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PHYSICAL CHEMISTRY OF EXCITABLE BIOMEMBRANES

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PERSPECTIVES AND SUMMARY

Excitable membranes are the carriers of electrical signal transmission in biology. Well-known examples of excitable biomembranes are the plasma membranes of nerve and muscle cells. Excitability of these cells is generally manifested in rapid transient changes (msec time range) of electrical properties. Nerve impulse and other membrane potential changes and the concomitant ionic crossmembrane currents result from changes in the electrical conductivity of the membrane. These conductivity changes reflect membrane permeability changes, in many cases selectively to Na^+ , K^+ , Ca^{2+} , or

Cl⁻ ions. The permeability changes mobilizing the metabolically maintained steady-state gradients of these ions can be brought about by physical and chemical changes in the membrane and the immediate environment.

The electrical properties of excitable biomembranes have been explored intensively and neurochemical investigations gain increasingly in interest. Yet, the *major unsolved problem of bioelectricity is the molecular mechanism underlying the permeability changes* in excitable biomembranes. We are thus faced with mechanistic questions that require biochemical techniques for the isolation and identification of permeability control systems, and physical-chemical methods for the molecular characterization of the regulatory apparatus and its components. At present this aim is yet a largely programmatic perspective. The problems cover a multidisciplinary field and require an integration of many results of various disciplines. In a situation where little is known about the nature of the observed bioelectrical phenomena, theoretical approaches are very useful as working hypotheses, provided they are general, and yet specific enough to differentiate between various alternatives, and stimulate and guide new experiments. Recent evidence shows that newer voltage-clamp data cannot be described with conventional model theories such as the Hodgkin-Huxley formalism. Very probably, only dissipative chemical models are adequate for the correlation between measured conductivity changes and intrinsic membrane permeability parameters.

A molecular interpretation requires chemical information on the components of the control systems. Several components of permeation regulation systems have been isolated and thermodynamic and kinetic properties have been measured. In a few cases comparison of physical-chemical properties between isolated macromolecules and the membrane-bound *in vivo* system is very suggestive. Yet the number values given have to be seen in the framework of the assumptions underlying the theoretical approaches. Most intensive physical-chemical investigations have been performed with the isolated and membrane-bound acetylcholine receptor; attempts to recombine this regulatory protein with artificial lipid membrane are just beginning. Therefore, the thermodynamic and kinetic properties of this control component are discussed in detail.

This review is necessarily limited to those studies in which physical-chemical methods were applied. We cover only those theoretical approaches operating with physical-chemical concepts relating to experimental systems. This limitation gives, therefore, a selective view of the large multidisciplinary field of excitable membrane research. The major outcome and interesting perspective is, however, the recognition that the rapid and transient permeability changes in excitable membranes appear to reflect special cases of a more general dissipative control principle that is based on activator-receptor interactions.

ELECTROPHYSIOLOGICAL PROPERTIES

The electrical property particularly relevant for a mechanistic approach to excitability is the electrical conductivity. This fundamental quantity can be derived from voltage-clamp (VC) experiments where electric current relaxation is measured during (and after) a stepwise change in the voltage across excitable membranes (1, 2). In connection with pharmacological methods (application of activators and inhibitors) this technique has yielded evidence that excitability generally involves two dominant, apparently independent ion flows (3–9). The two current contributions are associated with opposite ion gradients across apparently distinct pathways selective for certain ion types (9, 10). These selective pathways (ion-transport channels) are probably spatially separated and have different kinetic properties (3, 4, 8, 9). There is a rapid component usually carried by Na^+ ions and a slower one usually carried by K^+ ions. Under depolarizing VC conditions the rapid component is intrinsically transient, increases first, and then decreases, whereas the slower contribution increases to a steady-state level (3–6). The presence of Ca^{2+} ions appears to be essential for excitability (1, 2). Mg^{2+} ions interfere with Ca^{2+} action (11), suggesting that these ions do not just participate in simple ion exchange that influences the surface potential but probably are also coupled to structural properties of the membrane components. The calculation of conductivity contribution g_i for an ion type i is straightforward only when the ionic gradients do not change appreciably during the VC experiment. This condition may be met only for short clamping times.

Time Constants and Noise Analysis

The VC technique is widely applied also to synaptic parts of excitable membranes, and activators and inhibitors of the permeability control reactions have been used as diagnostic tools. If the perturbations introduced by voltage change and drug application are relatively small, the current relaxations can be described in terms of relaxation time constants. These fundamental experimental quantities in chemical systems are dependent on environmental conditions of temperature, ionic composition, and ion concentrations (12). Furthermore, the time constants of axonal as well as of synaptic parts of excitable membranes depend on the voltage across the membrane (3–6, 13–17).

The analysis of fluctuation spectra of electric currents and voltage changes in excitable membranes (channel noise) is in principle suited to derive average pathway parameters, e.g. the lifetime of an open pathway configuration (18, 19). However, mechanistic interpretations and numbers have to be seen in the framework of the specified assumptions made before analysis of the rather complicated noise spectra resulting from several

elementary processes. In a recent account, Neumcke concludes that the selective gating of K^+ ions may involve transient binding of these ions to the pathways (20).

The average lifetimes for the open configurations of synaptic pathways controlled by acetylcholine (AcCh) in nicotinic receptor systems are about 1 msec (21, 22). Recent experiments by Stevens and co-workers show that the time constants of the AcCh-induced productivity changes at neuromuscular receptor systems depend on the voltage across the postjunctional membrane (13–17). The lifetimes of the open configurations induced by AcCh binding are in the range of 1–10 msec.

Gating Concept

The measured time constants for the rapid conductivity changes in excitability are in the msec time range and are associated with a temperature coefficient of $Q_{10} \approx 2-3$ (1). Time range and Q_{10} together with the large heat changes accompanying the action potential (23, 24) suggest that the time course of the current relaxations cannot reflect simple electrodiffusion (1) but is controlled by slower gating processes (3–6) in the ion pathways of the membrane phase (25, 26).

If the conductivity changes in excitable membranes reflect rate-limiting processes of the permeability changes, the time constants of the conductivity changes are equal to the time constants of the permeability control system.

Recently, the so-called asymmetrical displacement-current contributions in squid giant axons (27) and at frog Ranvier nodes (28, 29) have been associated with intrinsic charge and dipole movements of a gating subunit in the higher-order structure of the rapid pathway. The interpretation of data on squid giant axon, for instance, is consistent with the assumed cubic dependence of the Na^+ conductivity contribution on specific subunits of the Na^+ gating system as proposed by Hodgkin & Huxley (3–6).

Due to lack of knowledge, mechanistic proposals for gating in axons are usually chemically unspecific. The biochemical and analytical results on excitable tissue of various origins led Nachmansohn to propose a chemically specific model (30, 31). This proposal, at that time necessarily only qualitative, is based on the activator-receptor concept that is now widely used for synaptic control reactions. Nachmansohn suggested the acetylcholine system as the general (dissipative) control device for the rapid regulation of transient ion flows in bioelectricity (30–33).

Without doubt, the gating in axonal and synaptic parts of excitable membranes involves membrane proteins, and the rate-limiting processes for the conductivity changes are potential-dependent. Besides these common properties there are many other similarities between axonal and synaptic

parts (25, 31, 32). There are, however, also a large number of electrical and pharmacological differences. In particular, the failure of externally applied tetrodotoxin to interfere with synaptic signal transmission and the failure of externally applied α -bungarotoxin to interfere with nonsynaptic signal conduction suggest differences. The differences by themselves are not surprising: there are obviously morphological differences in the environment of the excitable membranes of synaptic and axonal parts, probably providing different diffusion barriers for activators and inhibitors of the permeability control systems. There are also action potentials, which are not blocked by large doses of tetrodotoxin. At present, however, the mode of action of the activators and inhibitors is unknown; none of the observed differences is understood in molecular terms.

The phenomenological differences do not, therefore, necessarily indicate differences in the control principle for the gating of passive ion flows. Alternatively, different phenomena may be produced by structural and organizational variations of one and the same regulatory principle, or even control system. In particular, the synaptic parts of excitable membranes appear to be coupled to additional reactions modifying a basic control apparatus according to special requirements developed at later stages of cell differentiation and evolution. Many so-called putative transmitters may serve as modifiers of a general and unique control system (31). An integral view of excitability operating with one control principle but allowing modifications by additional factors has been outlined in a recent programmatic study (33). This alternative requires one general assumption, whereas the view of different mechanistic principles for each class of different electrical and pharmacological phenomena requires a whole set of different assumptions.

GATING THEORIES

In a situation where only very little is known about the nature of the observed phenomena, theoretical approaches are very useful as working hypotheses. Model theories should cover the basic facts with a minimum of complexity. Useful models must go beyond pure mathematical parametrization and operate with physical-chemical quantities that can, at least in part, be measured with independent methods (34).

Cooperativity

Many phenomena of excitation appear to reflect cooperativity. This cooperativity can be an intrinsic property of the separated pathways or, alternatively, may be caused by a cooperative coupling between the pathways. The Hodgkin-Huxley formalism of nerve excitation reflects intrinsic channel cooperativity (3–6). Tasaki proposes macromolecular cooperative transi-

tions coupled to ion exchanges in cooperative domain structures of the membranes (2). The molecular theory of Adam operates with cooperative pathway coupling through phase changes in the membranes (35). The lattice theory developed by Blumenthal, Changeux & Lefever is also an approach with inherent cooperativity in coupled membrane domains gating passive ion flows (36). In a recent theoretical analysis Hill & Chen stressed that there is no experimental evidence for cooperative channel coupling, and that cooperativity is more likely an intrinsic pathway property (37, 38). Cooperativity in dose-response curves does not necessarily reflect molecular cooperativity in the activator-receptor interaction. In this context, a remark of Magleby & Stevens (14) on the cooperative dose-depolarization response curve published by Katz & Thesleff (39) is very instructive. If the depolarizations are recalculated to conductivity ratios, the conductivity-dose curve shows no cooperativity (Hill coefficient of about 1). Thus, unless the response parameter cannot be independently shown to reflect liganded receptor in high permeability configurations, questions of molecular cooperativity in terms of ligand binding cannot be answered. Values given for the cooperative number of bound ligands for the elementary response must thus be seen within the specific assumptions (sometimes physically unrealistic) made to derive these values (16, 40, 41). Membrane-bound receptors show signs of cooperativity, but no molecular details can be deduced unequivocally (42, 43).

Theoretical Models

The majority of theoretical models for possible excitation mechanisms are conservative. Since, however, permeability control systems in biological membranes, like all biological regulatory devices, probably operate far from equilibrium, dissipative elements appear to be necessary for an adequate physical-chemical description of gating. Furthermore, the large heat changes accompanying the nerve impulse suggest dissipation of free energy, realized by "consumption of some substance." Thus, in particular the transient rapid conductivity component usually carried by Na^+ ions and responsible for the "negative-resistance" feature (1) in excitable membranes appears to be dissipatively gated by chemical means.

There is increasing evidence that two-state schemes using one open and one closed configuration for the gating system are not sufficient to describe newer VC data (44–48). On the next higher level of complexity are three-state schemes. In a theoretical analysis, Jakobsson concluded that three-state models are not superior to the Hodgkin-Huxley scheme, and that both fail, even in the mathematical description of newer electrophysiological data (48). It should, however, be recognized that this failure arises from a specific ad hoc assumption appearing in connection with common procedures of

analyzing VC data. The schemes examined by Jakobsson and other proposals (3–6, 44–50) are applied together with the assumption that the functional relationship between measured conductivity and intrinsic membrane parameters is independent of membrane potential. If this restrictive assumption is not made, there is at least one three-state model that accounts for the newer VC results (51).

The time constants and amplitudes of current relaxations at constant voltage for this model have recently been directly determined using a new and simple analytic technique. If this method is applied, for example, to the Na^+ -current component of axons, the functional relationship between Na^+ conductivity and the open configuration of the gating system is found to be dependent on membrane potential. It could be shown that a dissipative three-state model is able to reproduce all details of newer VC data including the peak current ratio (h_∞) shift with test potential; no other model has so far been able to accommodate the h_∞ -shift.

CHEMICAL GATING PROCESSES

The acetylcholine receptor system represents the most intensively studied gating system for ion flows in bioelectricity. For acetylcholine, the activator-receptor concept is clearly dissipative; the activator removal mechanism is based on one of the fastest enzymes present in biological cells (30, 31, 52). All mechanistic schemes proposed for the AcCh interaction with AcCh receptor reflect the inherent assumption of competition between the acetylcholine esterase (AcChE) and receptor for AcCh (16, 30, 39, 43, 53). In view of recent kinetic data on isolated AcCh receptor and AcChE, this assumption appears doubtful. It was found that the association rate constant for the binding of cationic ligands, like N-methylacridinium or quinolinium derivatives, is extremely high, $>10^9 \text{ M}^{-1} \text{ sec}^{-1}$ (54). On the other hand, for similar experimental conditions the association rate constant of AcCh binding to isolated receptor proteins is only $3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ (55). So, binding of AcCh to AcChE is about 100 times more rapid than the association of AcCh with the AcCh receptor. Even if substrate inhibition of the esterase is considered, the competition hypothesis suggests a very inefficient mechanism for the use of AcCh.

If, on the other hand, the reaction spaces for both proteins are spatially separated, a sequential processing of AcCh can occur (25, 54). The reaction space for the association of AcCh with the receptor may be the membrane phase, whereas the hydrolytic removal may be extramembraneous. In this hypothesis, the conformational transition of the receptor would not only cause the permeability change but would also translocate AcCh to the reaction space where AcChE hydrolyzes AcCh. Thus the permeability

increase is coupled to the flow of AcCh through the receptor to the esterase (25, 31).

Control concepts for activators like AcCh express conductivity increase in terms of AcCh bound to the high permeability configuration of receptors. However, the binding step as such cannot be reflected in the conductivity change because inhibitors bind too. Thus, some other process coupled to the binding step must participate. The measured time constant of the conductivity change is then necessarily a normal-mode time constant of a coupled reaction system (12). Now, depending on the coupling mechanism and the "concentrations" of the reaction partners, the normal-mode time constant may or may not be dependent on concentration. Thus the elucidation of mechanistic details requires the measurement of concentration dependencies. There may, however, be experimental conditions where the measured time constant has dominant contributions from only one reaction step. Such a situation may be reflected in the data of Stevens (16), who found opposite potential dependencies for the effectiveness of neurally evoked and externally applied AcCh, respectively.

According to the AcCh flow scheme (Figure 1), nerve stimulation will increase the AcCh concentration A_1 in space 1 and drive the cycle along the dominant reaction path $AR \rightarrow AR'$. The closure process represented by the transition $R' \rightarrow R$ will be independent of AcCh concentration, if the concentration of AcCh, $A_{(2)}$, in reaction space 2 is kept low by esterase activity. Thus nerve stimulation drives the AcCh cycle clockwise. On the other hand, if external application of AcCh increases $A_{(2)}$, the opening process is dominantly induced by the reaction $A_{(2)} + R' \rightarrow AR'$; i.e. the AcCh cycle is initially traversed counterclockwise. In this way, opposite potential dependencies would be based on the respective dominance of opposite pathways. If, in addition, receptor contributions to pharmacological desensitization have to be incorporated, a third receptor state R'' has to be considered (Figure 1). The AcCh flow scheme is physically consistent with the basic observations. It takes into account the "apparent inefficiency" of AcCh binding to (isolated) receptor protein compared with AcChE. The measured association rate constant for the AcCh binding to isolated receptor from *Torpedo californica* ($3 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) is very close to the value of $10^7 \text{ M}^{-1} \text{ sec}^{-1}$, derived by Sheridan & Lester from the concentration dependence of the time constants measured at 3 mM Ba^{2+} in voltage-clamped electroplax of *Electrophorus electricus* (17). The relatively low value thus appears not to be a special property of isolated receptors in solution. The kinetic data support the assumption that, as far as AcCh effects are concerned, the isolated receptor protein retains functional integrity when separated from the natural membrane environment.

An activator-receptor cycle like that in Figure 1 has been used to quantitatively describe VC data of axons (51).

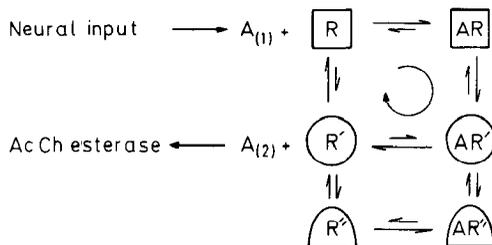


Figure 1 Reaction scheme for the *in vivo* translocation of acetylcholine (AcCh) through the cholinergic receptor system. $A_{(1)}$, AcCh in reaction space 1 separated from the acetylcholine esterase (AcChE); $A_{(2)}$, AcCh in reaction space 2 accessible to AcChE; R and AR, "nonconducting" states; R' and AR', high-permeability configurations of AcCh receptor. Neural input drives the cycle along the path $R \rightarrow AR \rightarrow AR'$; external application of AcCh populates AR' also via $A_{(2)} + R' \rightarrow AR'$.

The overall cyclic nature of a permeability control by AcCh in excitable membranes is already indicated in a reaction proposal developed 25 years ago (30). The present version of the acetylcholine cycle is shown in Figure 2, which displays the complexity of mutual coupling between the various cycles directly or indirectly involved in rapid electrochemical permeability control.

THE ACETYLCHOLINE RECEPTOR SYSTEM

A significant advance toward a more direct understanding of the molecular nature of the AcCh receptor system was made in recent years. It became possible to investigate membrane-bound and isolated receptor molecules by biochemical and physical-chemical means (56–60).

Studies with receptor-rich membrane fragments have provided information at an intermediate level of complexity between the *in vivo* system and the isolated receptor material. On the one hand, the kinetic analysis of tracer (^{22}Na , ^{40}K) efflux measurements (61–64) appears to be a good alternative means of investigating ion conductance changes induced by activators. This technique has further allowed a more refined way of determining classical pharmacological parameters. On the other hand, the study of the binding properties of radioactively labelled or fluorescent ligands, in conjunction with the similar studies performed with the isolated receptor, has yielded valuable correlative information about the respective mechanisms of action (65–68).

The methodological aspects of the procedures to isolate receptor proteins have been reviewed in several recent articles (68–73). Here we focus on examining the significance of the physical parameters that have been measured on the receptor material in various stages of preparation.

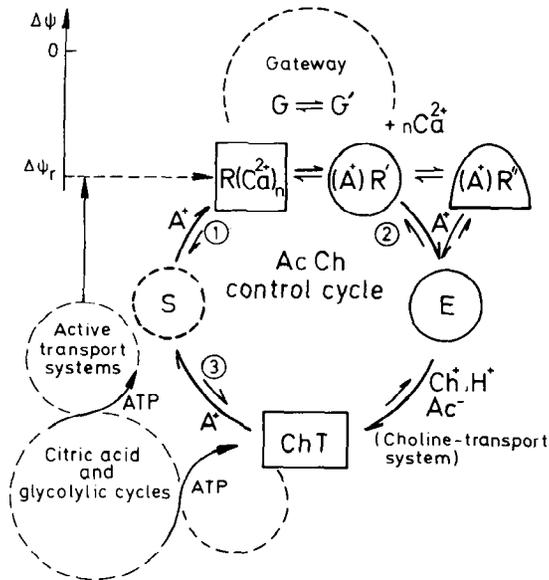


Figure 2 Acetylcholine (AcCh) cycle of the dissipative chemical control of stationary membrane potentials, $\Delta\psi$, and transient potential changes (and ionic cross-membrane currents). The AcCh control cycle for the (rapidly operating) gateway G (Ca^{2+} binding and closed) and G' (the open permeable configuration) consists of the AcCh receptor system (R), the AcCh esterase (E), the choline-O-acetyltransferase (ChT), and a (hypothetical) AcCh storage (S) system. The continuous flux of AcCh through a R - E - ChT - S control "subunit" is maintained by ChT [coupled to the choline (Ch^+) uptake system], and by the practically irreversible hydrolytic removal of AcCh ion, A^+ , from the reaction space ② by E .

The opening-closing process $G \rightleftharpoons G'$ is controlled by the overall receptor reaction $R(\text{Ca}^{2+})_n + \text{A}^+ \rightleftharpoons (\text{A}^+)R' + n\text{Ca}^{2+}$, with $n \leq 2-3$. R is the low, and R' the high, conductivity configuration of the receptor. R'' accounts for pharmacological desensitization. In the resting steady state, the membrane potential ($\Delta\psi_r$) reflects dynamic balance between the active transport (including AcCh-synthesis systems) and the flux of AcCh through the control cycles surrounding the gateway. Fluctuations in membrane potentials and ionic currents are amplified by fluctuations in the local AcCh concentrations. The AcCh control cycle is probably coupled to the electric field of the membrane by the receptor system. The encircled numbers refer to different microreaction spaces for the processing of AcCh. This picture summarizes the present knowledge and is a modified version of a former scheme [Neumann & Nachmansohn (25, 32)].

So far the main sources for the extraction of the receptor material have been homogenates of the electric organ of *E. electricus* and *Torpedo* species. Although other receptor-rich preparations have been investigated (74–78), the following discussion is limited to the AcCh-receptor system of *Electrophorus* and *Torpedo*, which have been studied more intensively. This receptor appears to be an integral membrane protein (79). Detergent solutions are required to isolate it from its membrane environment and to keep the protein in solution. The essential step of virtually all purification procedures presently used is the passage of a crude detergent extract through a suitably labelled affinity-chromatography column. The affinity-labelling compounds used have either been α -neurotoxins or substances containing a quaternary ammonium group.

Certain bulk physical-chemical properties of the receptor material have been determined. Some of the measured values reflect that the intrinsic properties of the isolated material vary according to the physiological origin of the preparation (see Table 1). However, the amino acid composition of *Electrophorus* and *Torpedo* receptors does not differ greatly from that of other membrane-associated proteins (e.g. AcChE) (71, 72, 80). The isolated material could be identified as a glycoprotein (63, 72). The isoelectric point of various receptor preparations is in the range of 4.5 to 4.8 (81, 85), indicating an effective negative charge at physiological pH values. It is significant that the isolated receptor contains free SH groups (84, 86), which appear to decrease upon storage. In the case of *T. californica* receptors, at least 2 SH groups per AcCh-binding site were found (84).

The multiplicity of the values, particularly for AcCh equilibrium binding constants and molecular weights, has led to a series of investigations particularly concerned with the possible modifications of the receptor protein during extraction and purification.

Heterogeneity of Receptor Properties

Several measured properties of the receptor at various stages of preparation and isolation have led to speculations about its functional role. The ability

Table 1 Summary of data on acetylcholine receptors

Species	Bands in SDS-gel electrophoresis	Molecular weight	Reference
<i>Electrophorus</i>	43,000; 48,000	—	72
	45,000; 54,000	230,000 \pm 15,000	124
<i>T. californica</i>	40,000; 50,000; 65,000	—	68
	—	330,000; 660,000	97
<i>T. marmorata</i>	45,000; 50,000	—	70
	—	330,000; 1,300,000	97

to bind AcCh and other cholinergic activators has been used to characterize the physical-chemical state of the protein, and changes in binding have been associated with conformational changes that may occur during receptor functioning. Furthermore, the relative position of components in the sedimentation profile of diverse receptor preparations has led to speculations about the aggregation properties of receptor units and the role of possible macroassociations of receptors necessary for excitability. The diversity in ligand-binding parameters and colligative properties has been attributed to a number of factors, such as the constraints present in the membrane and the modifying effect of the solvent in solubilized and purified preparations.

As the values in Table 2 reflect, equilibrium binding measurements indicate that the *Torpedo* membrane-bound receptor binds AcCh with a single high-affinity constant corresponding to an equilibrium dissociation constant $K \approx 10$ nM, whereas solubilization and purification are associated with the appearance of low-affinity binding sites, $K \approx 1$ μ M.

O'Brien & Gibson (87, 88) have proposed that the low-affinity form is the result of a denaturation of the high-affinity form, which is suggested to be the physiologically significant state of the receptor. They found that the membrane-bound receptor in fresh particulate fractions of *T. californica* electroplax tissue is relatively insensitive to heat treatment. In contrast, in the case of detergent extracts and purified receptor material, heating, aging, or treatment with certain SH-group oxidizing agents leads to a predominantly low-affinity form. This irreversible conversion was suggested to involve the oxidation of SH groups.

On the other hand, Sugiyama & Changeux (89) have reported that the different binding forms are interconvertible. They found that dissolution of receptor-rich fragments of *Torpedo marmorata* in cholate detergent led to the appearance of medium-affinity ($K \approx 0.1$ μ M) and low-affinity AcCh-binding sites. These forms were still present upon an exchange of detergents. The dilution of the detergent solution was accompanied by a partial recovery of the high-affinity binding sites. These results led Sugiyama & Changeux to conclude that an intrinsic heterogeneity of the structure of the receptor protein underlies the different binding forms, rather than detergent-receptor interactions.

Since upon aging and purification of the receptor preparation no significant recovery of high-affinity sites could be brought about (89), it is possible that the low-affinity form observed by O'Brien & Gibson results from a relatively severe treatment, and that the presumed interconvertible forms of Sugiyama & Changeux are present under milder conditions.

Several authors have discussed the difference between the thermodynamic dissociation constants (K) measured in binding studies for various

Table 2 Acetylcholine-binding properties of acetylcholine receptor

Species	Material	K (M)	Binding site	Reference
<i>T. marmorata</i>	membrane	1×10^{-8}	—	113
			<u>nmole/g tissue</u>	
<i>T. marmorata</i>	homogenate	8×10^{-9}	0.1	80
		6.8×10^{-8}	0.83	
<i>T. marmorata</i>	solubilized (crude)	1.4×10^{-9}	0.2	81
		2.2×10^{-8}	0.45	
<i>T. marmorata</i>	solubilized (crude)	2×10^{-8}	0.5	84
<i>T. marmorata</i>	solubilized (crude)	1.1×10^{-8}	0.04	100
	(1% Triton)	5.6×10^{-7}	0.2	
<i>T. marmorata</i>	solubilized (crude)	4.2×10^{-9}	0.087	
	(4% Lubrol)	3.3×10^{-7}	0.15	
			<u>% of toxin site</u>	
<i>T. marmorata</i>	solubilized (crude)	1.7×10^{-7}	40	89
	1% Na-cholate	2×10^{-6}	60	
<i>T. marmorata</i>	solubilized (crude)			
	2% Emulphogen	1.4×10^{-8}	110	
<i>T. marmorata</i>	solubilized (crude)	1×10^{-8}	57	
	5% Triton	3×10^{-7}	43	
<i>T. marmorata</i>	solubilized (crude)			
	0.2-0.03% Triton	3×10^{-8}	103	
<i>T. marmorata</i>	purified	3×10^{-8}	6.8	
		2×10^{-6}	107	
<i>T. marmorata</i> & <i>T. californica</i>	purified	2×10^{-8}	34.6	84, 85
		2×10^{-6}	65.4	
<i>T. californica</i>	membrane (fresh)	1.4×10^{-8}	—	87
<i>T. californica</i>	solubilized (crude)	1.7×10^{-8}	—	
	1% Triton			
<i>T. californica</i>	purified	2.3×10^{-6}	42	83
<i>T. californica</i>	purified	5×10^{-6}	52	67
<i>Electrophorus</i>	purified	6×10^{-8}	46	72

receptor preparations and the half-response constants inferred from in vivo electrophysiological or in vitro tracer-efflux binding studies. Since the half-response constant is generally in the range of the K -values for the low-affinity form, it has been proposed that the high-affinity form observed in binding studies with membrane fragments is not associated with the physiologically active state of the receptor (66, 43). In accordance with models proposed to explain pharmacological desensitization (39,90) Cohen et al (66) suggested that, in addition to a "resting" and an "active" state, the receptor can also exist in a "desensitized" form, which would have a high affinity for activators.

Weber et al (91) have produced evidence in support of this hypothesis. They found that the exposure of *T. marmorata* receptor-rich membrane fragments to activators leads slowly (minute time range) and reversibly to a 5- to 20-fold increase in affinity for activators. This property is lost after aging of the preparations.

Popot et al (92) have reported that preincubation of *T. marmorata* fragments with carbamylcholine causes a subsequent decrease of the amplitude of the response to the same activator as measured by ^{22}Na efflux. Recently Grünhagen & Changeux (93) have used a fluorescent ligand, the local anesthetic analog quinacrine, to investigate conformational changes of the membrane-bound *Torpedo* receptor. They interpreted their results within the framework of the model of Katz & Thesleff (39). A kinetically unresolved rapid initial increase in fluorescence intensity upon the addition of an activator is suggested to represent the activation of the receptor-ionophore complex, while a subsequent slow decrease (second time range) is thought to correspond to the generation of a desensitized state, exhibiting a high affinity for activators.

Michaelson et al (94) have reported the presence of interconvertible high- and low-affinity binding forms in *T. californica* membrane fragments. They suggested that the high-affinity form may correspond to a desensitized state, and that conditions used for the preparation of membranes prior to affinity chromatography in the isolation procedure could result in material largely converted to that form irreversibly.

It appears, therefore, that activators induce a slow modification of the membrane-bound receptor, which leads to a decrease of the electrophysiological response, and an increase in the affinity for activators. A corresponding effect has not been observed with solubilized or purified receptor preparations (66). These findings may have the implication that only studies with membrane-bound receptors will yield reliable information about the functional properties of the physiologically active form of the receptor [see, however, (17, 55)]. Furthermore, since several coupled kinetic steps appear to be involved, equilibrium dialysis studies on conformational equilibria yield only overall dissociation constants, which represent the ratio between free ligand and total bound ligand.

It should be noted that while the studies cited above have dealt exclusively with *Torpedo* material, relatively little has been reported concerning desensitization in other preparations. Hess et al (95) have reported that desensitization is not observed in efflux studies involving *Electrophorus* membrane fragments.

An alternative but formally similar explanation of the difference between the half-response constants and thermodynamic dissociation constants was offered by O'Brien & Gibson (88). Noting that at sufficiently low AcCh concentrations positive cooperativity is observed in equilibrium binding

studies, they proposed that the presence of two binding forms, of which only one is physiologically active, could account for the observed data within the framework of the Monod-Wyman-Changeux model (96).

A multiplicity of results has also been reported for the determination of the molecular weight of various receptor preparations (see Table 1). Chang (86) found that the relative number of components with a molecular weight higher than the main species observed in gel electrophoresis and gel exclusion chromatography increase during storage of the purified *Electrophorus* receptor, and proposed that an irreversible aggregation of the major component was caused by an oxidation process leading to the formation of intermolecular disulfide linkages. Edelstein et al (97) found that aside from a 330,000-dalton unit, *T. marmorata* preparations contain a 1,300,000-dalton unit, and *T. californica* preparations a 660,000-dalton unit. They also suggest that the heavier components may result from SH-group oxidation during storage. In this connection it is noteworthy that commercially available detergents often contain oxidizing agents as contaminants or bleaching materials that might promote the aggregation of solubilized receptor (98).

O'Brien and co-workers (88, 99) investigated the sedimentation profile of solubilized and purified *Torpedo* receptor preparations. Their results for *T. californica* protein indicate that a low-molecular-weight (L) form and a relatively heavier form (H) figure prominently. Heating had no effect on the L form but halved the peak for H, and led to the appearance of a heavy oligomer (HH). Similarly at high pH, the L peak was left unaltered but the H peak was again decreased, and a low-molecular-weight component (LL) was formed. Both H and L peaks were found to be stable at neutral pH. The authors proposed a tentative assignment of molecular weights, where the LL form is suggested to be a minimal binding unit (89,000 daltons), and the H form was identified as a dimer of the L form and a hexamer of the LL form. The HH form was interpreted to be a detergent artifact. The H form is considered a likely candidate for the physiologically active unit.

It appears that our present knowledge of the physical state of the receptor macromolecules in various preparations is marred by the possibility of artifacts associated with the presence of detergent, oxidizing contaminants in the detergent, and the absence of membrane constraints required to maintain a physiologically active configuration. Much careful work will be required to assure that the evidently quite labile protein is obtained in a functionally intact form, so that physiologically more relevant physical-chemical studies can be carried out.

Ligand Binding and Receptor Functioning

As was discussed above, *in vivo* investigations have revealed that a relatively complex chain of processes is involved that leads to the conductance changes induced by the binding of activators. Desensitization, cooperative

effects, the "control" properties of Ca^{2+} ions, the influence of the membrane potential, and the selectivity in the transfer of ions should all be accounted for in a full description of receptor functioning. Although at present our knowledge is still very incomplete, ligand-binding studies, and studies involving the chemical modification of the receptor, have yielded some information about the nature and disposition of receptor groups that take part in the molecular events underlying permeability changes.

Pharmacological investigations have shown that the AcCh receptors of eel and *Torpedo* electric organs have ligand-binding properties typical of nicotinic cholinergic receptors (82, 100, 101). There have been several attempts to deduce the nature of the nicotinic agonist-binding region (102–105). The site complementary to most monoquaternary nitrogen activators presumably contains an anionic moiety corresponding to the quaternary nitrogen groups, and a spatially restricted region capable of accommodating a relatively flat group, such as an aromatic ring or a carbonyl segment (106). Furthermore, it is probable that there is a proton donor group at the receptor complementary to the AcCh carbonyl oxygen (107).

Little is known about the intramolecular processes in the receptor that are connected with activator binding. However, since the tetramethylammonium ion itself is an activating ligand (108), an interaction involving the anionic binding site appears to be of fundamental importance. Whether further interactions with the nonquaternary portion of activators serve to promote conformational transitions triggered by binding, or whether they merely prolong the lifetime of the activator-receptor complex, remains to be determined.

Karlin et al have studied changes in receptor properties brought about by selected chemical modifications. They found that the reduction of at least one disulfide bond in the *Electrophorus* electroplax receptor leads to a change in the pharmacological specificity. The in vivo response to monoquaternary activators is decreased (109), and the response to bisquaternary agents is increased (108). Studies involving the covalent attachment of affinity-labelling compounds (108, 110) to the reduced receptor have led to conclusions about the spatial position of the labile disulfide bond relative to the anionic binding site. It has been suggested that the receptor-activator binding compartment in the active state is in a "shortened" conformation compared to that in the inactive state (111).

A number of investigations have revealed the existence of apparently distinct binding sites for several other low-molecular-weight ligands. Pharmacological studies indicate that local anesthetics such as procaine and tetracaine bind at a site that interacts noncompetitively with the monoquaternary activator-binding site (112–114). Cohen et al studied the effect of various local anesthetics on the binding of AcCh and the fluorescent ligand

dansylcholine to the membrane-bound *Torpedo* receptor (66). They found that these agents increase the affinity of the receptor for both cholinergic activators and inhibitors at about the same concentrations at which they are physiologically active.

Investigations of the competition of mono- and bisquaternary agents with the binding of the fluorescent ligand bis-(3-aminopyridinium) 1,10-decane diiodide to the purified *T. californica* receptor led Raftery et al (68) to a model for ligand binding that encompasses two anionic sites. Monoquaternary activators are thought to bind to one of the two subsites, while the other would preferentially complex inorganic ions. Bisquaternary agents are considered to be bound to both moieties and to interact competitively with the binding of monoquaternary agents at one site and with the binding of cations at the other. It should be noted, however, that the apparent binding properties of charged ligands are intrinsically dependent on the ionic strength of the solution. Changes in the apparent binding constants caused by direct competition with inorganic cations may therefore not be a necessary assumption.

It is not known how the auxiliary binding sites of the receptor system for bisquaternary compounds or local anesthetics are involved in the in vivo receptor functionings.

There have been several investigations of the binding of divalent cations to the receptor purified from *Torpedo* electric organs (25, 68, 115–117). Eldefrawi et al found that large amounts of calcium ions are bound to the receptor (115). They reported that Ca^{2+} can block the binding of [^3H]acetylcholine, and deduced an apparent dissociation constant of 7×10^{-3} M for this cation. RübSamen et al (117) studied the fluorescence enhancement that accompanies the binding of terbium ions to the receptor. Assuming a direct competition between Ca^{2+} and Tb^{3+} ions, they obtained an apparent dissociation constant of 1.1 ± 0.1 mM for Ca^{2+} . Their results further indicate that Tb^{3+} ions, bound to sites that interact with Ca^{2+} ions, are displaced by activators, but not by inhibitors.

RübSamen et al (118) have also proposed that the terbium-binding sites of the receptor are associated with the same polypeptide chain as the binding site for activators. However, it may be questioned whether the binding sites they report for receptor fragments under denaturing conditions (SDS treatment) are to be identified with sites present in the intact material.

Chang & Neumann (25, 55, 116) studied the calcium-binding properties of *Electrophorus* and *Torpedo* receptors by a number of methods. In the case of *Torpedo californica* receptors there are at least three types of binding sites with apparent dissociation constants ranging from mM to $< \mu\text{M}$. The results indicate that at least one of these sites is competitively affected by

the binding of AcCh ($K_{Ca} = 3 \times 10^{-3} \text{ M}$). In agreement with the dependence of the apparent AcCh dissociation constant on Ca^{2+} concentration, it was found that AcCh binding leads to a release of Ca^{2+} ions from the receptor. On the other hand, α -bungarotoxin causes an uptake of Ca^{2+} ions. It thus appears that neural activators and inhibitors have opposing effects on the Ca^{2+} binding of receptors (116).

In contrast to these findings for the purified receptor, Cohen et al (66) reported that receptor-rich *Torpedo* membrane fragments show increased affinity for cholinergic ligands in the presence of Ca^{2+} . This effect, which was not observed with detergent-solubilized or purified preparations (66), was suggested to be related to the increase in desensitization brought about by Ca^{2+} ions in electrophysiological studies in vivo (43, 66).

These findings indicate that the functionally significant ligand interactions with the intact receptor units are relatively complex. There is at present little information about the detailed molecular events that are elicited or controlled by the binding of Ca^{2+} ions. According to specific proposals (31–33) the binding of AcCh was suggested to lead to a release of Ca^{2+} ions that is concomitant with a change in structure and organization of the gateway controlling the permeability to passive ion fluxes. Indeed, recent kinetic results show that the Ca^{2+} -receptor interactions involve at least three conformational states and that there is at least one intramolecular (conformational) change in the isolated receptor upon AcCh binding (55). Thus the receptor activation would be inhibited by Ca^{2+} ions, whereas the process observed by Cohen et al, attributed to physiological desensitization, is favored by Ca^{2+} ions (39, 66).

At present little is known about a possible coupling of receptor sites to the membrane potential. However, the voltage dependence of conductance changes implies that such a coupling exists. This coupling could involve orientations of poly-ionic regions or dipoles of the receptor, in the transmembrane electrical field, or it may be indirectly connected with the counterion atmosphere associated with fixed charges, or the binding equilibrium itself may be dependent on the electrical field.

Structural Organization and Functional Role of Receptor Subunits

Since the intact receptor-ion translocation system must ultimately include a membrane-partitioned structure across which ionic gradients are maintained, certain functional properties of the native control system will necessarily not be measurable on the isolated receptor material. This absence of intrinsic properties clearly limits the possibility of examining the degree to which the isolation procedure modifies the receptor unit. Although the presence of a component responsible for the *recognition* of characteristic

ligands is virtually assured, since ligand binding can be used to register the success of the extraction procedure, components associated with the ion-translocation process may have been lost or modified. Obviously the reconstitution of the isolated materials in a membrane is required to investigate the full spectrum of functional properties observed in the *in vivo* system.

The investigation of receptor material using gel electrophoresis under denaturing conditions has indicated the presence of several possible subunits (see Table 1) in which a 40,000-dalton unit and a slightly heavier unit of 54,000 daltons figure prominently.

The structural organization of isolated receptor and of receptors in membranes has been studied by a number of techniques. Electron micrographs of purified *Electrophorus* (72) and *Torpedo* (71) receptors indicate the presence of ring-like particles, 8–9 nm in diameter, which appear to consist of 5–6 subunits surrounding an electron-dense core. Freeze-etching (119, 120) and negative-staining (121) electron micrographs, as well as X-ray diffraction studies (122, 123) of receptor-rich *Torpedo* membranes, reveal a closely packed, regular array of particles, with a diameter of approximately 8 nm.

These findings have stimulated the proposals of specific models for the organization of receptor subunits in membranes. Hucho & Changeux (124) have suggested that three possibly identical subunits may be involved in the binding of cholinergic ligands, while other different subunits coupled to these may be involved in ion-transport processes. Raftery et al (123) have proposed a model encompassing three pairs of subunits organized in layers, which would have an inherent structural asymmetry.

The presence of a unit associated with the transport of ions (ionophore) in isolated preparations, which is functionally coupled to the activator-binding region of the receptor, is strongly indicated by reconstitution experiments utilizing receptor-rich membrane fragments. Hazelbauer & Changeux (125) were able to show that solubilization of microsacs in cholate, and subsequent reconstitution in a membrane of crude lipids extracted from the native membranes, does not entail a loss of agonist-induced cation permeability properties. Michaelson & Raftery (94, 126) incorporated purified receptor material into vesicles made from extracted *Torpedo* lipids. They were able to show that these structures were in several cases excitable by acetylcholine and carbamylcholine.

Attempts to incorporate receptor material in black lipid films have been reported by several laboratories (127–131). It appears that the technique is very susceptible to artifacts. Experience has shown that minute amounts of bacteria, organic impurities, and dust particles can cause major electrical artifacts. The quantities that have been determined are the rate of conductance increase and miniature conductance changes.

AN INTEGRAL VIEW OF RECEPTOR-MEDIATED ELECTRICAL CHANGES

Receptor-activator interactions control the production of a physiological response in numerous systems. A common feature of receptor-mediated processes is that the binding of a specific ligand to the receptor leads to the activation of a further functional component, the effector.

Cuatrecasas and co-workers (132–135) have proposed the mobile receptor model as a mechanistic scheme whereby hormone receptor occupancy generates biological signals. Hormone receptors and effectors are thought to diffuse independently within the plane of the membrane. The receptor may associate reversibly with the effector to regulate its activity. The affinity of the hormone-receptor complex for effector is suggested to be greater than the affinity of the unoccupied receptor for the effector.

A general scheme for signal-transmitting processes has recently been proposed by Strange & Koshland (136). Receptors are designed to bind specific ligands with high affinity, to ensure sensitivity at low concentrations of ligand. Either the receptor may be structurally closely connected with the effector part, or it may associate with the effector system only after activator binding. In a complex system comprising several receptor macromolecules, each receptor may interact with a different effector ("parallel pathway"), or receptors may compete for an effector ("focused pathway"). In excitable membranes receptors are functionally coupled with effector units, controlling the membrane permeability to ions. The effector need not be a molecular entity separate from the receptor macromolecule, but it must comprise a functional component assuring a lower activation barrier for transmembrane ion transfer than that of the remaining membrane phase.

The electrical responses induced by neural activators at synapses and neuromuscular junctions may be classified according to their time course and the nature of the electrical polarization generated. Thus, at mammalian ganglionic synapses a fast excitatory postsynaptic potential (f-e.p.s.p.), a slow excitatory postsynaptic potential (s-e.p.s.p.), and a slow inhibitory postsynaptic potential (s-i.p.s.p.) have been discerned. The f-e.p.s.p. occurs within milliseconds, and is elicited by the interaction of AcCh with nicotinic cholinergic receptors. The s-e.p.s.p. is due to the AcCh-induced activation of muscarinic cholinergic receptor systems and has a minimum latency of ~100 msec and a duration of 0.5 sec or longer (137–139). The s-i.p.s.p. is elicited by dopamine. Evidence is accumulating that the dopaminergic response is mediated by the production of cyclic AMP, by an adenylate cyclase coupled to the dopamine receptor (140–144). It has been proposed that the physiological response is ultimately produced by the phosphorylation of a membrane protein through the agency of protein kinases (144). Similarly

there is evidence that the muscarinic response is mediated by cyclic GMP (142, 143, 145, 146) and that muscarinic cholinergic receptors are coupled to a guanylate cyclase.

Purves (147) has proposed that muscarinic receptors are specialized to allow efficient temporal summation of potential changes. On the other hand, the rapid responses controlled by nicotinic receptors are less suited for mutual summation. They operate with a shorter latency and a correspondingly greater temporal precision. It appears that systems involving production of a cyclic-nucleotide-mediated response may generally be associated with a relatively slow rate of onset. Their functional role in connection with nicotinic action is to "regulate" the potential across the synaptic membrane, and thereby to modulate nicotinic cholinergic processes.

The investigation of the cholinergic receptors of fish electric organs has not led to unequivocal evidence that the ion-translocation (effector) component is a part of the receptor macromolecule, although reconstitution studies with microsacs suggest this alternative. The rapid response of the nicotinic system to activators would therefore be attributable to the direct functional association of the activator-receptive unit with the effector component.

Another factor that may be connected with the special role of the nicotinic system is the relative lack of specificity of activator-nicotinic receptor interactions. Pharmacological studies indicate that the muscarinic activator-binding site is spatially much more restricted than the corresponding nicotinic site. The degree to which modifications of the chemical structure of the muscarinic activator affect pharmacological activity is correspondingly greater (107, 148). In the case of activators of the β -adrenergic receptors it appears that a certain portion of the activator functional groups are connected with the affinity of these agents, while another portion primarily determines the response-producing capacity (149).

The lack of this type of specificity at nicotinic activator sites may be connected with the kinetic steps required to produce a rapid response, e.g. rapid access to and departure from the binding region for activators.

Jencks (150) has recently discussed at length how the intrinsic binding energy that results from the noncovalent interaction of a specific substrate with the active site of an enzyme may be utilized to provide the driving force for the catalytic modification of the substrate molecule. In the case of receptor-activator interactions, the situation is somewhat reversed. The activator molecule A has the "catalytic" function of reducing the activation barrier for a transition to an altered form R' of the receptor molecule R. The intrinsic binding energy of the activator is therefore utilized to promote the population of a specific receptor form AR'. This process may predominantly occur either by means of an induced change via

$A + R \rightleftharpoons AR \rightleftharpoons AR'$ (upper pathway), or by means of a shift in the equilibrium involving the less populated form of the receptor R' (lower pathway) via $A + (R' \rightleftharpoons R) \rightleftharpoons AR'$:



Such a cyclic kinetic scheme adequately expresses essential features of the activation phase of activator-receptor interactions. In addition, a mechanism responsible for the removal of activator from the reaction compartment contributes to the overall dissipative nature of these processes. Although many hormone-receptor complexes are associated with a relatively long period of action, rapid removal of activator by a degradative protein such as AcChE, or by reuptake of activator, occurs in synaptic control processes. The same general activator-receptor mechanism can be used to describe quantitatively the rapid transient permeability changes in nonsynaptic (axonal) parts of excitable membranes. Such a regulatory principle, entailing fast dissipative changes, appears to be a distinguishing feature of electrical-chemical excitability. The functional significance of this special case of activator-receptor interaction is presumably to ensure fast repetitive responses of the receptor system in excitable membranes, by the rapid regeneration of active receptive units.

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This review is dedicated to David Nachmansohn, a pioneer in the biochemistry of excitable membranes.

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