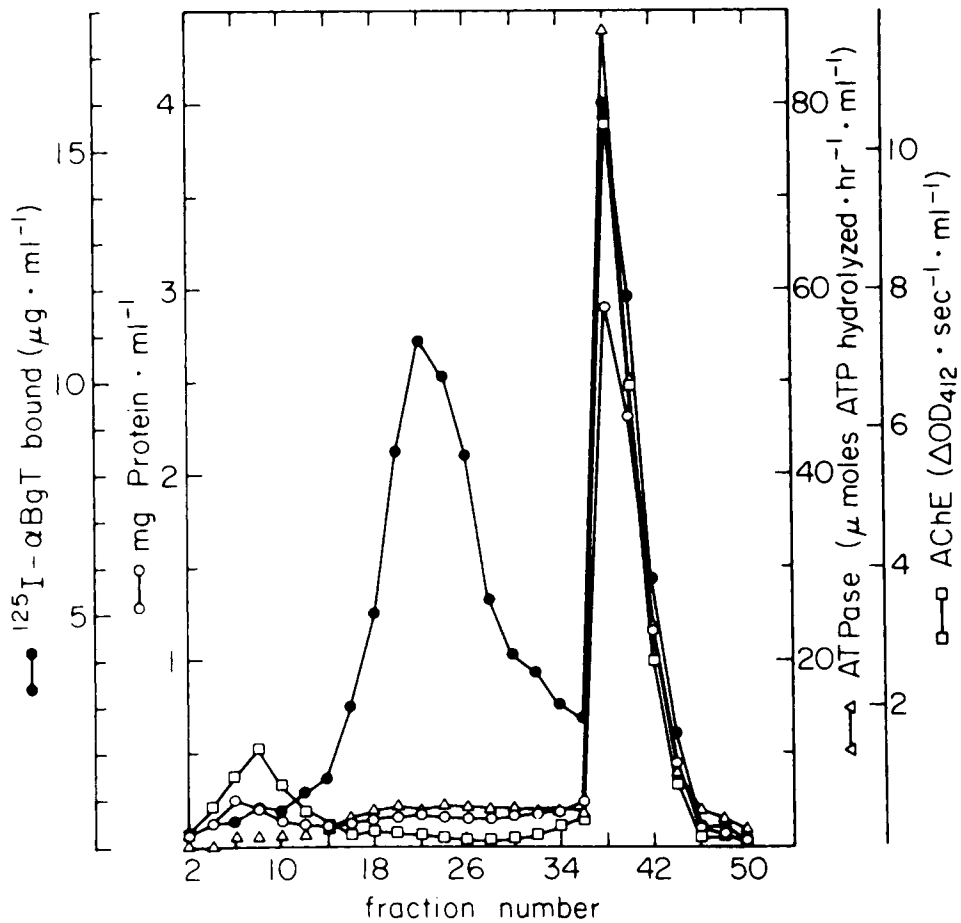

Fig. 1. Zonal sucrose gradient centrifugation of Torpedo californica electroplax membranes.

Electrophorus electricus. For the studies described here, the electric ray (Torpedo californica) was the source of AcChR.

For the results which are described in this communication, two preparations of AcChR are of importance: AcChR-rich membrane fragments and solubilized, purified AcChR. These preparations will first be briefly described.

AcChR-RICH MEMBRANE FRAGMENTS

Following homogenization of Torpedo electroplax and centrifugation of the membranes, a further dramatic fractionation of membrane particles can be achieved on a large scale in sucrose density gradients (Fig. 1). Two kinds of particulate fractions are of interest. At the top of the gradient (fractions 3–10), membrane fragments enriched in acetylcholinesterase activity but without significant binding of ^{125}I-α-Bgt are obtained,

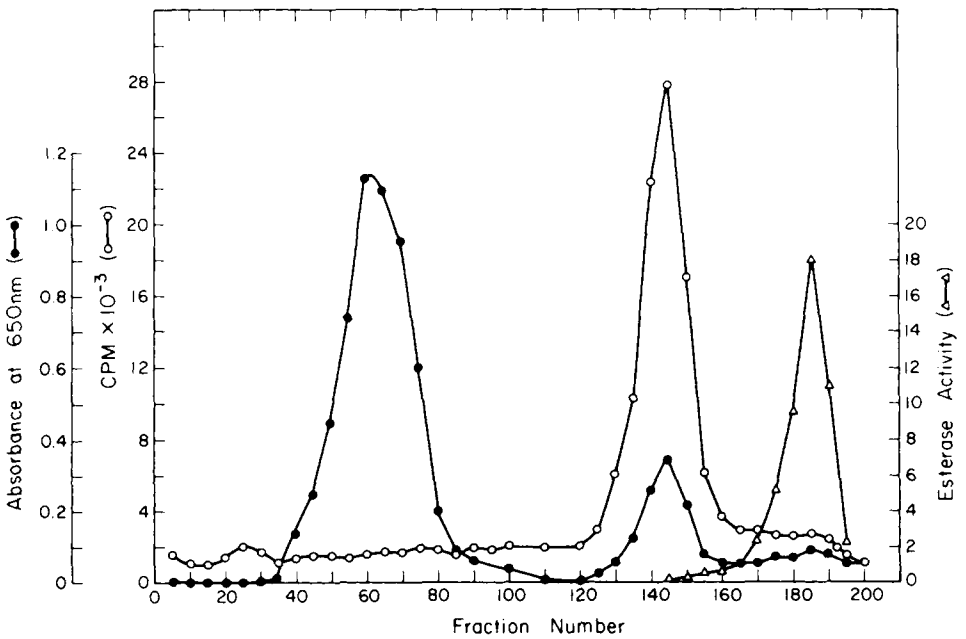

Fig. 2. Affinity chromatography of Torpedo californica membranes extracted with 2% Triton X-100 according to Schmidt and Raftery (12).

while in the middle of the gradient (fractions 18–30) membrane particles highly enriched in AcChR are obtained. The most dense fraction contains AcChE activity, AcChR, and all of the ATPase activity in the membranes.

Solubilization of membrane fragments by addition of 1–2% Triton X-100 immediately and quantitatively releases AcChR from its membrane-embedded state. Affinity chromatography procedures can then be used to fractionate the extracted protein species (Fig. 2).

A comparison of the polypeptide species present in the AcChR-rich membrane fragments and in the purified AcChR is instructive (Fig. 3). It is immediately obvious that one major difference exists between the two preparations: a component of MW 1.05×10^5 is present in the membrane fragments and absent in the purified receptor from the same fragments. Species of MW 40,000, 49,000, 60,000, and 67,000 are present in each preparation, with the relative amounts of each species being constant except for the 40,000 MW polypeptide, which seems to be present in a much larger quantity in the membrane fragments than in the purified receptor. Two interesting points may be made: (a) the patterns emphasize the high enrichment for AcChR in the membrane fragments (40–50% in our best preparations), and (b) if the isolated AcChR had been degraded by proteolysis so that the subunit molecular weights observed are not the true values, then such degradation would appear to have occurred quantitatively at the membrane stage, which we consider highly unlikely.

Our best preparations of solubilized/purified AcChR show a slightly less complex subunit pattern than that shown in Fig. 3. In Fig. 4 we present polyacrylamide gel electrophoresis under nondenaturing conditions (to the left) where it can be seen that the

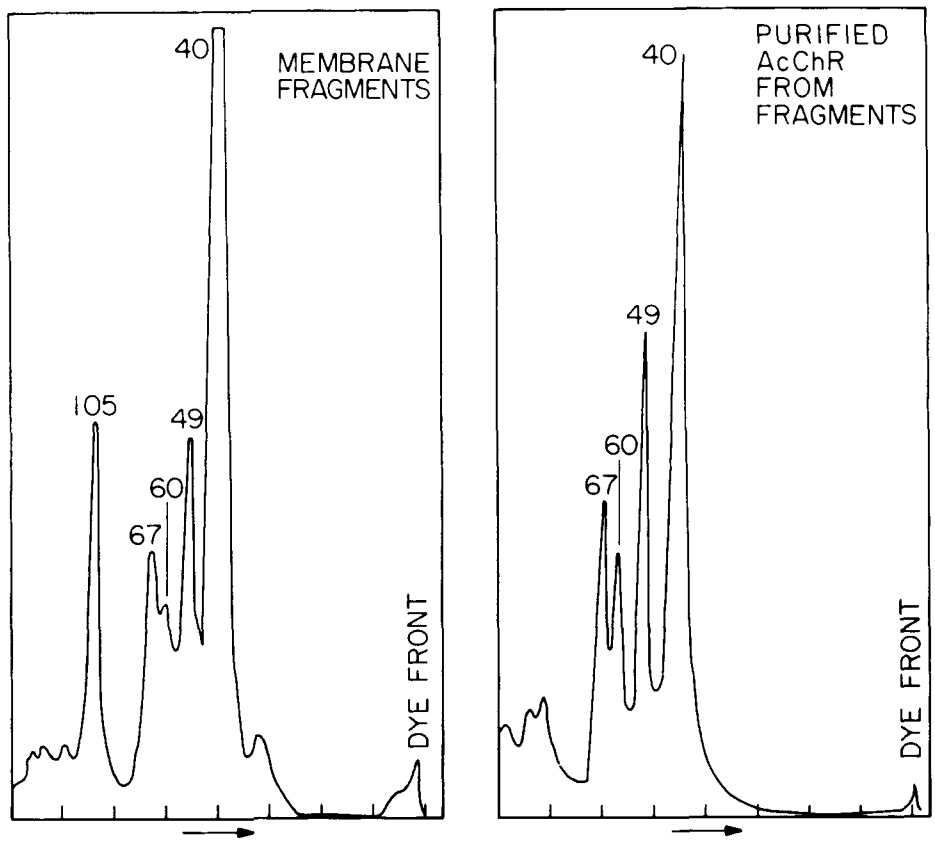

Fig. 3. Comparison of polypeptide components of AcChR-rich membrane fragments and purified AcChR from the same fragments.

purified material migrates as one major component. Lesser amounts of higher molecular weight material appear to be aggregates of the main component. Under denaturing conditions shown to the right, one major subunit of 40,000 MW is observed, with a smaller amount of 50,000 MW subunit also being present. To date, we have not been able to simplify this pattern any further.

LIGAND BINDING TO AcChR-RICH FRAGMENTS AND TO PURIFIED AcChR

We have conducted extensive binding studies with the isolated receptor and with the enriched fragments (2–5). The most pertinent binding data can be summarized as follows: The binding of acetylcholine to membrane fragments as shown in Fig. 5 yields a Hill coefficient very close to 1. This lack of cooperativity is in contrast to recently published studies, similar to these, on Torpedo marmorata electroplax membranes; very slight positive cooperativity was observed. We have found no indication of positive cooperativity for the binding of acetylcholine or carbamylcholine or any other cholinergic

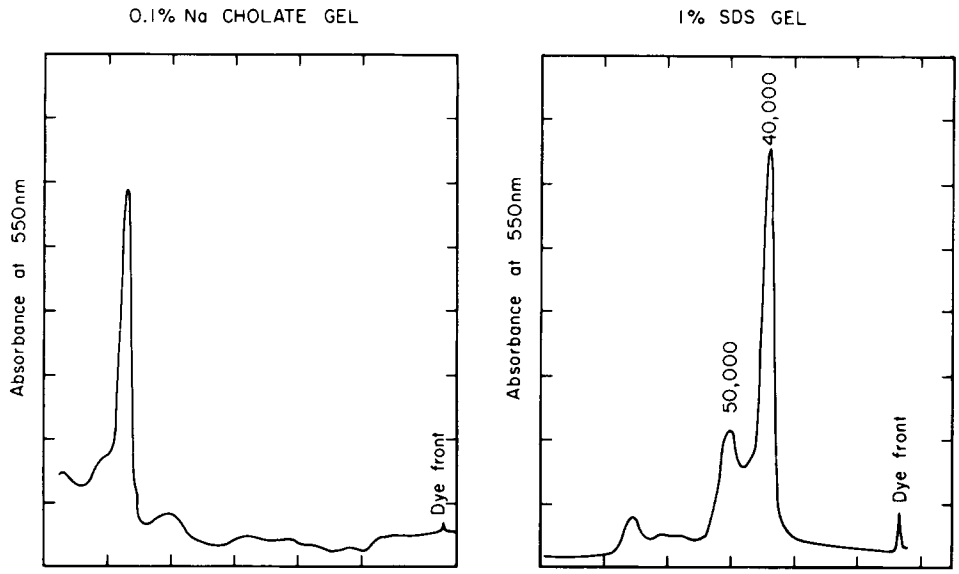

Fig. 4. Polyacrylamide gel electrophoresis of purified AcChR under nondenaturing (left) and denaturing (right) conditions.

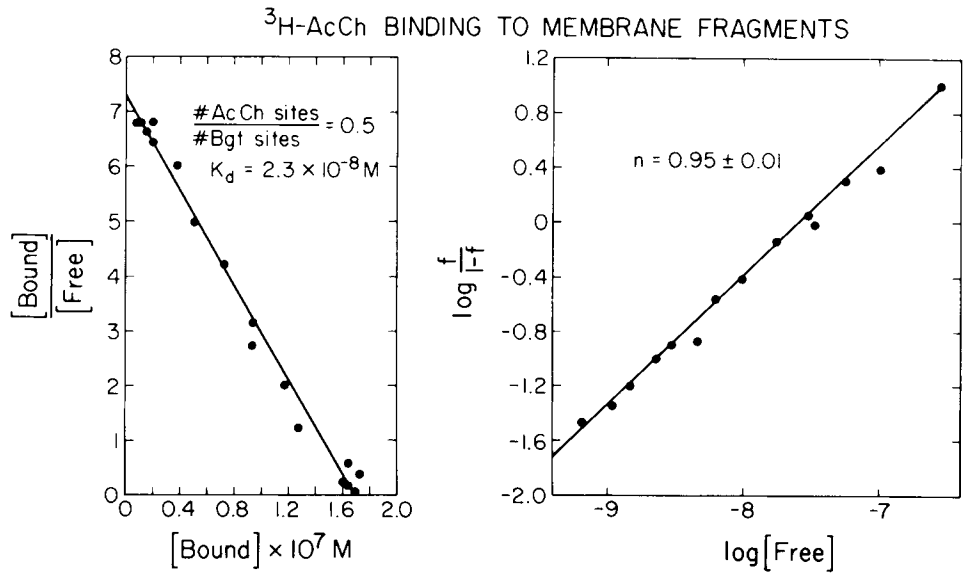

Fig. 5. Scatchard plot (left) and Hill plot (right) of acetylcholine binding to AcChR-rich membrane fragments.

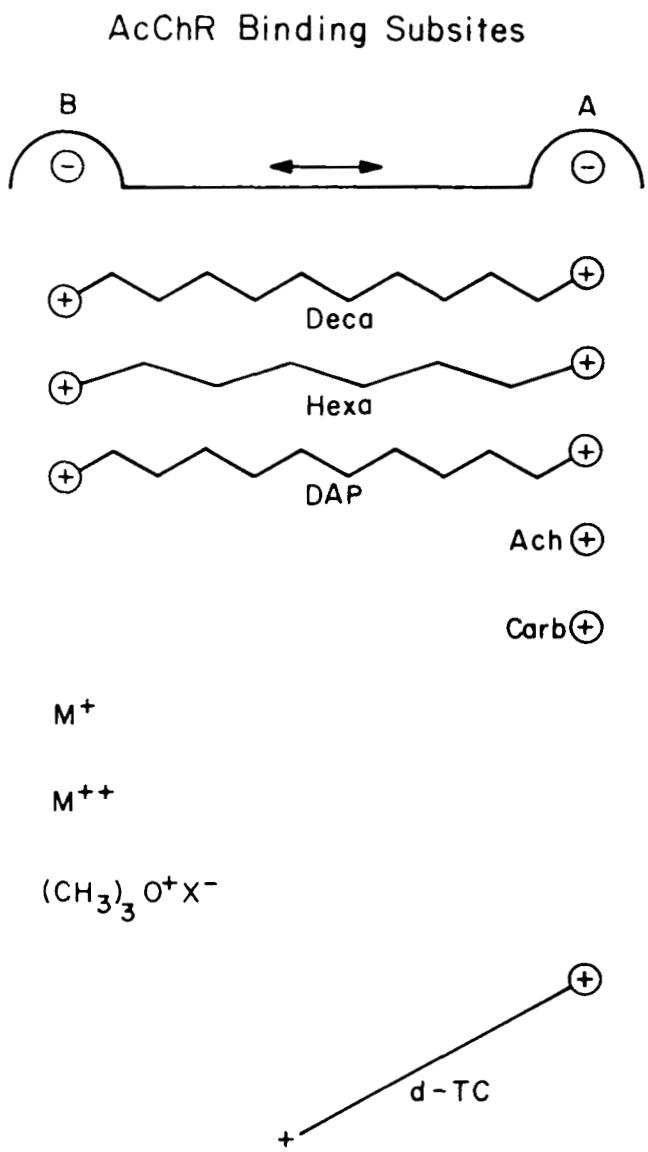

Fig. 6. Schematic model for binding of cholinergic compounds or cations to purified AcChR.

ligand to Torpedo californica AcChR-rich fragments or to isolated purified AcChR(2–5).

A second interesting feature is that we have consistently found half the number of ligand binding sites as toxin binding sites, as indicated in Fig. 5. This result may well mean that the receptor from Torpedo californica displays a phenomenon known as negative cooperativity in ligand binding (6).

Some further structural detail about AcChR has been elucidated by use of the fluorescent dye bis-(3-aminiopyridinium) 1, 10-decane diiodide (DAP) (3, 5, 7) and its competition with other cholinergic ligands. These results are summarized in Fig. 6.

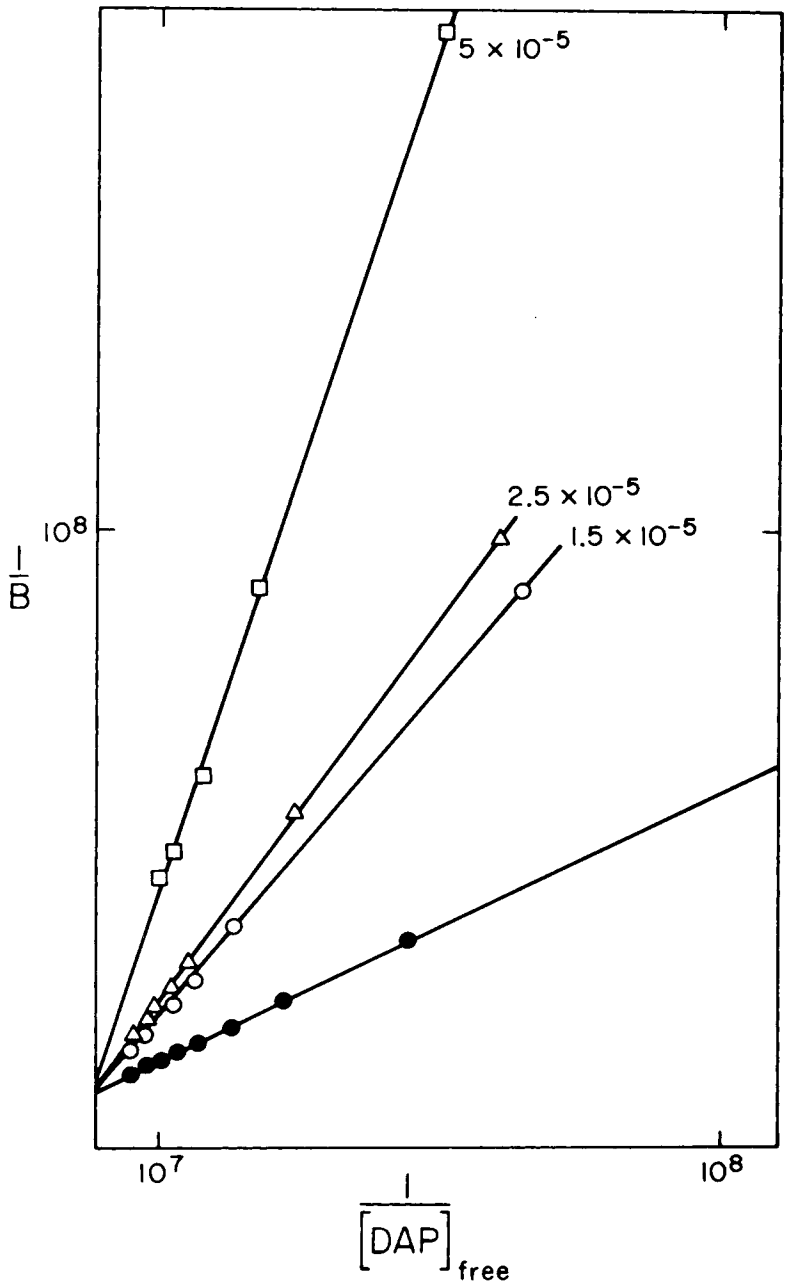

Fig. 7. Double reciprocal plot of DAP binding to purified AcChR and its competition with the indicated concentrations of acetylcholine.

According to this model, bis-quaternary cholinergic analogs bind to two negatively charged binding subsites on the receptor's surface. One of these is the recognition site for neurotransmitter, acetylcholine, or carbamylcholine since these two ligands are strictly competitive with DAP in their binding (see Fig. 7). The other binding of d-tubocurarine

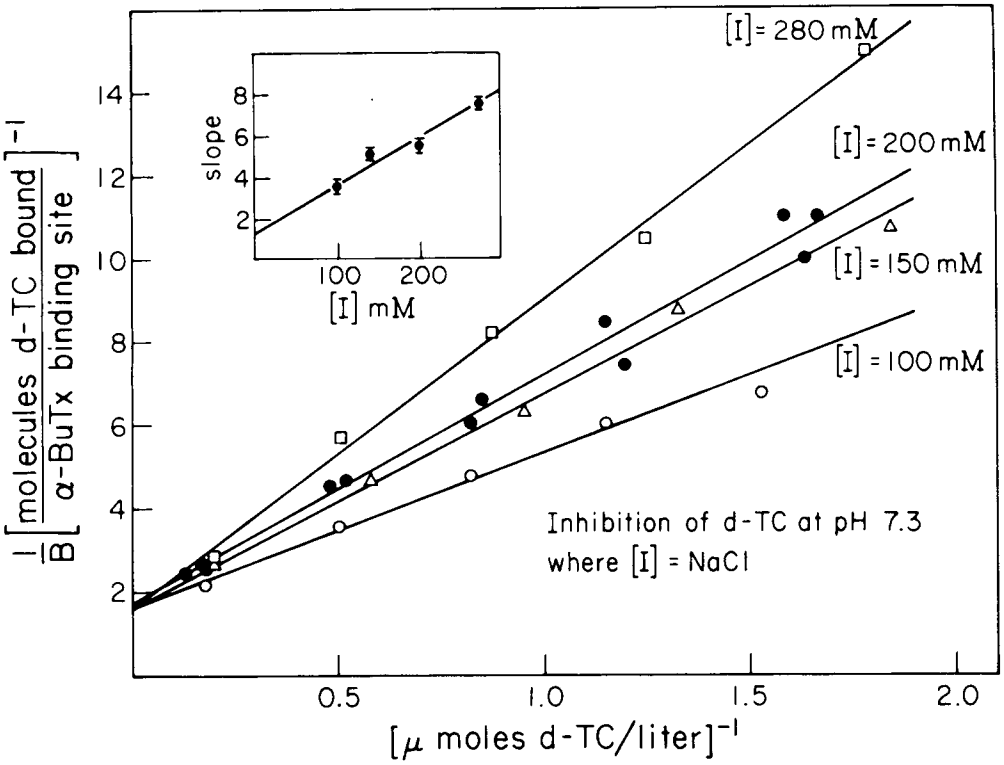

Fig. 8. Double reciprocal plot of the binding of d-tubocurarine to purified AcChR and its antagonism by NaCl.

is shown also in Fig. 6. It appears to interact with subsite A and may sometimes additionally occupy subsite B since its binding is competitive with sodium, as shown in Fig. 8; however, the inhibition constant for sodium competition is much higher than the value obtained from similar studies using decamethonium or DAP, which both appear to bind to subsites A and B. Additionally, subsite B can be preferentially inactivated by reaction with the alkylating agent trimethyloxonium fluoroborate, which appears to be an affinity reagent for this binding subsite, probably because of its similarity to methylated quaternary nitrogen compounds.

The rate of formation of ^{125}I-α-Bgt-AcChR complexes can be slowed down by preincubation with varying concentrations of cholinergic ligands. This effect is also observed when inorganic cations are present. Figure 9 shows the effect of varying concentrations of calcium chloride ($CaCl_2$). The midpoint of this inhibition curve corresponds almost exactly with the inhibition constants for calcium measured from competition studies with DAP (3). In Table I inhibition constants derived from initial toxin-binding rates for both membranes (AcChR-rich membranes) (8) and purified AcChR (3, 7) are compared. The results show that all ligands bind more tightly to AcChR-rich membrane fragments than to purified receptor. However, the most significant differences are observed with agonists, especially acetylcholine, carbamylcholine, and nicotine where the binding to the AcChR-rich membrane fragments is 10^2-10^3 higher. The difference for antagonists,

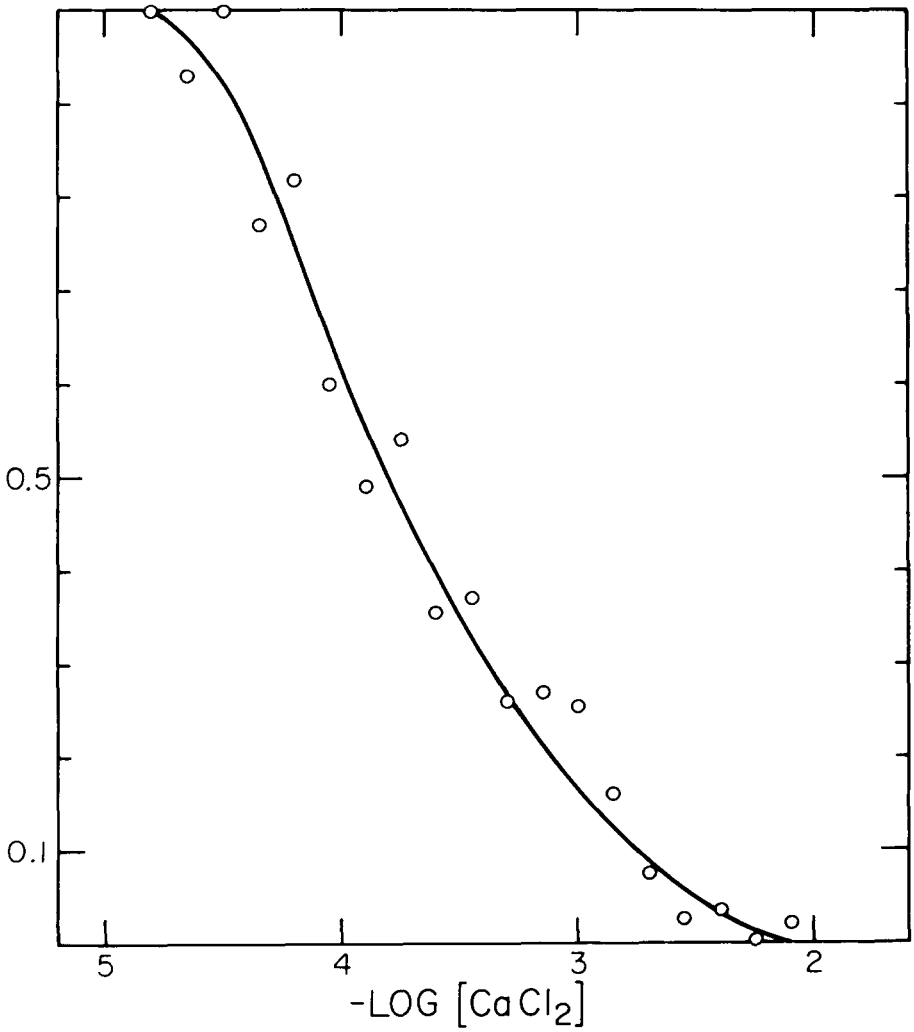

Fig. 9. The effect of $CaCl_2$ concentration on the initial rate of ^{125}I-α-Bgt AcChR complex formation.

such as hexamethonium, d-tubocurarine, and gallamine, are insignificant. We interpret this large difference as being due to a perturbation upon solubilization and purification which specifically affects binding subsite A. Subsite B is not affected by solubilization, since we have observed that cation binding is exactly the same in both preparations (8). Effects opposite to those observed here have been seen in comparative studies of ligand binding to AcChR-rich fragments and purified AcChR from Electrophorus electricus (9). The origin of the large differences we observe is not clear. However, it seems to be fairly localized in terms of the receptor macromolecule and involves only one of the two subsites shown in Fig. 6. It is possible that a specific lipid requirement is necessary for high-affinity binding to subsite A.

TABLE I. I_{50} Values for AcChR – Purified and Membrane-Bound

	Purified AcChR 5 mM Tris	AcChR membranes 20 mM NaCl – 5 mM Tris	Ratio
Acetylcholine	2.5×10^{-6}	6×10^{-9}	420
Carbamylcholine	4.5×10^{-5}	4×10^{-8}	1100
Nicotine	8×10^{-5}	1×10^{-7}	800
Phenytrimethyl-ammonium	8×10^{-5}	2×10^{-7}	400
Choline	1.7×10^{-3}	1×10^{-5}	170
Decamethonium	2×10^{-6}	9×10^{-9}	220
Hexamethonium	2×10^{-6}	5×10^{-7}	4.0
d-Tubocurarine	1×10^{-7}	3×10^{-8}	3.3
Gallamine	2×10^{-7}	8×10^{-8}	2.5
DAP	1×10^{-6}	2×10^{-8}	50

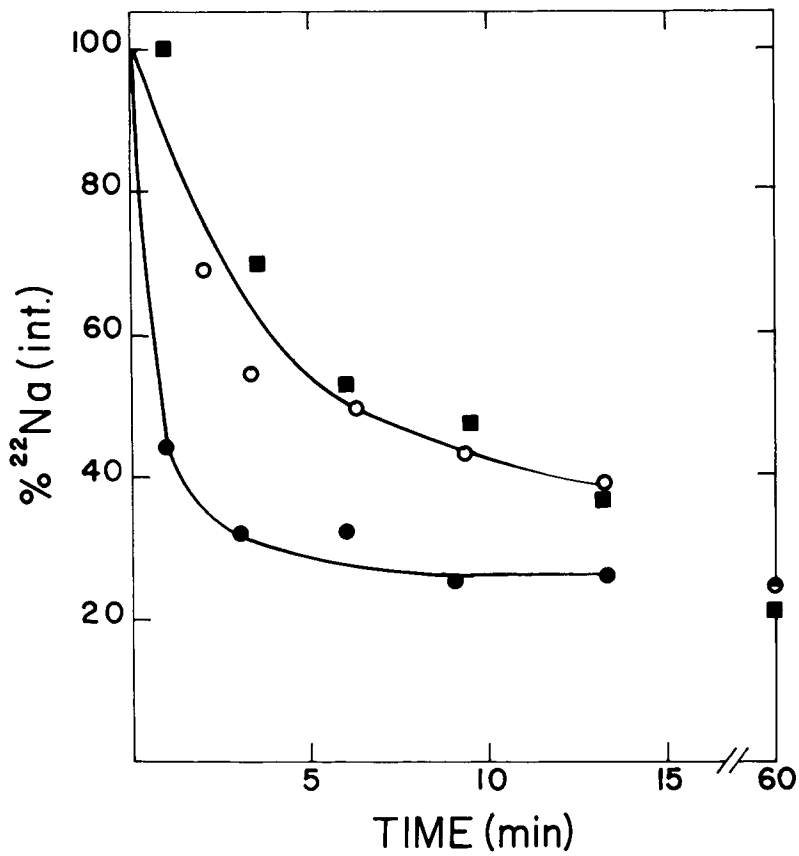

Fig. 10. ^{22}Na efflux from ^{22}NaCl-loaded AcChR-lipid reconstituted vesicles: ○—○, ^{22}Na leak in absence of additives; ●—●, with 100 μM carbamylcholine; ■—■, with 100 μM carbamylcholine following saturation with α-Bgt.

AcChR RECONSTITUTION

Recent experiments in our laboratory (10, 11) have yielded interesting results upon reassociation of Torpedo californica purified AcChR with phospholipids from the same source. A lipoprotein complex can be demonstrated by sucrose density gradient centrifugation. In addition, as shown in Fig. 10, the lipoprotein vesicles which are formed can be loaded with ^{22}NaCl, thus demonstrating an internal volume for the vesicles, and the rate of leakage of ^{22}Na from such vesicles can be followed. The vesicles are fairly leaky and their leakiness corresponds almost exactly with that observed for phospholipid vesicles (using Torpedo phospholipids) formed without addition of AcChR. A most interesting observation is that upon addition of carbamylcholine the vesicles become considerably more leaky. That this is a specific effect is also shown in Fig. 9; upon preincubation with α-Bgt the response to carbamylcholine is completely and irreversibly blocked. The significance of this experiment is that it is possible to reform a chemically excitable membrane from isolated purified AcChR and the phospholipids with which it naturally occurs. These results mean that the isolated macromolecular receptor for acetylcholine from Torpedo californica contains all the necessary elements to effect synaptic depolarization – that is, it contains both a specific neurotransmitter recognition site (binding subsite A) and the ion translocation apparatus. Further studies of such reconstituted systems should aid greatly in furthering our understanding of the molecular mechanisms involved in synaptic depolarization.

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