THE CHOLINERGIC RECEPTOR PROTEIN IN ITS MEMBRANE ENVIRONMENT[1]

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INTRODUCTION

The cholinergic receptor protein from fish electric organ is the first pharmacological receptor with a well-characterized physiological function to have been purified and isolated in milligram quantities (1–11). Nevertheless, the mechanism by which this regulatory protein (12) controls membrane permeability and the binding of acetylcholine remains largely unknown. Therefore, we focus this review on the receptor protein in its membrane environment. The electric organs from *Electrophorus,* a fresh water Teleost, and *Torpedo,* a sea water Elasmobranch, are still the most convenient systems for parallel electrophysiological and biochemical studies. We devote this review to work done on single cell preparations (electroplaques) and on membrane suspensions prepared from these organs. We often refer to ligands which, in addition to the typical nicotinic ligands, have been particularly useful for the selective labeling of cholinergic receptor sites: the snake α-toxins (13, 14) and affinity labeling reagents (15, 16). Several reviewers have discussed the anatomy and electrophysiology of the electric organs (17, 18) as well as the characterization, isolation, and purification of the cholinergic receptor from these organs (16, 19, 20).

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LOCALIZATION OF RECEPTOR SITES

Both *Electrophorus* and *Torpedo* electroplaques are asymmetrical cells which receive nerve terminals on only one face (the caudal one in *Electrophorus,* the ventral one in *Torpedo*), but the distribution of the synapses differs. In *Electrophorus* electroplaques (dimensions: 10 mm X 1 mm X 0.3 mm), the 10^5–10^6 synaptic contacts cover only about 1–2% of the total surface of the plasma membrane of the innervated face (21), rather than 30% as proposed previously (22). The lower value comes from a reanalysis of the stereology of the innervated membrane: microinvaginations (23) present almost exclusively between the synapses cause an increase of that surface by a factor of 10 (21). In *Torpedo* electroplaques (dimensions: 5 mm X 5 mm X 0.02 mm), the synaptic areas cover as much as 50% of the innervated membrane (24–26). Because of these morphological differences, *Torpedo* electric organ should be a richer source of subsynaptic membrane, and therefore of cholinergic receptor sites, than that of *Electrophorus.*

The *total number* of cholinergic receptor sites per single electroplaque has been measured, in the case of *Electrophorus,* with ^{3}H α-toxin from *N. nigricollis* (21). One to 2 X 10^{10} toxin sites per mg (wet weight) of electroplaque were found. Similar values (0.5–1.0 X 10^{10} sites per mg of electroplaque) have been obtained with a cholinergic affinity label, but after reduction of a disulfide bridge in the receptor protein (22). These numbers are smaller than those expected from the content of *Electrophorus* electric tissue in α-toxin sites determined from the amount of α-toxin bound to membranes isolated from tissue homogenates [50–100 nmol α-toxin sites per kg fresh tissue (1, 27, 28)]. The difference probably comes from the fact that the single electroplaques were dissected from the organ of Sachs which is morphologically distinct from the main organ used for the preparation of tissue homogenates. The total number of cholinergic receptor sites per single *Torpedo* electroplaque has not yet been measured. The content of α-toxin sites in that tissue after homogenization is much higher [1000 nmol per kg of fresh tissue (26, 28)] than that found in *Electrophorus* main organ.

Immunofluorescence studies using rabbit antiserum directed against *N. nigricollis* α-toxin (29) and autoradiography with ^{3}Hα-toxin (21) showed that in *Electrophorus* electroplaque the sites that selectively bind the α-toxin are almost exclusively on the innervated membrane. Upon taking into account the stereology of the cytoplasmic membrane, quantitative autoradiography at high resolution (21) reveals that the density of α-toxin sites under the synapses (30,000 $\pm$ 10,000 sites per μm^2) exceeds by a factor of 100 that found on the innervated membrane between the synapses and by a factor of 1000 that measured on the noninnervated membrane (which most likely represents the background of nonspecific toxin binding). Recent studies (30) indicate that because of an underestimation of the yield between disintegrations and silver grains, these values are
grains under the synapse, and because of the large fraction of extrasynaptic cytoplasmic membrane surface the ratio of extrasynaptic to subsynaptic receptor sites is about one in *Electrophorus* electroplaque.

At vertebrate neuromuscular junctions, the high density of toxin sites which exists under the nerve terminals has been strikingly visualized with a fluorescent derivative of α-bungarotoxin (31). Electron microscope autoradiography with radioactive α-toxins gives 5,000–10,000 α-toxin sites per μm^2, if one assumes a uniform distribution of toxin sites on the postsynaptic membrane (folds included) and an equal accessibility of all the sites to the toxin, even at the bottom of the folds (32–35). The first assumption has been questioned recently (35), but not the second. Further studies are therefore needed to clarify this point.

The purified receptor protein from *Electrophorus* appears on electron micrographs, after negative staining, as a ring-like particle 8–9 nm in diameter consisting of 5–6 subunits each 3–4 nm in diameter and surrounding an electron-dense pit (6, 36). That shape is quite different from the various molecular forms of acetylcholinesterase of *Electrophorus* seen in electron micrographs (51). For membrane fragments from *Torpedo*, in which on the basis of the α-toxin site concentration about 20% of the proteins consist of receptor protein (37), about 50% of the membrane surfaces show, after both freeze etching (36) and negative staining (36, 38), a high density of particles of similar dimension and shape to those of the receptor protein purified from *Electrophorus*. These particles make hexagonal arrays with a surface density of 10,000–15,000 particles per μm^2. This density is consistent with that found with the tritiated toxin in *Electrophorus* electroplaque if one assumes that each particle represents one receptor oligomer [about 250,000 mol wt (3, 39)] and that the receptor oligomer carries several toxin sites.

X-ray diffraction studies of *Torpedo* receptor-rich membrane fragments confirm the presence of an ordered structure in the plane of the membrane consistent with a 9 nm center-to-center distance of the diffracting units (40). Particulate arrays are also observed under the nerve terminal after freeze-fracture of a piece of electric tissue. Dense arrays of 6 nm particles are associated with the surface nearest the cleft while 8 nm particles are associated with the inner leaflet (41). If the fracture plane does split the postsynaptic membrane, the particles are associated with inner surfaces in vivo, while on the isolated membrane fragments the particulate structure becomes apparent only after deep etching and seems therefore to be directly exposed on the membrane surface. If the particles observed in both cases represent the receptor protein, then the differences noticed might be caused by a reorganization of membrane structure resulting from homogenization and fractionation.

Various cytochemical methods indicate that the enzyme acetylcholinesterase is located almost exclusively on the innervated membrane of the electroplaque both under and between the synapses (42–44). At least at vertebrate motor endplates, almost 90% of the sites for ^{3}H-diisopropyl fluorophosphate, a cholinesterase inhibitor, are located in postneuronal regions (45, 46). If in these regions the receptor protein forms an almost continuous lattice structure, it is likely that acetylcholinesterase is not integrated in the postsynaptic membrane in the same manner as the receptor protein (47). In fact, collagenase treatment releases esterase activity from skeletal neuromuscular junctions without impairing the resting permeability of the fiber and the physiological response to acetylcholine (48, 49). Also, salt washes

of electric tissue solubilize the esterase (50) but not the receptor (1), and membrane fragments purified from *Torpedo* electric tissue contain less than one catalytic site of acetylcholinesterase per 100 α-toxin binding sites (37).

RESPONSE OF THE ELECTROPLAQUE

Basic Physiology and Pharmacology

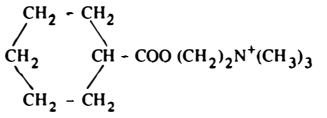
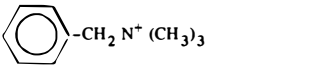
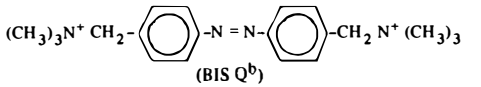
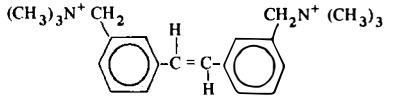
Electrical stimulation of the nerve terminals which cover the innervated membrane of both *Electrophorus* and *Torpedo* electroplaque evokes a transient (5–15 msec) excitatory postsynaptic potential (EPSP) which generates a tetrodotoxin-sensitive action potential in *Electrophorus* but not in *Torpedo* (18). The electroplaque EPSP resembles the endplate potential at neuromuscular junction: it is blocked by *d*-tubocurarine and snake α-neurotoxins, and at least in *Electrophorus* it involves an increase of permeability for Na^+, K^+ and perhaps Ca^{2+} (52). The total inward Na^+ current during the EPSP ranges between 75 and 100 mA/cm^2 of window area and the increase of conductance for Na^+ and K^+ ($\Delta g Na_s$ and $\Delta g K_s$) lies between 0.05 and 0.27 mho/cm^2 of window area with $\Delta g Na_s / \Delta g K_s$ close to unity, as at the neuromuscular junction (53, 54). Spontaneous depolarizations analogous to the miniature endplate potentials have been recorded in both *Electrophorus* (55) and *Torpedo* (26) electroplaques.

Few pharmacological experiments have been done with *Torpedo* electroplaques (56). On the other hand, individual electroplaques from *Electrophorus* can be dissected from Sach's organ, isolated, and studied in an artificial environment (57). The effect of cholinergic ligands has usually been studied by bath application of a solution of the compound tested on the innervated membrane (58), though iontophoresis can be used (55). Because of the large surface area and low resistance of the innervated membrane, voltage clamp techniques appear difficult to handle (see discussion in 18). Current pulse techniques have been used to determine membrane resistance (52, 59), but usually only transmembrane potential has been monitored. Bath application of carbamylcholine which is resistant to acetylcholinesterase causes within a few seconds a decrease of membrane potential to a plateau value that may last as long as an hour and is dependent on drug concentration (58). However, more fundamental parameters such as $^{42}K^+$ efflux (60) or membrane conductance (59) increase only transiently. For carbamylcholine concentrations above 30 μM, the conductance reaches a peak in about 1 min and then decreases to a plateau value (lasting at least 10 min) with peak and final plateau values being dependent on drug concentration (61). At 1 m*M* carbamylcholine the value of the plateau is about 20% of the maximum. For certain agonists, exposure of the innervated membrane to high concentrations causes a transient change of membrane potential (62–64). These transient changes of membrane properties are reminiscent of the phenomenon of "pharmacological desensitization" reported at the myoneural junction (65, 66). Such a phenomenon makes the interpretation of the physiological response of the electroplaque in terms of molecular events difficult. Additional difficulties include the following: 1. under conditions of bath application, both the subsynaptic and

extrasynaptic areas [which contain the cholinergic receptor at vastly different densities (21)] become exposed to the agonist; 2. during prolonged exposures, local changes of ionic concentration inside the cell or in the neighborhood of the excitable membrane might take place while the total ionic concentration inside the cell does not vary significantly (67, 68).

Nevertheless, empirical dose-response curves have been constructed using the steady-state depolarization as an index of the response to agonists (depolarizing agents) applied in the bath (58). Structure-function relationships for nicotinic ligands have been recently reviewed in the case of the neuromuscular junction (69) and of the *Electrophorus* electroplaque (70). Some relevant data for various compounds that act as agonists on *Electrophorus* electroplaque are given in Tables 1 and 2. Two characteristic parameters of the dose-response curve are shown: the maximal response and the apparent dissociation constant, K_{ap}, the concentration of agonist

Table 1 Drugs acting as agonists (depolarizing agents) at the innervated membrane of *Electrophorus* electroplaque[a]

Agonist	Ref.	K_{ap} (M)	Maximum depolarization (mV)
$CH_3CO_2(CH_2)_2N^+(CH_3)_3$	147, 148	2×10^{-6}	60
$C_3H_7CO_2(CH_2)_2N^+(CH_3)_3$	149	4×10^{-6}	60
	62	4×10^{-4}	15
$CH_3(CH_2)_3N^+(CH_3)_3$	63	5×10^{-6}	60
	63	10^{-5}	60
$(CH_3)_3N^+(CH_2)_{10}N^+(CH_3)_3$	71	2×10^{-6}	45
 (BIS Q[b])	150	7×10^{-8}	40
 (CT 5334[c])	151	2×10^{-6}	35

[a] The activities of the various ligands are characterized by the maximum depolarization they cause during bath application and by the drug concentration causing half the maximal depolarization (K_{ap}).

[b] The *cis*-isomer is apparently inactive (150).

[c] The 4,4'-isomer acts as a competive inhibitor, $K_I = 1 \times 10^{-6}$ M (151).

Table 2 Interaction of cholinergic ligands and local anesthetics with *Electrophorus* and *Torpedo* receptors in vivo and in vitro[a]

	Electrophorus		Torpedo		
	K_{ap} (in vivo) (*M*)	K_{ap} (in vitro) (*M*)	K_p (α-toxin) (*M*)	K_p (α-toxin) (*M*)	K_D (*M*)
Agonist					
Acetylcholine	2×10^{-6}	—	1×10^{-6}	8×10^{-9}	8×10^{-9}
Decamethonium	2×10^{-6} (1×10^{-5})[b]	1×10^{-6}	8×10^{-7}	8×10^{-7}	7×10^{-7}
Carbamylcholine	3×10^{-5} (3×10^{-4})[b]	4×10^{-5} (9×10^{-5})[c]	4×10^{-5}	5×10^{-7}	—
Antagonist					
d-Tubocurarine	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}[d]
Gallamine	3×10^{-7}	3×10^{-7}	4×10^{-7}	8×10^{-6}	1×10^{-5}[d]
Hexamethonium	3×10^{-5}	6×10^{-5}	6×10^{-5}	4×10^{-5}	
Anesthetics					
Procaine	1×10^{-4}	6×10^{-4}	1×10^{-3}	1×10^{-3}	5×10^{-3}[d]
Dimethisoquin	2×10^{-6}	—	5×10^{-5}	1×10^{-5}	6×10^{-5}[d]
Prilocaine	8×10^{-5}	—	5×10^{-3}	$>5 \times 10^{-3}$	$>5 \times 10^{-3}$[d]

[a] K_{ap} (in vivo): apparent dissociation constants determined from depolarization response of monocellular electroplaque (58, 71, 83, 147, 148, 152). K_{ap} (in vitro): apparent constants determined from the flux response of isolated *Electrophorus* vesicles (123). K_p (α-toxin): protection constants determined from the ability of drugs to decrease by 50% the initial rate of binding of the ^{3}H-α-toxin of *N. nigricollis* to isolated membranes (83). K_D: equilibrium dissociation constants from the binding of radioactive ligands to purified *Torpedo* membranes (83, 106, 145).

[b] K_{ap} from conductance response [monocellular electroplaque in presence of Ba^{++} and ouabain (61)].

[c] See (4).

[d] Dissociation constants determined from displacement of ^{3}H-acetylcholine.

that produces half of the maximal response. Different agonists often cause different maximal responses (Table 1). Since the ratio of the maximal depolarizations caused by decamethonium and carbamylcholine remains the same when the ionic composition of the solution bathing the innervated membrane was changed to high K^+ or low Na^+ concentrations the different maximal responses probably result from a difference in the quantity of ions transported rather than a difference in the ionic selectivity of the response (71). Different maximal conductance responses have also been determined for decamethonium and carbamylcholine (61). Although the *relative* values of K_{ap} for carbamylcholine and decamethonium do not change when slope conductances are measured instead of steady-state potentials, the absolute values of K_{ap} for both agonists increase by a factor of 10 (61) (Table 2). This difference is another indication that the depolarization response is not clearly related to the more fundamental conductance response.

As at the neuromuscular junction (72, 73) the dependence of the amplitude of the steady-state depolarization (71, 74) and the increase in conductance (75) on concentration does not follow an hyperbola but has a sigmoid shape (Hill coefficient: 1.8 for carbamylcholine, 1.6 for decamethonium). Furthermore, treatment of the electroplaque with agents that react with SH groups or reduce disulfide bonds results in a decrease of the sigmoid character of the curve (74, 76, 80). Presence of low concentration of the agonist decamethonium converts the dose-response curve for carbamylcholine from sigmoid to hyperbolic, while the presence of a competitive blocking agent, gallamine, causes a slight increase in the sigmoid character of the curve (59). These effects are reminiscent of some classical properties of regulatory

proteins (77). Different models have been proposed to account for such an apparent cooperativity (for a recent review, see 78), but it appears premature to decide in favor of any of them in the absence of a sufficiently precise comparison of the binding curve of the considered ligand and the appropriate change of membrane permeability. However, cooperative binding of acetylcholine has been shown to take place with receptor-rich membrane fragments from *Torpedo* (see later section).

Two major classes of agents reversibly block the response to the agonists: those that shift the dose-response curve towards higher concentration of agonists without changing the maximal response ("competitive antagonists") and those that reduce the maximal response without significant change of the apparent dissociation constant. Classic competitive antagonists of the action of carbamylcholine include *d*-tubocurarine (58), gallamine triethiodide (71), and certain benzoquinonium and ambenonium derivatives (79). Some quaternary ammonium antagonists have mixed effects, decreasing both the apparent affinity and the maximal response. This happens when *d*-tubocurarine is used to block the response to decamethonium (80), while a series of tetra(*n*-alkyl)ammonium derivatives modifies primarily the maximal response to carbamylcholine (63).

Aromatic amine local anesthetics have primarily a reversible effect on the maximal response to carbamylcholine and therefore are referred to as "noncompetitive" blocking agents, though their action is by no means specific for the response of the postsynaptic membrane to cholinergic agonists (81–83). At the vertebrate neuromuscular junction they modify both the time course and magnitude of the postsynaptic response (84–87), an observation that suggests they affect the response to acetylcholine at a site distinct from the cholinergic receptor site (perhaps the ion permeation site or a structure involved in its coupling with the receptor site). Histrionicotoxin, a neurotoxic alkaloid isolated from the skin of the Colombian frog *Dendrobates histrionicus*, shows similar properties as it modifies both the response to acetylcholine and muscle action potential at the murine motor endplate (88, 89). The binding of a radioactive derivative to endplates has been studied, and over the physiologically active concentration range the binding of the toxin appeared nonsaturable (89).

Compounds that bind irreversibly, or slowly reversibly, to the receptor site also affect the maximal response with little change of the apparent dissociation constant but obviously through an entirely different mechanism. This is the case for snake α-neurotoxins (90) and for several affinity reagents including compounds such as *p*-trimethylammonium benzene diazonium (91, 92) and those subsequently developed by Karlin and co-workers, which react irreversibly with the cholinergic receptor site only after reduction of disulfide bonds by treatment with dithiothreitol (DTT) (for a recent review see 15). As already mentioned, one of the latter, 4-(N-maleimido)-α-benzyl trimethylammonium iodide (MBTA), has been used to count the number of receptor sites per electroplaque (22) and also to identify a polypeptide chain associated with the receptor site during various stages of purification (5, 93). MBTA blocks the response to agonists without changing the membrane potential, while others (α-bromoacetylcholine or the *p*-nitrophenylester of carboxyphenyl trimethylammonium) depolarize the reduced electroplaque (94). The depolarization

caused by these compounds is temporarily reversed by high concentrations of *d*-tubocurarine and returns when the reversible antagonist is washed out (94). Only the depolarization caused by bromoacetylcholine could be reversed by a subsequent incubation of the electroplaque with the α-toxin from *Naja naja siamensis* (95). The simplest interpretation of these observations is that the reactive SH group exists at the periphery of the receptor-active site and that the trimethylammonium function interacts reversibly with a critical anionic subsite. Enough rotational flexibility exists in the molecule so that the covalent agonist can be displaced from the critical anionic subsite by the competitive antagonist.

Recently antisera have been prepared against purified receptor proteins from *Electrophorus* (96, 97) and *Torpedo* (98). The sera block by at least 70% and in a quasi-irreversible manner the maximal depolarization caused by carbamylcholine on *Electrophorus* electroplaque (97, 99). Some of the antibodies most likely react directly with the receptor site, others with different regions of the receptor molecule (97). They might be used to study the integration of the receptor protein in the excitable membrane.

Other classes of compounds modify the depolarization induced by agonists in *Electrophorus* electroplaque, though the data are insufficient to determine whether these compounds modify the apparent affinity or the maximal response. Agents that decrease the depolarization caused by agonists include 1. high concentrations of organophosphate acetylcholinesterase inhibitors such as diisopropyl fluorophosphate (DFP) (100) [in the motor endplate the reversible action of DFP on the time course of the endplate currents appears similar to that of local anesthetics (101)]; 2. low concentrations of the neutral detergent Triton X-100 (102, 103); 3. enzymatic digestion of the innervated face by hyaluronidase (103). Agents that potentiate the depolarization of the electroplaque surface caused by carbamylcholine include Ca^{2+} (104) and the alkaloid veratridine (105). The latter prolongs the time course of the permeability increase to Na^+ of electrically excitable membranes. While it acts as an antagonist at pH 7, at pH 9 it potentiates the depolarization caused by carbamylcholine.

Response Associated with Single Receptor Sites

To date, the conductance response associated with the binding of a single acetylcholine molecule has not been measured directly. However, different techniques permit an estimation of that value. First, it is plausible that during the bath application of high concentrations of agonists, conditions exist where all receptor sites are occupied. Under these conditions the observed steady-state conductance, g_{max}, measures the time average response associated with all the sites. For a known number of toxin sites, N_T, the average conductance response per site, $\rho = g_{max}/N_T$. [Actually, the number of acetylcholine binding sites may be the same as (possibly one half) the number of α-toxin sites for electric tissue (cf 6, 11, 106–108).] For the isolated *Electrophorus* electroplaque, $g_{max} = 0.3\ \Omega^{-1}$ per cm^2 of window area, whether measured by neural stimulation (52) or by bath application of carbamylcholine (52, 75). Such agreement appears surprising since there is no a priori reason that upon

neural stimulation all receptor sites should be occupied. However, using that value for g_{max} and a value of 0.5–5 X 10^{12} per cm^2 apparent surface (21, 22) for N_T, $\rho = 10^{-13}$–10^{-12} Ω^{-1}. For the frog motor endplate $N_T = 3 \times 10^7$ sites (47), and $g_{max} = 5 \times 10^{-6}$–$2 \times 10^{-5}$ Ω^{-1} (109, 110) for neural stimulation. No g_{max} has been determined by bath application of agonist, though values as large as 10^{-6} Ω^{-1} are far from the maximal response (73). Hence, if the endplate potential is a measure of g_{max}, $\rho = 10^{-13}$–10^{-12} Ω^{-1}.

Second, for the frog motor endplate Katz & Miledi (111, 112) have deduced values of the magnitude (γ) and duration (τ) of elementary conductance events from a statistical analysis of the random voltage fluctuations occurring during the iontophoretic application of acetylcholine and other cholinergic ligands. They concluded (111) that the observed root mean square fluctuations (30 μV for a 10 mV depolarization) resulted from the summation of elementary depolarizations of about 0.3 μV and that for acetylcholine $\gamma \approx 10^{-10}$ Ω^{-1}. The temporal analysis of the fluctuations gave an average duration of 1 msec, with five different agonists having values ranging from 0.1 msec (acetylthiocholine) to 1.6 ms (suberyldicholine) at 20°C (112). Analysis of current fluctuations at voltage-clamped endplates yields $\gamma = 3 \times 10^{-11}$ Ω^{-1} (113).

The magnitude of the average steady-state conductance (ρ) can be compared with that of the elementary conductance unit, γ. If the observed conductance increase represents the summation of elementary events of magnitude γ, then the conductance per receptor site must reflect the number of receptor sites per conductance unit, α, and the probability P that occupied receptor sites are associated with active conductance units: $\rho = \gamma P/\alpha$.

The average conductance increase per toxin site, ρ, is at least 30 and may be 1000 times less than the elementary conductance γ. Trivial explanations are possible, though not probable: γ for *Electrophorus* is not that of frog; g_{max} has not been measured (because of desensitization or problems with the voltage clamp). However, if the discrepancy between ρ and γ exists in fact, two different factors may be involved. 1. The duration of the conductance event is only a small fraction of the time that an agonist occupies a receptor site. 2. Several receptor sites and or receptor oligomers are involved in a single "elementary" conductance event. There may be more fundamental events associated with single sites or oligomers, or there may be an interaction between oligomers necessary to control permeability.

Although not properly the subject of this review, the results obtained to date from the statistical analysis of the endplate noise indicate that new approaches can be used to modify our picture of drug receptor interactions. The different characteristic times for different agonists may reflect a molecular basis for drug efficacy. Also, Katz & Miledi observed (114, 115) that while *d*-tubocurarine and bungarotoxin modify the number but not the duration of the conductance events, atropine modifies the duration of these events. Further studies should clarify whether γ is the same for all agonists and whether γ is the same for sub- and extrasynaptic regions where receptor densities are very different. Also, studies of the concentration dependence of the characteristic times will provide information about the molecular events determining these times. It may be possible to separate the contribution of the

duration of receptor occupancy from that of the duration of the structural changes that account for the permeation event. It will be important to establish whether the sigmoid shape of the steady-state conductance-concentration curve (73) reflects a similar concentration dependence of the root mean square of the voltage fluctuations.

SUBCELLULAR FRACTIONATION OF ELECTRIC ORGAN

The homogeneity and richness of cholinergic synapses in electric tissue make it a particularly useful material for subcellular fractionation. In *Electrophorus,* cytochemical methods reveal both acetylcholinesterase and the α-toxin sites on the innervated face of the electroplaque, while, by electrophysiological criteria, Na/K-ATPase activity appears associated with the noninnervated face (67). The procedures used to fractionate electric tissue include classical methods of tissue homogenization, differential centrifugation, and sedimentation in sucrose density gradients.

In early studies with *Electrophorus*, when no suitable assay for the cholinergic receptor site was available, only acetylcholinesterase and ATPase were followed during fractionation, and they were shown to be associated with membrane fragments of different hydrodynamic properties (116, 117). More recent experiments with frozen electric tissue from the same fish have shown that the α-toxin binding sites are carried by membrane fragments which band at 30% w/v sucrose and are partially distinct from those which contain acetylcholinesterase (21). When apparently similar procedures are used on fresh electric tissue a second band labeled by the α-toxin is found close to the peak of ATPase (35% w/v sucrose) but still quite distinct from the acetylcholinesterase-rich membrane fragments (10, 118). It is possible, but not shown, that the two populations of membrane fragments labeled by the α-toxin correspond to extra and subsynaptic areas of the cytoplasmic membrane from the innervated face. The membrane fragments isolated around 30% (w/v) sucrose contain 10–30 nmol of α-toxin sites per g of protein and similar concentrations of acetylcholinesterase (28).

Fractionation methods have been developed for *Torpedo* electric tissue which clearly separate membrane fragments containing toxin sites from those containing acetylcholinesterase (10, 37, 38, 119). Homogenization of fresh electric tissue in either distilled water (37) or 0.4 *M* NaCl–0.1 *M* $MgCl_2$ (38) followed by ultracentrifugation in sucrose gradients yields membrane fragments which make a band at 39% (w/v) sucrose and which contain 30% of all the tissue toxin sites. Those fragments contain 2000–3000 nmol α-toxin sites per g protein and less than 1% of that amount of acetylcholinesterase (37). As much as 20–40% of their protein is the cholinergic receptor. These membrane fragments constitute a particularly useful preparation for study of the physical properties of the receptor protein in its membrane environment (36, 38, 40, 120). Under the same fractionation conditions and at least after homogenization in distilled water, the band of membranes containing ATPase activity was found close to that of acetylcholinesterase (25% sucrose) (121). Recently, it has been shown by sodium dodecyl sulfate–polyacrylamide gel electro-

phoresis that the polypeptide composition of the different fractions rich in α-toxin sites, acetylcholinesterase, or Na/K-ATPase remain relatively simple and also distinct from each other (119). The fraction rich in α-toxin sites contained two dominant components: one dominant in the total homogenate and in all fractions studied and another of molecular weight close to 4×10^4 [close to the values reported for subunit molecular weights of the purified receptor from *Torpedo* (9, 10) and *Electrophorus* (3, 4, 6) and that determined by MBTA labeling (5)].

PERMEABILITY RESPONSE IN VITRO

Using a preparation of *Electrophorus* membranes rich in acetylcholinesterase, Kasai & Changeux (122–124) demonstrated that, in the absence of energy sources or concentration gradients, the isolated membranes which reseal into closed vesicles or microsacs (diameter: 0.1–1 μm) respond in vitro to cholinergic agonists by a change of permeability to $^{22}Na^+$. The pharmacology of the in vitro response agrees remarkably with that of the live electroplaque (122). Apparent affinities are the same within a factor of 2 or 3 (see also 4), and the sigmoid shape of the dose-response curve persists. The significant flux rates of various permeants through the resting microsacs indicate a leak more important than through the resting innervated membrane of the electroplaque (52). Carbamylcholine increases the rates of efflux (and of influx) of $^{22}Na^+$, $^{42}K^+$, $^{45}Ca^{2+}$ but not of anions, organic cations, and neutral permeants (123). The maximum increase of $^{22}Na^+$ efflux (a factor of 2 to 4) was not limited by the assay procedure itself since gramicidin increases the rate of $^{22}Na^+$ efflux by a factor of 10–20 greater than the efflux at rest.

On the basis of the observed efflux rates (123) and the number of receptor sites (28, 124), the flux response per receptor site was estimated assuming: 1. the ion efflux was from a population of vesicles of radius $r = 0.1$ μm (116, 125); 2. significant transmembrane potentials did not exist; 3. all toxin binding sites were involved in the response. With these assumptions, the conductance increase per area (123) or per receptor (10^{-16}–10^{-14} Ω^{-1}) appears about 10^3 less than the values determined in vivo. Such significant differences suggest that either the calculation is way off or the receptor in vitro behaves quite differently than in vivo. 1. It was observed (Table 4 in 123) that the simple assumptions relating the observed effluxes to permeabilities gave values for the K^+ permeability at rest about 10^2 or 10^3 less than that observed in vivo (60). 2. It is possible that only 0.1% of the receptors are involved in the permeability response, a problem that will have to be considered in any quantitative studies in vitro. This could happen if most receptors were in unsealed vesicles or were concentrated in only a small fraction of the vesicles contributing no important volume. 3. Electrophysiological data in vivo is consistent with the possibility that the conductance response approaches zero as the transmembrane potential approaches zero. For the electroplaque (52, 61), unlike the frog motor endplate (54, 110), membrane potentials have not been directly observed that are associated with the reversal of the sense of current flow during synaptic activation. Also, conductance desensitization occurs within minutes (61), the time scale of the flux experiments.

Kasai & Changeux (123) observed no clear correlation between the initial ionic gradients established across the membrane and the observed fluxes. Hence, they assumed that under their experimental conditions the transmembrane electrical potential was negligibly small. Recently, McNamee & McConnell (126) utilized electron spin resonance techniques to establish that diffusion potentials were generated by Na_2SO_4 concentration gradients between the outside and inside of *Electrophorus* vesicles, and that they last for at least 3 hr. No studies have yet been reported of $^{22}Na^+$ efflux under conditions where the transmembrane potential is known. The same authors were able to incorporate spin-labeled lipids into the vesicles without modifying the response to carbamylcholine. However, the presence of cholinergic ligands did not modify the spectral properties of the spin-labeled lipids. As in early fluorescence experiments (127), the probes might have been incorporated into membrane areas or even vesicles not containing receptor protein.

One of the limitations of the excitable microsac preparation from *Electrophorus* is that the receptor makes up only about 0.1% of the membrane proteins. The flux data (see Figures 1 and 6 in 123) demonstrate that a sizable fraction of the sealed vesicles (surely more than 10% of the enclosed volume) respond to agonists. If the α-toxin sites (10–30 nmol/g protein) are distributed uniformly over the microsacs [average diameter 0.2 μm (116, 125)], their density would be no more than several hundred sites per μm^2, the density of α-toxin sites on the extrasynaptic areas of the innervated face. Alternatively, if 99% of the α-toxin sites were present in sealed vesicles derived from subsynaptic areas (50,000 α-toxin sites per μm^2), they would enclose only 1% of the apparent volume and remain undetected in the flux assay. Hence, it is probable that the preparation of excitable microsacs used by Kasai and Changeux derive from extrasynaptic areas of the innervated face and contain between 0 and 10 receptors per vesicle.

Receptor-rich (1000 nmol α-toxin sites per g protein) membrane fragments from *Torpedo* respond to carbamylcholine by an increase of the rate of $^{22}Na^+$ release (K_{ap} = 10–100 μM) and α-toxin blocks that effect (128). Also, carbamylcholine increases by a factor of 10 (K_{ap} = 80 μM) the rate of $^{22}Na^+$ uptake by cultured muscle cells from chick embryo in the presence of ouabain (129). Apparently, the solute flow per receptor (10^6 ions/min) exceeds that calculated for *Electrophorus* microsacs (125) by 500, and for this system, there was evidence for a rapid desensitization (half-time 0.5 min) of the stimulated flux (129).

BINDING OF LIGANDS TO THE MEMBRANE-BOUND RECEPTOR

A major goal of the studies on the excitable microsacs in vitro is to relate the permeability response to the binding of cholinergic agonists. The reversible binding of cholinergic ligands to membrane fragments can be measured directly by centrifugation and equilibrium dialysis or indirectly via their interaction with radioactive α-toxins. With crude membrane preparations isolated from electric tissue, cholinergic ligands are expected to bind to several categories of sites in addition to the physiological receptor site: surely the catalytic site of acetylcholinesterase and other

less well-defined sites. O'Brien and co-workers, with both *Electrophorus* (130, 131) and *Torpedo* (131–133) electric tissue, have shown that the binding of individual ligands was generally characterized by multiple affinities, including affinities as much as three orders of magnitude greater than the apparent affinities observed in vivo (for a review, see 20). An important step in the classification of these various classes of sites was the finding that the α-toxins selectively block only certain of them (124), first postulated (14) [and subsequently confirmed with the purified protein (107, 108)] to belong to the cholinergic receptor protein.

Reversible Cholinergic Ligands

ELECTROPHORUS Kasai & Changeux (124) measured the binding of ^{14}C decamethonium to *Electrophorus* microsacs over concentrations ranging from 10^{-7} to 10^{-5} *M*. About half the binding is blocked by the α-toxin from *B. multicinctus,* and the binding curve of decamethonium to these sites [$K_D = 0.9 \pm 0.5$ μ*M* (134)] follows closely the flux-response curve of the same preparation of microsacs. The precision of the data was insufficient, however, to decide if the binding curve deviated from an hyperbola. Actually the remainder of the decamethonium binding (insensitive to α-toxin) was associated with a similar dissociation constant ($K_D = 2$ μM) and was probably associated with the acetylcholinesterase. Hence for this preparation the two affinities for decamethonium are close to one of the four affinities observed by Eldefrawi et al (130). The relative concentrations of the two higher affinity sites in the crude particulate preparation are so low that they would have remained undetected if present in the excitable microsac preparation. Kasai & Changeux (124) estimated binding constants for several cholinergic ligands from the displacement of decamethonium.

Indirect methods also show that the cholinergic response of the microsacs is directly related to the binding of cholinergic ligands. Weber & Changeux (106) characterized the manner in which cholinergic ligands decrease the initial rate of α-toxin binding, and the protection curves obtained were found to coincide with the binding and flux-response curves. For each ligand a protection constant K_p was defined as that concentration decreasing the initial rate of toxin binding by 50%. For agonists and antagonists, the value of the dissociation and protection constants coincide (Table 2) with the *apparent* dissociation constants determined either in vitro ($^{22}Na^+$ efflux) or in vivo (steady-state depolarization). On the other hand, Weber & Changeux (83) observed that the protection constants for a variety of local anesthetics exceed by one or two orders of magnitude their *apparent* dissociation constant determined in vivo following the decrease of the maximal response of the isolated electroplaque to carbamylcholine. They concluded that although the local anesthetics show a weak affinity for the cholinergic receptor site itself, their site of action as noncompetitive blocking agents must be distinct from the cholinergic receptor site, although presumably in its vicinity.

TORPEDO The direct binding of ^3H-acetylcholine and ^3H-decamethonium has been determined precisely by Weber & Changeux (106) on purified receptor-rich membrane fragments from *Torpedo.* Over the concentration range studied

(5×10^{-9}–2×10^{-7} M), all acetylcholine bound in the presence of tetram, a potent cholinesterase inhibitor, can be displaced by α-toxin and is characterized by a single dissociation constant ($K_D \approx 10$ nM).

This binding constant was the same as that accounting for 10% of acetylcholine binding sites in a crude particulate preparation, while the lower affinity sites observed by Eldefrawi et al [$K_D \approx 68$ nM (132)] were not found with this purified preparation. For the receptor-rich membranes, the binding curve, still determined in the presence of tetram, was slightly sigmoid in shape (Hill coefficient (n_H) = 1.3–1.5). No cooperative binding for decamethonium sensitive to the α-toxin was detected ($K_D = 0.8$ μM), and there was evidence for binding sites with lower affinity. In the same study, protection constants determined for a variety of cholinergic ligands were found to be identical with dissociation constants measured directly. The displacement of decamethonium or acetylcholine by *d*-tubocurarine was consistent, in a first approximation, with a competitive antagonism ($K_D = 0.2$ μM).

Fluorescence techniques have also been used to follow the binding of cholinergic ligands to receptor-rich membrane fragments (120) or to the purified receptor protein in detergent solutions (135). Cohen & Changeux (120) utilized DNS-chol [1-(5-dimethylaminonaphthalene-1-sulfonamido)-ethane-2-trimethylammonium iodide], an environmentally sensitive probe (136), which has only a moderate affinity for the receptor site ($K_D = 20$ μM). Its binding to receptor-rich membranes can be easily followed by energy transfer in the presence of physiological saline solution. When 2% of DNS-chol is bound to the receptor site it contributes as much as 50% of the detected fluorescence. The receptor affinities of nonfluorescent cholinergic ligands determined from the displacement of DNS-chol agree with those determined by radioactive ligand assays. Martinez-Carrion & Raftery (135) have shown that bis-(3-aminopyridinium)-1,10-decane diiodide, a known cholinesterase inhibitor (137), binds with a high affinity ($K_D = 10^{-7}$ M) to the isolated receptor in detergent solution but at low ionic strength (20 mM salt solution).

Because of the structural similarities between the purified *Torpedo* membranes and the postsynaptic membrane in vivo, it would be desirable to relate the observed ligand dissociation constants with the membrane permeability response. The ligand binding affinities bear little relation to those of the excitable *Electrophorus* microsacs (see Table 2) with acetylcholine and carbamylcholine affinities 100 times greater, *d*-tubocurarine and decamethonium the same, and gallamine 100 times less for *Torpedo* than *Electrophorus*. The flux assay data presently available for the *Torpedo* membranes (128) suggest that carbamylcholine increases the rate of $^{22}Na^+$ efflux when present at concentrations above 10^{-5} M, a concentration 10^2 in excess of that necessary to protect against α-toxin binding (106) or to displace DNS-chol from the receptor site (120). Further data is necessary to clarify this situation, but we may ask what interpretations are possible for this result. 1. For *Torpedo,* the high-affinity binding sites that are α-toxin-sensitive bear no relation to the physiological cholinergic receptor! 2. Over 95% of all receptors must be occupied before there is a permeability response when the receptors are present at very high surface density. 3. During the preparation procedure, the properties of most of the physiological receptors have been modified, and they bind agonists with high affinity but do not

show a permeability response. However, a small fraction of the receptors binds ligands with lower affinity, is associated with the flux response, and is not detected by the binding assay. We discuss later results that show that the binding function for cholinergic receptors can be controlled by various environmental factors, a situation that makes the last hypothesis plausible.

Snake Venom α-Toxins

Snake α-toxins block synaptic transmission in a quasi-irreversible or slowly reversible manner at neuromuscular junctions with the degree of reversibility depending both upon the α-toxin and synapse considered (13, 138, 139). At the electroplaque synapse, the action of the various toxins studied has not been reversed by at least 1 hr of washing (26, 90, 95). The mechanism of binding of radioactive derivatives of several α-toxins has been studied in detail with various membrane preparations. Dissociation constants reported with *Electrophorus* membrane fragments range between 2×10^{-11} *M* and 2×10^{-7} *M* (4, 27, 141). As already mentioned, cholinergic ligands decrease the initial rate of association of the toxin with the membrane fragments as well as the amount bound at equilibrium, which suggests, in a first approximation, a mutually exclusive binding of cholinergic ligands and snake α-toxins at the same class of sites (106). However, detailed analyses of these interactions show that the situation is more complex. The formation of the α-toxin-receptor complex does not follow a simple bimolecular mechanism. In the work of Weber & Changeux (106) with ^{3}H-α-toxin of *N. nigricollis,* a slow monophasic dissociation of the membrane-toxin complex was observed for low toxin and receptor concentrations (each about 0.2 n*M*), while at concentrations 10 times greater, the rate of dissociation was clearly biphasic with a more rapid component becoming significant. Fulpius et al (140) and Klett et al (2) studied the interaction of a pyridoxal phosphate derivative of *N. naja siamensis* with detergent solutions of *Electrophorus* receptors. With receptor concentrations around 10^{-9} *M* and α-toxin about 10^{-8} *M,* the binding was characterized by $K_D \approx 10^{-9}$ *M* on the basis of 80 min incubation, but after longer incubation periods there was apparently formation of a higher-affinity complex characterized by slower dissociation rates. Furthermore, for both the membrane-bound (4, 106) and the solubilized, purified *Electrophorus* receptor (2), the presence of certain cholinergic ligands such as decamethonium or hexamethonium increased the rate of dissociation of both slow and fast dissociating complexes. However, this effect cannot be correlated simply with an interaction of the cholinergic ligands with the receptor site.

The kinetics of association of ^{125}I-bungarotoxin with *Electrophorus* microsacs has been analyzed in terms of the formation of a reversible complex [$K_D = 7 \times 10^{-8}$ *M* (27) or 2×10^{-7} *M* (141)] followed by an "irreversible" reaction. In their studies at 1°C, Bulger & Hess (141) observed that the antagonist *d*-tubocurarine interfered with the formation of the reversible complex while two agonists interfered with the subsequent reaction. However, the apparent bimolecular rate constant they would have observed at low concentrations of toxin and toxin sites ($k_{obs} \approx 2 \times 10^5 M^{-1}$ min^{-1}) is two orders of magnitude lower than that observed for the interaction of the same α-toxin with *Torpedo* membranes (142). Furthermore, the bimolecular

association rates of other toxins with *Electrophorus* membranes generally vary less than a factor of 2 or 3 between 5 and 25°C, and the magnitudes of these rate constants are about 10^7 M^{-1} min^{-1} (4, 28, 140). Further studies will be necessary to clarify the mechanism of formation of the toxin-receptor complex and the effect of cholinergic ligands on this process.

REGULATORY PROPERTIES OF THE RECEPTOR IN VITRO

The major regulatory interaction mediated by the cholinergic receptor protein in vitro and in vivo is the control by acetylcholine of the membrane permeability. It is now known that the acetylcholine binding function for a membrane-bound receptor is sigmoid in shape (106). Also, it is now established that the binding properties of that membrane-bound receptor are controlled in vitro by pharmacological agents such as local anesthetics or calcium and also by the membrane environment, as evidenced by the effect of detergent solubilization. Finally, as postulated for years (65, 143), there is good physical-chemical evidence that a change of membrane structure occurs upon binding of cholinergic agonists to the physiological receptor site. However, it remains possible that the membrane isolation procedures modify the important molecular interactions, and it will not be possible to understand the significance of the control of the ligand binding function or of membrane structural features until we can relate those parameters directly to the permeability response in vitro.

Local Anesthetics and Ca^{2+}

Local anesthetics (xylocaine, prilocaine, dimethisoquin) cause an increase of affinity of the cholinergic receptor site in purified *Torpedo* membranes for agonists and antagonists that are both high- (acetylcholine) and low-affinity (decamethonium, DNS-chol) ligands [maximal increase two- to threefold (144, 145)]. The anesthetics tested produce this effect in vitro at concentrations where they act in vivo on *Electrophorus* electroplaque as noncompetitive blocking agents. Only at higher concentrations do these anesthetics displace cholinergic ligands from the receptor site. In the range of concentrations where prilocaine increases the affinity for acetylcholine, it causes a change of shape of the acetylcholine binding curve from sigmoid ($n_H = 1.4$) to hyperbolic ($n_H = 1.0$) (144, 145), an effect similar to that reported for allosteric ligands in the case of regulatory enzymes (77). Ca^{2+} causes a similar increase of affinity but with little effect on the cooperative binding of acetylcholine. Its effect is not additive to that of the local anesthetics. Since in vivo both local anesthetics and Ca^{2+} increase the rate of receptor desensitization (66), it is possible that the enhancement of affinity of the receptor site reflects that desensitization.

Solubilization

The dissociation constants of cholinergic ligands for the receptor site differ when the receptor protein is in the membrane or in detergent solution. In the case of *Electrophorus*, the receptor protein solubilized by anionic or neutral detergents binds agonists with affinities an order of magnitude greater than the membrane-

bound receptor, but the affinities for the antagonists do not change (107). In the case of *Torpedo,* detergent solubilization of crude (142) or purified (144, 145) membrane fragments causes a decrease of affinity more pronounced for the agonists than for the antagonists. Upon solubilization of receptor-rich membranes, acetylcholine no longer binds to a homogeneous population of sites but to at least two classes of sites with different affinities, with the lower ($K_D = 1\ \mu M$) a factor of 100 less than that of the membrane-bound protein (145). Neither local anesthetics nor Ca^{2+} control the affinities of either of these sites (145).

In detergent solutions, the relative concentrations of the different forms of the *Torpedo* receptor can be changed by various factors including the procedures used during purification (108, 146, 153). Different purification procedures have resulted in preparations binding acetylcholine with only a single affinity [$K_D = 2\ \mu M$ (108)] or with several [30% of the binding sites having $K_D = 0.02\ \mu M$ (8)].

Structural Changes Associated with the Binding of Agonists

While studying the interaction of the fluorescent ligand DNS-chol with purified receptor-rich membranes from *Torpedo,* Cohen & Changeux (120) observed that DNS-chol interacted with other "secondary" membrane sites, in addition to the cholinergic receptor site. These sites were characterized by a lower affinity for DNS-chol than the receptor site and by a lower efficiency of the energy transfer between membrane proteins and DNS-chol bound to those sites. The spectral properties of DNS-chol interacting with these sites depend on the pharmacological activity of the cholinergic ligand bound to the cholinergic receptor site. When the receptor sites were occupied by ligands that depolarize *Electrophorus* electroplaque, the wavelength of maximal emission ($\lambda_{max} = 522$ nm) was shifted to the blue in comparison with the wavelength observed ($\lambda_{max} = 537$ nm) either when antagonists or α-toxin occupied the receptor site or with emission of DNS-chol bound to the receptor site itself. The blue shift characteristic of the presence of agonists disappeared when the agonist was displaced by an antagonist at the level of the receptor site, and it was concluded that the spectral properties of DNS-chol bound to the secondary sites reflected changes of membrane structure associated with the physiological response of the membrane.

The nature of the secondary binding sites remains uncertain, and it is conceivable that they are located close to the receptor site so that the spectral shift reflects the fact that agonist and antagonist do not interact with exactly the same binding groups on the active site. However, there is no spectroscopic evidence of the secondary sites after solubilization of the receptor protein by detergents, although agonists and antagonists still bind to the receptor site (145). In addition, in vivo DNS-chol acts both as an agonist and as a noncompetitive antagonist on *Electrophorus* electroplaque (145). Furthermore, local anesthetics cause a change of spectral properties of DNS-chol bound to the secondary sites: the blue shift associated with agonist binding to the receptor site is abolished by just those concentrations of anesthetics that block in vivo the depolarization of *Electrophorus* electroplaque or increase the affinity of the receptor site in vitro (145). At higher concentrations of anesthetics, the fluorescence intensity of DNS-chol decreases as if it were displaced from the

membrane. Hence, it is possible that the secondary sites of DNS-chol binding are related to the sites of binding of local anesthetics.

CONCLUDING REMARKS

It is not yet clear that the receptor protein recently purified from detergent solution retains all the structural elements required for the regulatory control of ion translocation and is in a functional state. For instance, is the ionophore a component of the 250,000 mol wt oligomer (3, 39)? In addition, membrane environment evidently controls the binding properties of the receptor site and most likely of the ion translocation mechanism. Are specific lipids and/or proteins responsible for this membrane constraint? Does the high density of receptor sites under the synapse lead to functional interactions between receptor oligomers? Further comparison of the properties of the receptor protein in its membrane-bound and purified forms as well as reconstitution of an acetylcholine-sensitive membrane (128) from well-defined biochemical components should provide an answer to such essential questions.

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