The Acetylcholine Receptor: A Model For Allosteric Membrane Proteins

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Introduction

The cells of the nervous system, or neurons, possess a property unique in the organism in their ability to form multiple finely branched extensions (called axons and dendrites) by which they assemble into networks of extreme complexity. Electron microscopy reveals that at the level of their contacts (or synapses), the membranes of these cells juxtapose each other while separated by a distance of tens of nanometers. Chemical substances, or neurotransmitters, serve as relays between the electrical signals propagated along the axon of one neuron and those produced by the next cell. The propagation of the action potential down the axon stimulates the release of neurotransmitter at a high concentration (around 1 mM) for a very brief period of time (around 1 ms) [1]. This ‘chemical impulse’ passes across the synaptic cleft in a fraction of a millisecond, and triggers an electrical signal on the postsynaptic cell as a result of the neurotransmitter binding to a ‘receptive substance’ or receptor, the former being postulated by the British pharmacologist John Newport Langley as early as 1905 [2]. Among neurotransmitter receptors, the open ion channel is selective either for cations (Na+, K+, Ca2+), producing an excitation, or for anions (Cl−), producing inhibition. In all cases, the transduction of the chemical signal into the electrical signal involves the opening of an ion channel (resulting in activation) which occurs with a time course of 1 µs to several milliseconds. When the neurotransmitter is applied on to the postsynaptic membrane in a prolonged manner, and at a concentration which can be lower than that which elicits activation, the amplitude of the response slowly decreases. A desensitization of the response takes place within a period of several hundred milliseconds to several minutes.

In 1964, in my Ph.D. thesis, I mentioned that the processes involved in neurotransmitter recognition and signal transduction at the synapse might plausibly be found, in the future, to be relevant to allosteric mechanisms. I therefore decided to embark on a research programme based on an explicit theoretical model, and took advantage of a well defined experimental system. The theoretical model [3] was that neurotransmitter receptors were

Abbreviations used: TDF, phenyltrimethyl ammonium benzene diazonium difluoroborate; DDF, similar to TDF, but with a dimethyl ammonium group in place of the quaternary ammonium group; GABA, γ-aminobutyric acid.

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specialized regulatory proteins, or allosteric proteins. Such molecules, typified by haemoglobin and diverse regulatory enzymes, are characterized by the presence of several topologically distinct binding sites, and by a particular structural flexibility that mediates coupling between these sites [4–9] (Figure 1). These proteins were assumed to be composed of several subunits organized in the form of a symmetrical 'microcrystal'. They were further postulated to spontaneously undergo a reversible conformational transition between at least two discrete states which preserve their properties of symmetry, while having different affinities for substrates and/or regulatory ligands, as well as different biological activities. In the particular case of the channel-linked neurotransmitter receptor, this is the state of opening of an ion channel.

The experimental system which led to the first identification of a neurotransmitter receptor, the acetylcholine receptor, as well as providing support for the suggested theoretical model, was the electric organ of the electric fish (Electrophorus and Torpedo). In 1937, David Nachmansohn [10] had recognized the advantages offered by this system: an exceptional richness of homogeneous cholinergic synapses, as well as the possibility of isolating a single cell, or electroplaque, from the electric organ and of studying its pharmacological properties, which later proved to be quite similar to those of the human neuromuscular junction. A brief postdoctoral stay in the laboratory of David Nachmansohn at Columbia University in New York permitted me to verify, with the help of Tom Podleski, that receptor agonists (acetylcholine, phenyltrimethyl ammonium, decamethonium) and competitive antagonists (curare, flaxedil) behaved in accordance with the predictions of the two-state allosteric model [11]. At the same time, I demonstrated that an affinity label previously used by Leon Wofsky and Wolf Singer for identifying the active site of specific antibodies, phenyltrimethyl ammonium benzene diazonium difluoroborate (TDF), behaved as an irreversible competitor of acetylcholine with the electroplaque, and could thus potentially be used for the same purpose with the acetylcholine receptor [12].

**Electrochemical transduction in vitro**

The analysis of the basic mechanisms of electrochemical transduction had until this time occurred through the recording of the electrical activity of cells. I quickly realized that the identification of a receptor, and the analysis of the molecular mechanisms involved in signal transduction, would require the establishment of an *in vitro* 'chemical' system using which structure/function relationships could finally be assessed in a direct manner. This step was advanced by the demonstration by Michiki Kasai and myself [13] that membrane fragments of the electric organ of the eel, when purified as a homogenate, spontaneously formed closed vesicles, or microsacs, which responded to acetylcholine with an increase in permeability to Na⁺ and K⁺ *in vitro* (Figure 2). In agreement with the suggested allosteric mechanism,
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Demonstration of the ionic response in vitro of membrane fragments purified from the electric organ of Electrophorus electricus

(a) Description of the filtration method used for measuring the efflux of $^{22}\text{Na}^+$ in a suspension of membrane fragments forming closed vesicles (microsacs). (b) When microsacs rich in acetylcholinesterase and apparently derived from the innervated face of the electroplaque are used, (i) the nicotinic agonist carbamylcholine increases the efflux of $^{22}\text{Na}^+$, and this effect is blocked by $d$-tubocurarine. With microsacs not enriched for acetylcholinesterase and apparently derived from the non-innervated face of the electroplaque, (ii), no response is measured [13,14]. Symbols: $\bullet$, control; $\Diamond$, 10 $\mu$M $d$-tubocurarine; $\times$, 100 $\mu$M carbamylcholine; $\Delta$, 100 $\mu$M carbamylcholine + 10 $\mu$M $d$-tubocurarine.

Identification of the receptor protein

A second, no less decisive, step in this direction was the use of a label that was highly selective for the active site of the receptor. This was $\alpha$-bungarotoxin, a polypeptide of 74 amino acids, initially characterized and purified by the Taiwanese pharmacologist Chen Yuan Lee [16] from the venom of the snake Bungarus multicinctus. This toxin has a paralysing effect, similar to that of curare, but acts at extremely low concentrations and in a slowly reversible manner. $\alpha$-Bungarotoxin inhibits the electrophysiological response of isolated electric eel electroplaque, blocks the increase in ion permeability caused by acetylcholine in excitable microsacs, and displaces already bound, radioactively labelled decamethonium in a microsac.
preparation solubilized by the detergent deoxycholate [15] (Figure 3). The protein which binds α-bungarotoxin and the nicotinic agonist decamethonium in a mutually exclusive manner has a very large size, and could be separated from acetylcholinesterase.

The receptor protein was hereafter purified at the Institut Pasteur and in other laboratories, and was then observed for the first time in the electron microscope in 1973 by Jean Cartaud, using my preparations [20-22]. Arthur Karlin and Michael Raftery [23] independently showed that the receptor had a molecular mass of the order of 300,000 Da, was composed of four types of subunit assembled into a heteropentamer (α2βγδ), and that the two α subunits each contributed a binding site for acetylcholine or α-bungarotoxin.

A biochemist cannot be satisfied on discovering a new type of molecule without knowing if the purified protein retained all its functions that were related to electrochemical transduction. In particular, did it function as an ion channel? The strategy adopted by Gérald Hazelbauer and myself [24] was to re-incorporate a purified fraction of the receptor protein into artificial lipid vesicles and use them, like the microsacs of the electric organ, to study ion fluxes as a method of assessing their transduction function. This experiment succeeded first with a purified fraction, and then with the purified protein [25, 26]. Finally, the electrical activity of individual ion channels was recorded by Mauricio Montal and his collaborators in 1980 [27] with the receptor protein integrated into lipid bilayers. The oligomer (α2βγδ) thus contained the binding site for acetylcholine and the ion channel, as well as all the structural elements engaged in activation and desensitization. The critical physiological step of transducing a chemical signal into an electrical signal at the synapse was thus ‘reduced’ to the properties of a single molecular species.

**Figure 3**

Identification of the acetylcholine receptor with the aid of α-bungarotoxin (α-Bgt), a toxin from the venom of *Bungarus multicintus*

(a) Quasi-irreversible inhibition by α-Bgt of the electrical response to carbamylcholine (Carb) of the isolated electroplaque from *Electrophorus electricus*. R, rinsing with buffer.
(b) The ionic response of excitable microsacs is blocked in vitro by α-Bgt. ● Control; ×, 100 μM carbamylcholine; ○, 10 μM d-tubocurarine. (c) α-Bgt displaces the binding of the radioactively labelled nicotinic agonist decamethonium measured by equilibrium dialysis with a membrane extract of *Electrophorus electricus* solubilized by the detergent sodium deoxycholate (after [15]).
The contribution of biotechnology

At the end of the 1970s, progress in protein microsequencing and in genetic engineering provided access to the partial and then complete primary amino acid sequence of the receptor. The sequence of the 20 amino acids of the N-terminal domain of the α subunit of Torpedo marmorata was established by Anne Devillers-Thiéry, Donny Strosberg and myself in 1979 [28]. Then, in the following year, the partial N-terminal sequence of the four chains of the receptor from Torpedo californica was determined by Michael Raftery and his collaborators [29], revealing a 35–50% sequence identity among the subunits. This sequence homology was interpreted on the basis of an evolutionary relationship among the genes encoding each of the four chains of the receptor. These sequence data also suggested a pseudo-symmetrical organization of the receptor oligomer (α2β2γδ) around a five-fold axis of rotation perpendicular to the plane of the membrane.

Based on these biochemical data, the cloning of the cDNAs encoding the chains of the receptor was reported by four laboratories, including mine [30]. Then, the complete sequence of the four chains of the receptor of Torpedo californica was established by Shosaku Numa and his collaborators [31], and that of the α chain of Torpedo marmorata by my group at the Institut Pasteur [32]. An analysis of neighbouring hydrophilic and hydrophobic amino acids along the length of each of the four chains revealed a remarkable compartmentalization into functional domains (see [32]). On this basis, a transmembrane organization of each polypeptide subunit was suggested: the large, hydrophilic N-terminal domain would face the synaptic cleft, the small hydrophilic C-terminal domain would be in contact with the cytoplasm, the four hydrophobic segments of about 20 amino acids each would cross the membrane, and the C-terminal tail would sneak into the synaptic cleft.

The same scheme applies to the sequences of the four chains of the human muscle receptor and to those of the neuronal nicotinic receptors, of which there are known to be seven types of α subunits and three types of non-α subunits (reviewed in [33]). Among these, the α7 subunit possesses the remarkable property of being able to associate with itself and form functional homooligomers when expressed in Xenopus oocytes [34,35]. This particularly simple system will permit progress in the analysis of the functional properties of the receptor. But the list is not yet finished. It has recently extended to include receptors for other neurotransmitters: the glycine and γ-aminobutyric acid (GABA) receptors contain an ion channel selective for chloride ions, and play a role as inhibitory receptors. All these neurotransmitter receptors comprise a superfamily of ligand-gated ion channels to which belong, as distant cousins, the subfamily of glutamate receptors [33]. The work initially developed for the nicotinic receptor of the electric eel has thus opened the way for a targeted molecular pharmacology of neurotransmitter-gated ion channels involved in fast cerebral communication.

Structure of the active site

The identification of the amino acids comprising the active site has benefited from the use of affinity labels which, like TDF [12], serve as ‘self-sticking labels’ by binding selectively to the active site in a covalent manner. DDF, a compound very similar to TDF, but with a dimethyl ammonium group in place of the quaternary ammonium group [36], acts as a reversible competitive antagonist in the dark. Furthermore, it can be activated by energy transfer, i.e. by light emitted by aromatic amino acids present in the binding site when they are irradiated by ultraviolet light. This results in a considerable increase in the specificity of labelling. Work towards identifying the amino acids labelled by DDF, which involved the participation of Michael Dennis, Jérôme Giraudat and Jean-Luc Galzi from my laboratory, provided evidence for the presence of three loops (loop A, Tyr-93; loop B, Trp-149; loop C, Tyr-190, Cys-192, Cys-193, Tyr-198) comprising, as expected, a large hydrophilic N-terminal domain [37,38]. The amino acids labelled in loop C indicated cysteines 192 and 193, initially identified by Arthur Karlin and his collaborators [39] in the reduced receptor by using an affinity label reacting selectively with cysteines. With the exception of these two cysteines, all the amino acids labelled were aromatic amino acids (Figure 4). Moreover, several groups (reviewed in [41]), using different affinity labels, have confirmed the contribution to the active site of Tyr-93 (acetylcholine mustard), Tyr-190 (lophotoxin, nicotine, d-tubocurarine) and Tyr-198 (nicotine). Furthermore, the amino acids labelled by DDF in the Torpedo receptor are conserved in the nicotinic receptors of the central nervous system. Finally, work performed in collaboration with Daniel Bertrand of the Centre Médical Universitaire of Geneva, has shown that a substitution by phenylalanine of the amino acids homologous to Tyr-93, Trp-149 and Tyr-190 of the
Figure 4
Model of several loops of the binding site for nicotinic ligands

The sphere represents a molecule of DDF in all possible orientations. The amino acids labelled by DDF, lophotoxin, MBTA, acetylcholine mustard and nicotine are indicated. The symbols in bold refer to mutated amino acids. The residues labelled by d-tubocurarine (dTC) in the γ and δ subunits are Trp-55 and Trp-57 respectively (see the text). The amino acid sequences of the hydrophilic N-terminal domain of several peripheral and central nicotinic receptors show that residues labelled by DDF in Torpedo are highly conserved (modified from [40]).

α7 subunit expressed in Xenopus oocytes [42] results in a decrease in the apparent affinity of this receptor for acetylcholine and nicotine. These amino acids thus contribute to the recognition of the neurotransmitter. They form some kind of aromatic basket in which negative charges are delocalized to accommodate the positively charged quaternary ammonium group of acetylcholine. Recently, Nigel Unwin [43] has presented high resolution electron microscopic images which reveal that, in the synaptic portion of the α subunit, three segments are folded into an α helix, which may correspond to the three loops of the active site.

Identification and structure of the ion channel
Affinity labels opened the way towards identifying the structure of the ion channel. For several decades, pharmacologists and physiologists had noted that a rather heterogeneous group of pharmacological agents, which include local anaesthetics and the frog toxin histrionicotoxin, blocked the permeability response of the acetylcholine receptor in a manner different from that of curare. These agents blocked the permeability response without significantly inhibiting the binding of acetylcholine. The idea was put forward that these non-
competitive inhibitors enter into the ion channel and block the passage of ions in a steric fashion [44]. One of these inhibitors, chlorpromazine, was used by Thierry Heidmann, Robert Oswald and myself for labelling the ion channel. It was used for at least three reasons: (1) Chlorpromazine, among its other effects, brings about a reduction in the mean open time of the channel in response to acetylcholine in cultured mouse myotubes [45,46], and inhibits ion fluxes in excitable microsacs in vitro. (2) Chlorpromazine binds to a single high-affinity site in each receptor oligomer [α,βγδ]; furthermore, when chlorpromazine is bound to this unique site, UV irradiation results in a labelling of all the subunits, suggesting an arrangement of this site in the axis of symmetry of the receptor oligomer [47]. (3) A rapid mixing of receptor-rich excitable microsacs with acetylcholine, followed by brief UV irradiation, results in a 100-1000-fold increase in the rate of covalent attachment of chlorpromazine to four of the subunits [48,49]; these rates of incorporation diminish during exposure to a fixed concentration of acetylcholine with kinetics consistent with those of rapid desensitization of ion flux measured with the same membrane fragments [50].

In other words, chlorpromazine binds freely and reacts covalently with its high-affinity site under the precise conditions where acetylcholine causes the opening of the ion channel.

Following a particularly difficult fractionation of the hydrophobic peptide labelled with chlorpromazine, Jérôme Giraudat, Michael Dennis, Thierry Heidmann, Jui-Yoa Chang and I arrived at the conclusion that chlorpromazine bound to the serine at position 262 of the δ subunit within the putative segment M2 [51]. Equivalent serines were found to be equally labelled in other subunits, as well as the corresponding leucines at positions 257 and 260 in the β and γ subunits respectively, and threonine-253 in the γ subunit [52-54] (Figure 5). Following the initial work begun in my laboratory, Ferdinand Hucho and his group identified by the same method the same serine-262 in the δ subunit, using another non-competitive blocker, triphenylmethylphosphonium [55,56]. These results were in accordance with models already mentioned in which (1) the binding site of chlorpromazine is located in the axis of pseudo-symmetry of the molecule [47], and (2) this site is bordered by a segment common to each subunit, organized in the form of α-helices (see [32]).

Figure 5

Model of the high-affinity site for non-competitive blockers within the ion channel and a comparison of the amino acid sequences of the M2 segment of several channel-linked receptors

The numbers on the left refer to the sequence of amino acids of the γ-subunit of Torpedo. Based on [41] and [54].

Cleft

Outer ring 271
Valine ring 264
Leucine ring 260
Serine ring 257
Threonine ring 253
Intermediate ring 250
Inner ring 246

Cytoplasm
This work performed in my laboratory with chlorpromazine [51] showed for the first time the contribution of the M2 segment to the walls of the ion channel. Mutagenesis experiments carried out by the groups of Shosaku Numa and Bert Sakmann, and by Henry A. Lester and Norman Davidson, confirmed the notion that the M2 segment [51–56] contributes to the inner surface of the ion channel [57–59].

The analysis of the correspondence between the selectivity properties of the ion channel and the three-dimensional structure of the receptor molecule is but the beginning. A collaboration with the group of Daniel Bertrand has pursued this theme. The ion channels of the neuronal nicotinic receptors, particularly α7, are relatively more permeable to Ca²⁺ compared with the muscle receptor. Mutations at two levels of M2, at the 'intermediate' ring of negatively charged residues on the cytoplasmic side [57,58] and at the double ring of leucines on the synaptic side, selectively abolish Ca²⁺ permeability without interfering with Na⁺ or K⁺ permeability [60]. The receptors for GABA and glycine possess an ion channel that is selective for anions. Exchange of the two rings of critical amino acids and insertion of a novel ring at the N-terminus of M2 suffices to convert the ion channel of the α7 receptor from cationic to anionic [61]. As in the case of immunoglobulins, the diverse members of the superfamily of ion-channel-linked receptors possess a similar molecular architecture. However, they differ in the nature of amino acids situated in critical positions of the protein, which are sufficient to determine the specificities for both neurotransmitter recognition and ion transport.

**Slow allosteric transitions and the regulation of synaptic efficacy**

A brief 'chemical impulse' of neurotransmitter assures the rapid transmission of a signal from one neuron to another across the synaptic cleft. Neurotransmitter application in a continuous manner, and at a concentration much lower than that which activates the ion channel, results in a slow, yet reversible, decrease in the amplitude of the permeability response, known as desensitization. In 1957, Sir Bernard Katz and Stephen Thesleff [62] proposed, on the basis of electrophysiological data, a strict theoretical scheme for activation and desensitization, according to which stabilization at equilibrium of the receptors produces a 'refractory' state with a high affinity for acetylcholine (Figure 6). The opening of the ion channel would occur through a transient state of low affinity. Biochemical experiments have demonstrated the general validity of this scheme, but with several significant differences.

Soon after identification of the receptor, direct equilibrium-binding measurements with receptor-rich microsacs from Torpedo revealed an affinity for acetylcholine of the order of 10⁷M, about 1000–10000-fold higher than the concentration which results in the opening of the ion channel with the same membranes [64–67]. The high affinity observed at equilibrium could not have corresponded to the active site. Rapid mixing experiments, first carried out 'by hand' by Michel Weber [68], and then later with the aid of stopped-flow
apparatus by Hans Grunhagen [69,70] and then Therry Heidmann [71–73] in my laboratory, revealed without ambiguity that, at rest, around 80% of the receptor molecules are in a state of low affinity. Placed in the presence of a fixed concentration of acetylcholine, these receptors slowly interconvert towards a high-affinity state at equilibrium. The system of excitable microsacs from Torpedo thus gives access to the measurement, in parallel, and in the same experimental system, of both ion permeability and the binding of agonists which activates these permeabilities [50]. The data demonstrated for the first time, in a direct manner, that at least two states of high affinity ($K_d$s around $10^{-11}$ to $10^{-9}$M) result in a closed ion channel and thus correspond to desensitized states of the receptor [50].

The receptor, when purified and reconstituted into an activatable form, maintains its capacity to desensitize (see [24–26]). This is thus an intrinsic feature of the receptor which is also found in the neuronal receptors. A single molecule thus possesses two classes of regulatory properties: elementary properties for transduction of a chemical signal into an electrical signal, and higher-order properties which regulate the efficiency of this transduction process. The systematic analysis, by site-directed mutagenesis, of the role of amino acids labelled by chlorpromazine, singled out a few of them, in particular leucines from segment M2, that are specifically involved in this regulation. Mutations into threonine (or serine) of the equatorial leucine ring present in the M2 segment of the $\alpha_7$ subunit (L237T) results in several simultaneous modifications of the electrophysiological response: the appearance of a new open channel type, a loss of desensitization, and an increase in the apparent affinity of the permeability response to nicotinic agonists. In accordance with the four-state model of allosteric transitions, these results are interpreted on the basis of a permeabilization of the desensitized state. Mutation of the leucine ring unblocks the ion channel from its desensitized state. In agreement with this conclusion, competitive antagonists such as dihydro-β-erythroidine or curare, which we know favour the desensitized state [69,70], stabilize in the mutant an ‘open desensitized’ state and thus behave as agonists [74,75].

Neurotransmitter receptors, such as those for GABA, glycine and glutamate, share several structural properties with the nicotinic receptor [33]. They undergo desensitization, but in a manner which varies with the subunit composition. It seems plausible, though it has not been demonstrated, that the ‘desensitization mutants’ of the type mentioned for the nicotinic receptor offer one plausible model of this diversity [74,75].

Finally, physiological signals other than neurotransmitters regulate the rate and amplitude of desensitization of the muscle nicotinic receptor: membrane potential, $Ca^{2+}$ and phosphorylation makes possible a regulation of desensitization by neuronal activity (reviewed in [76]).

All the elements are there for proposing that desensitization, and in a general sense all structural transitions of the receptor other than the activation reaction, play an essential role in the regulation of synaptic transmission at the postsynaptic level. The transmembrane organization of the receptor molecule confers on it the capacity to recognize signals coming from the exterior as from the interior of the cell. It allows for the integration of multiple signals and the reading of their coincidence in time. The allosteric properties of the channel-linked receptor offer plausible biochemical mechanisms for the learning law postulated by the Canadian psychologist Donald Hebb [77,78]. The relationship between these elementary properties and learning at the level of behaviour, however, remains to be established.

In conclusion, the data and interpretation of work begun with the nicotinic receptor from the electric eel electric organ can be expanded to diverse members of the superfamilly of ligand-gated ion channels present in the central nervous system. A targeted pharmacology for each particular type of receptor which takes into account conformational flexibility becomes henceforth plausible, with the hope that treatments for a select number of neurological and psychiatric disorders will be possible.

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