ISOLATION OF ACETYLCHOLINE RECEPTORS[1,2] 6526

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When ACh acts in an excitatory way, it increases the conductance of a post-synaptic membrane for Na^+ and K^+, leading to membrane depolarization. We shall use the term acetylcholine receptor (AChR) for those macromolecules involved in this transduction that bear the recognition site(s) for ACh and other cholinergic ligands. It is now widely believed that cholinergic receptors can be isolated, using techniques that parallel those employed by enzymologists. Early attempts (1–4) failed because it was not recognized that isolated receptors must possess rather precisely specified properties. These earlier attempts were thoroughly reviewed (5–9), therefore we shall restrict our treatment of the subject to current attempts.

The key to the problem is the discovery of the right indices to follow as purification proceeds. Enzymologists can follow the catalytic activity in the various fractions they isolate. Receptologists have no such single index. Instead they must search for macromolecules that bind correct (but not incorrect) ligands with the correct affinity and correct reversibility, and that are present only in the appropriate amount in appropriate tissues. By "correct" we mean corresponding to the physiological response.

The question of what quantity of AChR one should expect to find is an important and difficult one. Waser (10) used autoradiography of dried diaphragm (1 mm thick) of mice after exposure to curare and found it to accumulate in the end-plates. Its concentration was estimated to be 4×10^6 molecules/end plate. This number was employed by several workers to estimate the concentration of AChR in different tissues. For example, Trams (11) estimated that 1.1 nmole/g of AChR should be found in eel electroplax. We combined the Waser data with Nachmansohn's estimate of 50,000 synapses per electroplax and calculated a concentration of 0.01 nmole/g for

[1] The following abbreviations are used in this review: ACh (acetylcholine); AChR [acetylcholine receptor(s)]; AChE (acetylcholinesterase); DMTC (dimethyl-*d*-tubocurarine); α-BGT (α-bungarotoxin, a polypeptide from venom of the snake, *Bungarus multicinctus*); MBTA (4-maleimidobenzyl trimethylammonium iodide); DTT (1,4-dithiothreitol); TDF (*p*-(trimethylammonium)-benzenediazonium fluoroborate); EEDQ (*N*-ethoxycarbonyl 2-ethoxy-1,2-dihydroquinoline); SDS (sodium dodecyl sulfate).

[2] Experimental work from this laboratory was supported by U.S. P.H.S. grants NS 09144, GM 07804 and Training grant ES 98.

eel electroplax. Applying even rougher calculations (also based on Waser's data) to rat brains, O'Brien & Gilmour (12) estimated that 18 nmole/g should be found. These discrepancies illustrate the difficulties involved in making such estimates.

Tissues and Preparations Examined for AChR

AChR should be found in high concentration in tissues rich in ACh and AChE. In vertebrates, the highest concentrations of ACh and AChE are found in neural tissues, so AChR has been searched for in cerebral cortex from several species (9, 13, Farrow & O'Brien, unpublished). The brain of the housefly, *Musca domestica* which contains a concentration of AChE 30 × higher than that found in mouse brain (14) has been used in three studies (15–17). The richest known sources of AChE are the electric tissues of certain fish, which are regarded as modified skeletal muscles (18). That of the electric eel, *Electophorus electricus,* is chemically as well as electrically excitable and has relatively few synapses, whereas that of the electric ray, *Torpedo,* is only chemically excitable and has a larger number of synapses (19). Consequently *Torpedo* electroplax contains higher concentrations of ACh and AChE than eel electroplax (20–24) (calculated to be 8–20 and 8–12 times higher, respectively). Table 1 lists the tissues, preparations and techniques used in recent attempts to identify and isolate AChR.

Techniques

A. Binding of Cholinergic Ligands in Aqueous Media:

Two techniques have been used to monitor AChR as isolation proceeds. One is the "affinity label technique" in which a radioactive agent is intended to bind irreversibly to the active site of the AChR. Because the label is maintained throughout purification, monitoring is very simple. Disadvantages are that binding may irreversibly inactivate the receptor and it is uncertain whether the label is attached to the whole receptor or only a fragment. Some of these problems can be surmounted by testing for binding in samples taken after each purification step.

The other technique uses reversibly bound ligands to detect and identify AChR in vitro. A major advantage is that the active receptor can be recovered at any time simply by washing. Also, the commonly used drugs (and the transmitter itself) for which extensive in vivo data are available, can be employed and a ligand concentration selected such that binding occurs primarily to the receptor. In comparison with the affinity label technique, it is more laborious because for each sample a dialysis or other assay rather than a simple count must be done.

Irreversible binding.—α-Bungarotoxin was found to block specifically the depolarizing action of ACh at vertebrate neuromuscular junction (25, 26) and the innervated membrane of the electric tissue of *Torpedo marmorata*

(*27*). This does not prove that α-BGT cannot bind in vitro to neural components which are, in physiological preparations, inaccessible to the toxin; or to components whose binding to α-BGT gives no electrophysiological response. So far, it has been used in two in vitro studies to label macromolecules suspected to be AChR. Miledi, Molinoff & Potter (*27*) homogenized electric tissue of *Torpedo* and studied binding of ^{131}I-α-BGT to the resuspended pellet of 23,000 × *g* and to the 1% Triton-solubilized pellet. Evidence that the α-BGT binding macromolecules were AChR, was that its binding in membrane suspensions was retarded by preaddition of curare or carbachol and by the latter when in solution. The effect of other cholinergic or noncholinergic ligands on binding was not determined, so that specificity of this binding was not clearly defined. The concentration of binding sites equalled those previously found by others using equilibrium dialysis of the same tissue with the reversibly binding ligands ACh, muscarone, and nicotine (Table 1) (17, 28–30).

In another study (13) ^{3}H-acetylated α-BGT was found by ultrafiltration to bind to the homogenate of guinea pig cerebral cortex in 0.1% Triton and 0.1 M Tris. Binding was slowly reversible (75% in 90 hr), and was relatively specific; as demonstrated by its inhibition by *d*-tubocurarine, ACh, carbachol, gallamine, decamethonium, and hexamethonium, but not by atropine, choline, propanpheline, and serotonin. Additional evidence for specificity was that the P_2 synaptosomal fraction bound twice as much α-BGT per unit weight as the total homogenate; and of the former, the synaptosomal membrane fractions were the richest in the binding macromolecules (representing 65% of total binding to P_2 fraction). Unfortunately, boiling the protein fraction that bound α-BGT reduced its binding by only about 60%; this "boiled" binding did not saturate with increasing concentration of α-BGT, indicating it involves nonspecific adsorption.

Dibenamine is another irreversible blocker of AChR which was used to attempt in vitro labeling (31). Smooth muscle strips of dog small intestine were exposed to atropine sulfate followed by unlabeled dibenamine, and thus the nonatropinic sites were bound irreversibly. Then the reversibly binding atropine was washed off, and the muscle exposed to ^{3}H-dibenamine to label the atropine binding sites. The highest concentration of label was found in the supernatant from centrifugation at 1000 × *g* and the pellet of 45,000 × *g*. No attempt was made to confirm further the identity of the binding macromolecules as AChR. Since atropine is known to bind to serotonergic receptors as well (32), some of the dibenamine-labeled sites may not be on AChR; this would explain the large number of labeled sites that one can calculate from the data (Table 1): about 70 nmoles/g as compared with the physiological estimate by Paton & Rang (33) of 0.88 nmoles/g for the sum of the two sites they found.

Karlin and associates (22, 34, 35) obtained relatively specific labeling of AChR in the Sachs organ of the electric eel with disulfide and sulfhydryl agents. Findings on the single cell preparation suggested that ^{3}H-MBTA

TABLE 1. Concentrations of macromolecules suggested to be AChR in various tissue preparations.

Tissue preparation	Ligand and Technique	Amount found nmoles/g original tissue	Ref.
	Electrophorus electroplax		
Pellet of 45,000×*g*	^{3}H-muscarone, ^{3}H-nicotine, ^{14}C-DMTC, ^{3}H-deca/ equilibrium dialysis	.021–0.033	Eldefrawi et al (45)
Sachs electroplax	^{3}H-MBTA to DTT treated electroplax *in situ*	0.01–.02	Karlin et al (22)
Pellet of 28,000×*g* of innervated membrane (deoxycholate solubilized)	^{3}H-deca and interference by α-BGT/equilibrium dialysis	0.19–0.38[a]	Changeux et al (44)
Proteolipid (chloroform-methanol extract)	^{14}C-ACh./Sephadex LH-20 chromatography	0.57[b]	De Robertis et al (61)
	Torpedo electroplax		
Lyophilized pellet of 12,000×*g*	^{3}H-ACh, ^{3}H-muscarone, ^{3}H-deca, ^{3}H-nicotine, ^{14}C-DMTC/equilibrium dialysis, centrifugal assay	0.54–1.3	O'Brien et al (28) Eldefrawi et al (17) Eldefrawi et al (29) Eldefrawi et al (30)
Pellet of 23,000×*g* also Triton solubilized	^{131}I-α BGT/centrifugal assay, gel filtration	1.1	Miledi et al (27)
Proteolipid (chloroform-methanol extract)	^{14}C-hexa/Sephadex LH-20 chromatography	162.5[c]	La Torre et al (57)
	Brains		
Rat-synaptic membranes	^{3}H-atropine/equilibrium dialysis	0.045–0.7	Farrow & O'Brien (unpublished)
Cat-synaptic membranes[d]	^{14}C-DMTC/centrifugal assay, Sephadex LH-20 chromatography	1.0[e]	De Robertis et al (38)

[a] Calculated on the assumption that 1 gram electroplax yields 7.5–15 mg protein in the final preparation.

[b] Calculated from the authors' estimate of 1.2×10^{18} proteolipid molecules/single electroplax weighing 35 mg.

[c] Calculated from the authors' value of 1.3×10^{-10} moles hexa bound/mg protein, while 1 g electroplax tissue yields 1.25 mg/proteolipid proteins.

[d] Two studies on the binding of ^{14}C-DMTC and ^{14}C-hexa to rat brain extracts (37, 59) could not be used due to insufficient information.

[e] Calculated from the dpm given for ^{14}C-DMTC bound/g tissue and specific activity of DMTC used.

TABLE 1—*(Continued)*

Tissue preparation	Ligand and Technique	Amount found nmoles/g original tissue	Ref.
Guinea pig cortex homogenate	^{3}H-ABGT/ultrafiltration	17.5	Bosmann (13)
Housefly brain-supernatant of 100,000×*g*	^{3}H-muscarone, ^{3}H-atropine	12–16[f]	Eldefrawi & O'Brien (15)
	^{3}H-nicotine, ^{3}H-deca/equilibrium dialysis		Eldefrawi et al (17)
	Muscle		
Dog intestine-pellets of <77,000×*g*	^{3}H-dibenamine/*in situ*	≃70[g]	Takagi & Takahashi (31)

[f] Calculated on the estimate that the brain of the housefly weighs about 15% of the total head weight.

[g] Calculated on the assumption that the final preparation contained 20 mg protein/g muscle tissue, and calculations of an average bound value of 3.35 nmoles/mg protein from the values of dpm in (test-control) and the specific activity of dibenamine.

bound to an anionic subsite of AChR with its quaternary ammonium group, while its maleimide group alkylated the sulfhydryl group formed by prior reduction by DTT (*22*). Binding of ^{3}H-MBTA was therefore studied by treating intact membranes and then digesting them. Binding was reduced by the competitor hexamethonium, as well as by the sulfhydryl reoxidizing agent, cholinedisulfide. The fact that hexamethonium was a more effective blocker than cholinedisulfide was unexplained. The concentration of AChR was suggested to represent 20–60% of the MBTA binding sites and was calculated to be 0.01–0.02 nmoles/g tissue.

An important criterion of in vitro identification of AChR is that ligands whose pharmacologic action is reversible should bind reversibly to AChR in vitro. De Robertis and co-workers (36–38) isolated nerve ending membranes from cerebral cortex of cat and rat, and studied binding of ^{14}C-DMTC and ^{14}C-hexamethonium by centrifugal assay and column chromatography. But binding of DMTC was "not displaced by rehomogenization and repeated washings" (*37*), which would indicate that this curare binding was irreversible, though its action is reversible in vivo. Furthermore, Triton X-100 did not solubilize the curare-binding material, in disagreement with the findings of several other groups who employed labeled ACh or α-BGT to identify AChR (13, *27*, Eldefrawi, unpublished). The irreversibility and the Triton-insolubility therefore suggest that this DMTC binding is not to AChR. This view is corroborated by the fact that the DMTC bound to the synaptosome fraction (36) can be calculated as only 14 cpm/g pellet, but in the myelin fraction it was 124 cpm/g pellet. Nevertheless, one can calculate

(Table 1) that the amount of DMTC bound to synaptosomes was about 1 nmole/g, which is a reasonable value for receptor concentration.

Reversible binding.—Two classes of membrane fractions were separated by Changeux and co-workers from crude homogenates of electric organs of *Electrophorus* by ultracentifugation in sucrose gradient: one rich in AChE and presumed to be derived from the innervated face of the electroplax, and the other rich in ATPase, presumably derived from the noninnervated membrane (39). These membrane fragments formed vesicles (microsacs) in solution in which they were able to measure influx and efflux of Na^+, K^+, and Ca^{++}. The most striking property of these microsacs was that the rate of $^{22}Na^+$ efflux from the first class of microsacs was considerably increased by the cholinergic agonists carbachol and decamethonium, and blocked by *d*-tubocurarine (40, 41). The apparent binding constant of several cholinergic ligands was determined by this technique and found to be almost identical to those determined from electrophysiological experiments on the monocellular electroplax preparation (42). This was evidence for the presence of AChR in this in vitro preparation.

Equilibrium dialysis has been used to study the binding of several ligands to preparations from eel electroplax. Changeux et al (43) found that ACh binding to the "innervated-face" membranes was anomalous in that it showed no saturation. It has been suggested (29) that the anomaly could be the result of dialysis at low ionic strength, a Donnan equilibrium artefact, or incomplete inhibition of the AChE present. Satisfactory binding of ACh to homogenates of *Torpedo* electroplax will be described below.

Using the deoxycholate-solubilized membranes, Changeux et al (41, 42) found that decamethonium bound with a single binding constant (0.8 μM) in an amount calculated as about 0.3 nmoles/g (Table 1). This K value compares favorably with that of 1.2 μM measured either physiologically on electroplax cells or by block of Na^{22} efflux from microsacs. The binding was blocked by five cholinergic ligands. Even through reversibility was not established, nor were noncholinergic ligands shown to be ineffectual, these findings very strongly implied that decamethonium bound to one macromolecule, namely AChR. However later work did not support this conclusion. Thus α-BGT blocked only 72% of binding of 0.6 μM decamethonium, and evidence suggested that the other 28% was bound to the ubiquitous AChE (44). Another study (45) on 28 randomly selected proteins showed that 0.01 μM decamethonium bound to ten of them (by contrast, DMTC bound to two, nicotine to one, and muscarone to none). Furthermore, we studied decamethonium binding to a particulate preparation from eel electroplax (45) and found four binding constants with K values ranging from 0.1 mM to 2.5 nM. One of them (K_3)had a dissociation constant of 2.5 μM and an amount of 0.25 nmoles/g; values close enough to Changeux's to suggest that they involve the same binding.

We also explored the binding of three other ligands to eel electroplax.

Two agonists, muscarone and nicotine, bound reversibly with Ks of 0.05 μM to a single site in the amount 0.021–0.033 nmoles/g electroplax (45). These values are very close to the values of 0.01–0.02 obtained by Karlin by measuring binding of ^{3}H-MBTA to DTT-treated electroplax cells (22). In addition since binding of muscarone and nicotine was blocked by three nicotinic ligands (45), it seems likely that the binding was to AChR. Binding of the antagonist DMTC indicated at least three affinities, of which two were very apparent; the high-affinity site was at 0.05 nmoles/g. Although this number corresponds well with that for AChR as judged by muscarone or nicotine binding to the same preparation, this DMTC binding differed in being insensitive to phosolipase C (45). It is clear that for several reasons decamethonium or DMTC binding are unsuitable indices of AChR activity.

Electric tissues of *Torpedo* and electric eel are generally similar pharmacologically (19–21, 27), but the former is probably a better source of AChR because it is richer in all components of the cholinergic system (see above). We studied binding of several cholinergic ligands to a particulate fraction (pellet of 12,000 x g) from electric tissue of *Torpedo* using equilibrium dialysis (17, 28–30). Multiple sites for reversible binding of the various ligands were revealed, two each for muscarone, nicotine, and DMTC and three for decamethonium. The two agonists muscarone and nicotine each showed a low affinity binding of approximately 0.5 nmole/g and a high affinity binding of approximately 0.1 nmoles/g. The effect of hydrolases and the antagonism of binding by other cholinergic ligands led to the suggestion that binding is to two different sites, which exhibit binding properties similar to AChR. Decamethonium and DMTC were found to bind to other distinct sites and maybe also to the sites binding muscarone and nicotine. The additive concentration of these sites is 20–50 times higher than their counterparts in *Electrophorus* (Table 1). This is in line with the 8–20 × higher concentration of AChE found in electric tissue of *Torpedo* (22–24).

The major problem in using binding of the transmitter ACh to identify AChR in vitro was that enough AChE was present in preparations used for in vitro studies to hydrolyze ACh rapidly. This was overcome when we found that 0.1 mM concentrations of several organophosphates could irreversibly inhibit all the AChE present in electric tissue of *Torpedo* without interfering with binding of muscarone, nicotine, or ACh. At higher concentrations, organophosphates reversibly blocked binding of these agonists to the proposed AChR sites (46), in good agreement with the pharmacological effects of organophosphates on AChR of the monocellular preparation of eel electroplax (47). We were thus able to study binding of ^{3}H-ACh to the subcellular preparation of *Torpedo* electroplax, which also lacked choline acetyltransferase (unpublished). Two high affinity sites (K_1 = 8 nM and K_2 = 68 nM) bound ACh reversibly and binding was blocked by nicotinic drugs (29). Characteristics and concentrations of these sites were similar to the ones binding muscarone and nicotine, which led to our proposal that the

same two sites bind the three ligands. The additive concentration of the two binding sites (0.93 nmoles/g tissue) for ACh was remarkably similar to that obtained by measuring the irreversible binding of α-BGT to the same tissue (27) (Table 1).

The multiple binding affinities observed in electric tissue of *Torpedo* may be due to one or more of the following alternatives. There may exist more than one macromolecule that binds cholinergic ligands; DMTC was suggested to fit this category (30). Alternatively, the two affinities found for agonists may result from binding of two sites on the same macromolecule which may either not interact or be antagonistic (48, 49). Another possibility is that the affinities observed are due to binding of ligands with different configurations to similar sites, according to the model recently proposed by Laiken & Némethy (50).

An apparent discrepancy in our data is the fact that binding constants of cholinergic ligands are much lower than those reported from physiological experiments (51, 52). This discrepancy may be due to one or more of the following reasons: Responses measured in vivo reflect both the combined effect of affinity for the ligand and its ability to depolarize or block. Also, barriers to permeability are known to exist for ions, so that actual concentration of the agonist or antagonist at the active site is unknown. Such factors required making many assumptions in calculations of in vivo values (51, 53), e.g. agonists elicit a response of only one type in the effector and that the effect measured is directly proportional to occupancy. On the other hand, the in vitro binding constants are thermodynamically feasible; e.g. one coulombic bond plus one hydrogen bond plus one hydrophobic bond can give at 4°C a value of K = 4 n*M*. Differences between in vitro and in vivo dissociation constants in the same direction and of similar magnitude (140–510) have also been found for AChE of the eel electroplax (54). However, all this does not mean that the physiological findings are irrelevant. If occupation theory is basically correct, the directly measured true dissociation constants must always be equal to or lower than the physiological values. And we believe that occupation theory does hold for AChR, because agonists as well as antagonists both have dissociation constants in vitro in the same range (29, 30, 42, 45) in opposition to the rate theory which "predicts low affinities for agonists compared to antagonists" (55)

Brain is undoubtedly the most interesting source of AChR, but also the most difficult from which to obtain physiological and pharmacological information. Unfortunately, vertebrate brain tissue has a variety of transmitters and their receptors and a relatively low content of the cholinergic macromolecules as compared to electric tissues. However the brain of the housefly equals the eel electroplax in its content of AChE (14). We found that the supernatant (100,000 × *g*) from homogenates of heads of houseflies bound muscarone reversibly and with high affinity (15). Binding was blocked by cholinergic but not by noncholinergic drugs, and exhibited both nicotinic and muscarinic characteristics as compared to the pure nicotinic nature of

electric tissues of fish. Binding of four other cholinergic ligands to the same preparation suggested that muscarone, nicotine, decamethonium, and atropine bound to a common site on AChR present in the amount of 0.4 nmole/g brain (Table 1). DMTC was nonspecific and bound to sites 20 times higher in concentration. Further evidence that the agonist-binding macromolecules were AChR was the good correlation found between the toxicity of nicotine and five analogs to houseflies, and their ability to block binding of muscarone and nicotine (16).

In the membrane fraction derived from synaptosomes of whole rat brain, Farrow & O'Brien (unpublished) found that atropine binds to two sites totalling 1.0 nmoles/g, one K was 0.9 μM and the other was 0.6 nM, agreeing amazingly well with the two values reported by Paton & Rang (33) for guinea pig ileum, K = 0.5 μM, and 1.1 nM. The high-affinity atropine binding was blocked by scopolamine, but not by eight other cholinergic and seven noncholinergic drugs. But even at 0.01 μM, atropine binding was observed with liver fractions (yet little with kidney and lung) perhaps due to an atropinesterase. By contrast, muscarone at 0.01 μM did not bind to preparations from liver, kidney, or lung. Even more important, 0.01 μM muscarone did not bind to synaptic vesicles, so that these were not contributing to the reported binding, even though the vesicles' roles as ACh storage sites would raise that possibility.

B. BINDING OF CHOLINERGIC LIGANDS IN ORGANIC MEDIA:

A different approach of great interest even though it produces results which are not easy to compare with those of other laboratories is that of De Robertis and co-workers (9, 36, 56-61) who extracted proteolipids from brains and electric tissues into chloroform-methanol, and studied their binding of cholinergic ligands. When proteolipids and radiolabeled ligands were chromatographed on Sephadex LH-20, the label was eluted along with proteolipid peaks, which were therefore suggested to be AChR. Several cholinergic ligands were used, ^{14}C-DMTC for brains, and ^{14}C-ACh, ^{14}C-hexamethonium, and ^{3}H-TDF for electric tissues of eel and *Torpedo*. They also used other methods to measure binding to proteolipids from cerebral cortex. Atropine sulfate was found to increase polarization of fluorescence of the proteolipid from bovine cerebral cortex, and this effect was blocked by ACh and homatropine bromide (58). Atropine sulfate also increased light scattering by the proteolipid from cat and ox brains in chloroform-methanol, and this effect was inhibited by ACh, DMTC, succinylcholine, and hexamethonium (56). But, the fact that they found "the light scattering phenomenon is not stereospecific since it may be obtained with other bivalent amines such as sulfates of eserine, amphetamine, dibenzylamine, and strychnine," and not by atropine base and monovalent salts of atropine, casts doubt on the claim that these proteolipids exhibit properties of muscarinic AChR. They postulated "that in central synapses there is a receptor proteolipid which has group specificity rather than stereospecificity for the various

amines," and that probably "once the receptor proteolipid has been separated from the membrane, its stereospecificity is lost." This is contrary to the finding by others studying binding of cholinergic ligands to soluble brain extracts; that AChR-like macromolecules retained their stereospecificity (*13, 15*).

Binding was measured in all the work of De Robertis and associates on proteolipids in an organic phase, where the change in polarity of the microenvironment around the active site should affect binding properties (*62*). They did not demonstrate reversibility of binding of the ligands whose pharmacologic action is reversible. The fact (Table 1) that the concentrations of binding sites calculated from their data for different tissues were much higher than those determined by others for the same tissue (in electric tissues 2–50 × for eel and 160 × for *Torpedo*), suggests that most of the binding is not to AChR. Confirmation of this view is provided by the finding that binding activity of AChR (as measured by equilibrium dialysis) was destroyed by treatment with chloroform-methanol and other organic solvents (*28*); furthermore, the α-BGT macromolecular complex from *Torpedo* electroplax or brain was not extractable by this solvent mixture (13, Potter, personal communication).

Purification of AChR Macromolecules

Purification of the macromolecules suspected to be AChR is in progress in several laboratories. The deoxycholate-solubilized protein from eel electroplax was extracted from the innervated membrane, thereby achieving some purification (*44*). Approximate values can be calculated for purification of α-BGT-binding AChR from *Torpedo* electroplax (*27*): about 180-fold by extracting synaptic membranes followed by solubilization by Triton ×-100 and SDS.

The macromolecules suggested to be AChR were found in units of varying molecular weight. In *Torpedo* electroplax, about 80% of the macromolecules had a molecular weight higher than 300,000 daltons and 20% lower, as judged by membrane filtration in presence of Lubrol XW (Eldefrawi, unpublished). AChR obtained using Triton X-100 and ultracentrifugation in density gradients and gel filtration, had a molecular weight between 250,000 and 600,000 daltons, but smaller units of 180,000 and 88,000 daltons were found in presence of SDS (*27*). The following smaller values for the molecular weight of AChR were also reported when SDS-disc gel electrophoresis was used: 40,000 in eel electroplax (*63*) and 76,000 in guinea pig brain (*13*). There is as yet no evidence that the smaller units are functional AChR, i.e. that they can bind ACh. SDS is known to dissociate polypeptides from their macromolecular components (*64–66*).

Acetylcholinesterase and Acetylcholine Receptor

AChE and AChR have many properties in common, e.g. presence in the same tissue fractions, similar molecular weights, and comparable effects by

chemical reagents. DTT, TDF, EEDQ, and dibenamine inhibit hydrolysis of ACh by AChE and bind or block binding of cholinergic ligands to AChR (31, 34, 67–70, Eldefrawi, unpublished). These similarities led to suggestions that they are identical macromolecules (71, 72). Yet there is now overwhelming evidence against this and also against the suggestion that the depolarization caused by ACh results from its hydrolysis by AChE and the accumulation of choline cations that induce the permeability changes (73).

The following data demonstrate the dissimilarities between the catalytic site of AChE and the ACh binding site of AChR: (a) ACh agonists and antagonists (e.g. muscarone, nicotine, decamethonium, curare, and atropine) have much higher affinities for AChR than AChE (30, 41, 74–76). (*b*) ACh, acetylthiocholine, and acetylselenocholine are hydrolyzed by AChE at similar rates, but have widely different depolarizing potencies (77). (*c*) Several organophosphates phosphorylate AChE irreversibly at low concentrations without affecting depolarization of the innervated membrane of eel electroplax; only at much higher concentrations do these cause its reversible repolarization and block binding of cholinergic ligands in vitro (46, 47). (*d*) Analogs of benzoquinonium and ambenonium derivatives have effects on depolarization of eel electroplax that differ markedly from their effects on the inhibitory action on AChE in vitro (78). (e) AChE is degraded by papain and not phospholipase C, whereas the opposite is true for AChR measured by binding of muscarone to *Torpedo* extracts (28). (*f*) After toluene extraction of the fraction from *Torpedo,* there is no binding of muscarone, but AChE is still active (28). (*g*) They have different sensitivities to pH (12). Exposing AChE to 48°C for 20 min destroys it, whereas about 30% of the suspected AChR, as judged by the specific decamethonium binding, survives (79).

Differences found between the active sites of AChE and AChR do not exclude the possibility that a single molecule carries both sites, especially since the ratio of catalytic sites of AChE to binding sites of AChR is near unity. For eel the ratio is calculated to be 0.8 (44) or 0.5 to 1.3 (22), and for *Torpedo* 0.3 (12). Several experiments have shown that ACh agonists and antagonists (e.g. curare, gallamine, TEA, decamethonium) affect kinetics of hydrolysis of ACh (80) or decarbamylation (81) or sulfonylation (82) of AChE, to which they must therefore bind. Also, the potency of series of alkyltrimethylammoniums and polymethoniums related to decamethonium on AChE parallel their depolarization or blocking potencies (82, 83). These led to the suggestion by Zupancic (80) that the anionic center of the catalytic site of membrane-bound AChE is the active site of AChR, and to the use by Belleau of AChE as a model for AChR (83), and to the suggestion by Changeux, Podleski & Meunier (68, 84) that a peripheral anionic site on AChE (that seems to bind TDF and decamethonium) may function as AChR.

Physical separation of a macromolecule that binds α-BGT but does not hydrolyze ACh, has been achieved by gel filtration or density gradient frac-

tionation in the presence of SDS (27). Using a Lubrol XW-solubilized preparation from *Torpedo* electroplax, we recently achieved partial separation on Sepharose 6B of AChE from AChR, judging the latter by appropriate ACh binding (unpublished). Also when most of AChR in the deoxycholate extract of eel electroplax bound irreversibly to α-BGT coupled to Sepharose 4B, it sedimented in a low speed pellet, leaving most of AChE in solution (79). These partial separations of AChR activity from AChE support the suggestions that they are separate macromolecules. Also, whereas muscarone and ACh at 1 μM bound to the macromolecules suggested to be AChR in different tissue preparations, they did not bind at this concentration to purified AChE from eel electroplax or erythrocytes (15, 29). Final proof awaits isolation of pure AChR.

Chemical Nature and Drug Profile

Nachmansohn (85) has long proposed, on theoretical grounds, that AChR is a protein; and this proposal has been strengthened by data from in vitro studies. Degradability of AChR by hydrolytic enzymes gives crude estimates of its gross chemical nature. Since binding of suspected AChR from brains of houseflies (15) and guinea pigs (13) was affected by trypsin, chymotrypsin, but not by phospholipase C or other hydrolases, the AChR macromolecules were classified as proteins. On the other hand, we classified AChR of electric organs of eel and *Torpedo* as phospholipoproteins because binding of ACh, muscarone, nicotine, and decamethonium was reduced by prior treatment with these three enzymes (28, 30, 45). The α-BGT binding macromolecules from *Torpedo* were classified as proteins without evidence (27). The dibenamine binding macromolecules from muscles were determined to be proteins by the degrading effect of pronase and trypsin (31). By contrast, De Robertis considers that AChR as well as other receptors from several sources are proteolipids which are extractable by chloroform-methanol (36, 56–61).

The use of active-site-directed reagents that react with specific functional groups in proteins, provides partial but more specific chemical identification of the receptor. It was shown both with the monocellular eel electroplax and with in vitro reduction of binding of ACh to *Torpedo* electroplax, that AChR in electric tissues has disulfide bonds, sulfhydryl and carboxyl groups, and one or more of the amino acids vulnerable to diazotization by TDF (34, 35, 68, 84, 86, 87, Eldefrawi, unpublished).

Comparing the effect of pretreatment with several reagents on binding of ACh and decamethonium to macromolecules in a particulate preparation from electric tissue of *Torpedo,* we found that they were affected differently by DTT and Tetram (unpublished). Reduction of binding of ACh to AChR by different concentrations of decamethonium, suggested that there are sites on AChR that bind decamethonium with high affinities, and are distinct from ACh-binding sites; the latter sites also bind decamethonium but with lower affinities.

An important matter is the extent to which binding to isolated receptors mirrors the drug sensitivity of the physiological receptor. Those drugs whose physiological interference is with the agonist binding step should show blockade of agonist binding to the isolated receptor. By contrast, if a drug that is inactive physiologically blocks binding to isolated material, the receptor identity of that material is in question.

It is pleasant to report that in fact the drug profile of several isolated AChR preparations follows closely the physiological profile. Thus AChR isolated from electric tissues were shown to be of a neuromuscular nicotinic type, as evidenced by the effectiveness of several nicotinic drugs and the ineffectiveness of nonnicotinic and noncholinergic ones in reducing their binding of muscarone, ACh, nicotine, and decamethonium (17, 28–30, 45); and also the effect of curare and carbachol in retarding binding of α-BGT (27). On the other hand, AChR from brains were either muscarinic (Farrow & O'Brien, unpublished) or nicotinic (13) when binding of atropine and α-BGT were studied, respectively; indicating the possible existence of both kinds of AChR in brains. Pharmacology of the brain of the housefly is unknown, and its AChR represent a different picture. Binding of muscarone, nicotine, decamethonium, and atropine was reduced by both nicotinic and muscarinic drugs, suggesting that their AChR are of mixed nicotinic-muscarinic nature (15–17); maybe of a kind similar to that of crayfish stretch receptor neurons (94).

Desensitization and Sensitization

Desensitization is the phenomenon whereby treatment with high concentrations of depolarizers can cause inactivation of AChR. It has been observed with many agonists and many preparations, and also by repetitive stimulation (88–93). Using equilibrium dialysis, we found that in vitro binding of ACh to *Torpedo* AChR saturated at 1 μM and at higher concentrations there was reduction in binding. This inhibitory effect was reversible upon removal of the excess ACh from the dialysis medium (95). Thus it was suggested that this autoinhibition of ACh binding to AChR in vitro parallels the physiological phenomenon of desensitization, and results from ACh binding to regulatory sites on AChR. This causes AChR to take a new and inactive conformation, and in the process reject ACh molecules bound to a larger number of active sites. We suggested that binding of small cations (possibly Ca^{++}) to AChR is also involved (physiological desensitization is known to be Ca^{++}-dependent (96, 97)), If this relationship holds, then it would provide a mechanism for the conformational change in AChR that causes desensitization. Supporting evidence for the reduced binding to a desensitized AChR, is the finding that ACh-desensitized-muscle bound less α-BGT (27). With another technique, photo-affinity labeling with two quaternary ammonium aryl azides, AChR at neuromuscular junctions of whole-frog sartorius muscles were irreversibly inactivated; and at higher concentrations the azides produced less inhibition of AChR (98). The authors sug-

gested that the effect was due to competition by photolysis product; but it could also be explained by an autoinhibition effect of the aryl azides themselves, similar to that described above for ACh.

Denervation of muscle leads to sensitization, i.e. a spreading increase of sensitivity to ACh and other agonists (99). After denervation of diaphragm muscle, there were about 20- to 200-fold increases in the amount of α-BGT bound to end plates and outside end plates, respectively (Potter, personal communication). In these experiments, binding of α-BGT occurred in vivo before extraction of the binding macromolecules.

Conclusion

The parallelism between in vivo and in vitro data is a convincing argument that AChR is extractable and retains its properties in vitro. With certain exceptions already noted, it appears that several laboratories, using different tissue sources and techniques, are converging upon the solubilization and preliminary fractionation of several AChR. None has yet prepared a highly purified AChR, and consequently characterization is in the future, but perhaps only a year or two away. Then will come the real task, that is, the full explanation of the molecular basis of the transduction of ACh-binding to ion-conductance. It may turn out that what is defined herein as AChR, i.e. the ACh-recognizing molecule, will be but one component, teamed up with an ionophore or perhaps integrated with a whole matrix that responds to configurational changes in the AChR (84, 100). But these are only speculations at present.

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