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Chemical Events in Conducting and Synaptic Membranes during Electrical Activity

(protein assembly/acetylcholine/Ca^{++}/α-toxin/electroplax Na^+ efflux)

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ABSTRACT **Evidence has accumulated in recent years for the central role of proteins and enzymes in the function of cell membranes. In the chemical theory proposed for the generation of bioelectricity, i.e., for the control of the ion permeability changes of excitable membranes, the protein assembly associated with the action of acetylcholine plays an essential role. Support of the theory by recent protein studies in which the excitable membranes of the highly specialized electric tissue were used will be discussed. A scheme is presented indicating the possible sequence of chemical reactions that change ion permeability after excitation. A sequence of chemical events within the excitable membranes of the synaptic junctions, i.e., within the pre- and postsynaptic membranes, similar to that proposed for the conducting membranes, is presented in a second scheme as an alternative to the hypothesis of the role of acetylcholine as a transmitter between two cells.**

In view of their crucial function, cell membranes have been in recent years among the most actively explored fields in biological sciences. While a decade ago the notion of the unit membrane (1), based on the Danielli–Davson model, appeared at first attractive to many investigators, it soon proved inadequate to account for a variety of observations (see e.g., 2–6). In spite of the many great efforts to elucidate the membrane structure, the topography and the precise molecular arrangements of phospholipids and proteins is at present still under lively discussion. Quite a few interesting models have been proposed in the last few years (7–10). However, the most pertinent feature of recent developments is the conceptual change in respect to function. As it is now widely recognized, membranes are highly dynamic and well-organized structures, the site of a great number of different proteins and enzymes and of intensive chemical reactions. Whereas the importance of phospholipids is not contested, e.g., in their role as barriers, the emphasis in the present notions of functional activities of membranes, whatever the structural arrangements may be, has shifted to the central role of proteins and enzymes. Proteins account more readily than phospholipids for the great diversity of the function of different cell membranes, their high degree of specificity, and their remarkable efficiency (4, 8, 11).

The excitable membranes, i.e., the plasma membranes surrounding nerve and muscle fibers, have the special ability of controlling the rapid and reversible changes of permeability to ions that move across a membrane and carry the electric currents that propagate nerve impulses. Hodgkin assumes that a simple diffusion process accounts for these ion movements; he explicitly rejects any chemical reactions in bioelectrogenesis (12, 13). The theory is contradicted by many experimental data. For instance, drastic changes of ion concentrations on the inside or the outside of the axon have little effect on the electrical parameters, contrary to the prediction of the theory (14). The strong heat production and absorption that coincide with electrical activity (15) and the production during the rising and the absorption during the falling phase of the action current (16) cannot be explained by ion mixing or ion friction, as proposed by supporters of the theory (12, 16); there is no alternative to the assumption, as was stressed by A. V. Hill (17), that this heat produced and absorbed must be attributed to chemical reactions effecting the permeability changes. Moreover, the concepts of the Cambridge group were developed on the basis of the Planck–Nernst equations, which can only be applied to systems in equilibrium, whereas cell membranes—as is today widely accepted—necessitate the use of nonequilibrium thermodynamics (18).

A chemical theory has been developed during the last 3 decades, based on an approach that centered on protein and enzyme chemistry and on the use of bioenergetics. A large amount of evidence has accumulated for the presence of a special protein assembly in excitable membranes that controls the permeability changes during electrical activity (19–24).

CHEMICAL EVENTS IN CONDUCTING MEMBRANES

The theory is schematically presented in Fig. 1. Acetylcholine (AcCh), long believed to be a "neurohumoral transmitter" between nerve and nerve or nerve and muscle, is the trigger that starts a series of reactions resulting in a permeability change of excitable membranes to Na^+ and K^+ ions. The release and the action of AcCh are taking place *within* the membrane. AcCh is bound in resting condition in some storage form; it is released on excitation. Whether K^+, H_3O^+ ions, protons, or the electric field are the active agents is an open question. The free AcCh acts on the AcCh-receptor protein (R) to induce a conformational change, as was first suggested in my Harvey Lecture in 1953 (25). This change may release, by allosteric action, Ca^{2+} ions bound to carboxyl groups of the protein (the second valence may be possibly bound to $P\text{-}O^-$ groups of phospholipids). Ca^{2+} ions have long been associated with excitability; they are known to have a strong effect on the conformation of phospholipids. Change of conformation of these compounds and other polyelectrolytes

Abbreviation: AcCh, acetylcholine.

may markedly increase the membrane permeability to ions. The end result of this series of reactions is the amplification of the signal to permit the movements of thousands of ions, possibly as many as 20,000–40,000, in each direction, per molecule of AcCh released. While Ca^{2+} ions may strongly affect that element of the membrane that is referred to as ionophore by Changeux (26), they must be controlled by specific reactions. Only proteins have the ability to recognize ligands, which act as specific signals. The ion movements start a new circuit, stimulating adjacent points; there the same processes are repeated and the impulse is thus propagated along the axon. The free and bound forms of AcCh and receptor are in a dynamic equilibrium. The free ester is attacked and hydrolyzed by AcCh-esterase (EC 3.1.1.7; *E*, see Fig. 1) in microseconds, thereby permitting the receptor to return to its original conformation and restoring the ion barrier. The whole system will probably be structurally highly organized, such as is known to be the case with other protein assemblies in membranes, which would account for the high speed, the precision, and the efficiency of the process.

Instrumental in these developments has been the availability of a tissue highly specialized in bioelectrogenesis: the electric organs of fish. There are few examples in nature of such a high degree of specialization. This is indicated by the low protein (3%) and high water content (92%) of the tissue. When the writer discovered, in 1937, that 1 kg of electric tissue (fresh weight) hydrolyzed 3–4 kg of AcCh per hour, this absolutely amazing figure immediately suggested that here was a uniquely favorable material for the biochemist to investigate the proteins and enzymes associated with the function of AcCh and for the aim of correlating biophysical and biochemical phenomena. This hope was borne out by subsequent developments. In the 3 decades after this discovery, the use of this tissue was decisive for the isolation, characterization, and analysis of the proteins associated with AcCh function and their role in bioelectrogenesis (23, 27).

The exclusive localization of AcCh-esterase in excitable membranes of a great variety of different types of preparations, by means of electron microscopy in combination with histochemical staining techniques, confirmed the suggestion made 3 decades ago based on biochemical data, which were of necessity indirect (21, 22). Recently, Changeux and his associates (28) separated the excitable and nonexcitable membranes of eel electroplax, either by mechanical separation or by the use of differential centrifugation and discontinuous saccharose density gradients; they found virtually all the enzyme in the excitable membrane (Fig. 2). The enzyme is uniformly distributed along the entire surface of the excitable membrane; there is no difference between synaptic and conducting parts of the membrane. Applying newly developed histochemical techniques, Drs. Nancy Tomas, Richard Davis, and George B. Koelle have obtained electron micrographs of the electroplax that demonstrate the exclusive and uniform localization of the enzyme in the pre- and postsynaptic, as well as conducting parts of the membrane, in a particularly elegant and striking way (personal communication). In the light of these new data, the AcCh-esterase concentration in electric tissue must be referred to the excitable membranes only: 1 g of excitable membrane hydrolyzes 30 kg or more of AcCh per hour. The enzyme protein forms approximately 5% of the total volume of the

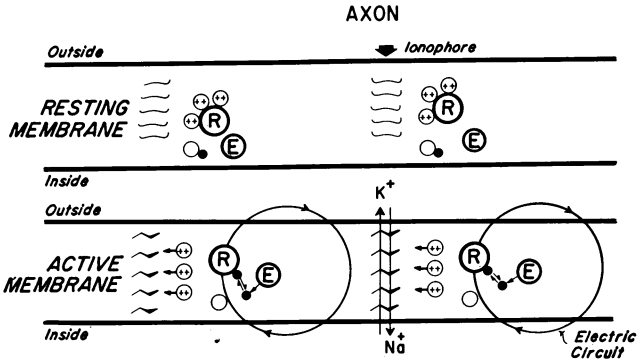

FIG. 1. Schematic presentation of the role of AcCh postulated to act as a signal within excitable membranes and to initiate a series of reactions resulting in increased ion permeability. In the resting condition, AcCh (●) is bound to a storage form. On excitation, AcCh is released within the membrane and acts on the receptor (*R*) inducing a conformational change. Ca^{2+} ions (⊕) bound to the protein, may be released by allosteric action and act on the ionophore inducing conformational changes of phospholipids and other polyelectrolytes and permitting, thereby, accelerated ion movements. The amplification of the signal initiates a new electrical circuit that stimulates adjacent points; there the processes are repeated. AcCh is rapidly hydrolyzed by AcCh-esterase (*E*); the receptor returns to its resting condition and the barrier for ions is reestablished. ●, AcCh; ⊕, Ca^{++}.

excitable membrane of the electroplax. The number of enzyme molecules in the membrane of a single electroplax has been estimated to be about 1 to 5 × 10^{-13} mol [Changeux *et al.* (29) and unpublished data of Dr. T. L. Rosenberry, personal communication].

The monocellular electroplax preparation of *Electrophorus*, developed in this laboratory about 15 years ago (30), offered a uniquely favorable material for the analysis of many characteristics of the receptor protein. Some of the results (active site, cooperativity and allosteric effects, affinity labeling of the receptor, etc.) have been discussed in the preceding communication and elsewhere (21, 22, 24). Just as for the enzyme, the electric tissue offers a uniquely favorable material for isolation of the receptor protein, especially in combination with the information obtained about some of its characteristics. In the last few years, several groups have devoted great efforts to isolate and characterize the receptor protein from electric tissue, applying a variety of procedures (29, 31–39). A few aspects pertinent to this presentation may be mentioned. An important new tool for these studies is the use of snake-venom toxins, applied first by Changeux, Lee, and their associates, in particular the α-bungarotoxin prepared from the venom of *Bungarus mutlicinctus* (29), and the α-toxin of *Naja nigricollis* (32). When solubilized extracts were prepared from electric tissue that contained both receptor and AcCh-esterase, and α-toxin of *Naja* coupled to Sepharose was added, 75–100% of the receptor was absorbed, whereas the enzyme remained in solution. This successful separation shows that the enzyme and receptor are two different proteins. The difference between the two active sites has been apparent for a long time, but the problem of whether two different proteins are involved had been an open question and has now been answered. The α-toxin of *Naja* was also used for testing the localization of the receptor in the electroplax. Antibodies to the toxin were prepared, coupled with

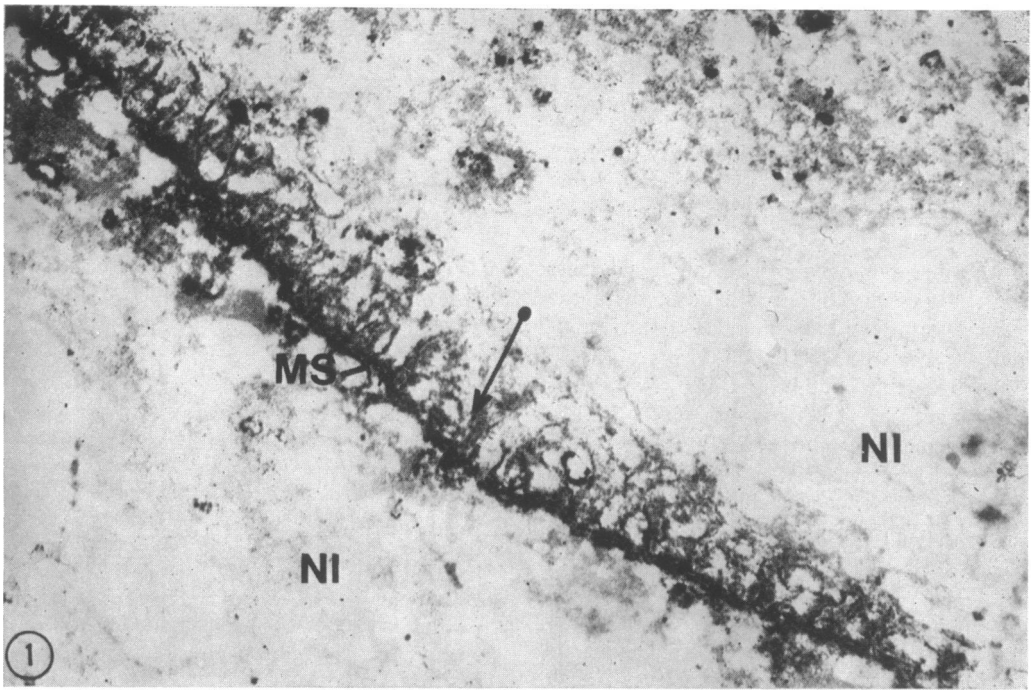

FIG. 2. Electron micrograph of an isolated excitable membrane fragment of eel electroplax. Picture shows a striking uniformity of the AcCh-esterase distribution in the innervated surface (*MS*) of the membrane; no staining in the noninnervated membranes (*NI*). Acetylthiocholine was used as the substrate in the staining procedure. (From Changeux, Gautron, Israel, and Podleski, ref. 28.)

immunoglobulins, and conjugated with fluorescein (33). It was found that the toxin binds exclusively to the excitable membrane (Fig. 3). The distribution of the receptor (Fig. 3*A*) is not quite as uniform as that of the enzyme (Fig. 3*B*), shown on an equivalent segment of the excitable membrane. For the interpretation of this electron micrograph and for the question of whether the receptor is uniformly distributed in synaptic and conducting parts of the excitable membrane, several factors must be considered. First, the total surface area of the synaptic junctions, in spite of their great number, forms less than 5% of the total surface area of the excitable membrane; the estimate of 5% was originally based on electron micrographs taken some 15 years ago, which did not show the strong infoldings of the innervated membrane as they are apparent in recent electron micrographs. The synaptic surface area would thus hardly account for the large part of the fluorescent segments. Secondly, the physicochemical data clearly indicate the presence and functional activity of the receptor in both conducting and synaptic parts of the membrane (21, 22). Moreover, in contrast to the procedures demonstrating the presence of AcCh-esterase, worked out and improved over 2 decades, that for the localization of the receptor is quite new and the authors indicate that they are working on its improvement and its adaptation to electron microscopy. Considering all these factors, it seems to the writer that the electron micrographs presented favor the assumption of the localization of the receptor parallel to that of the enzyme.

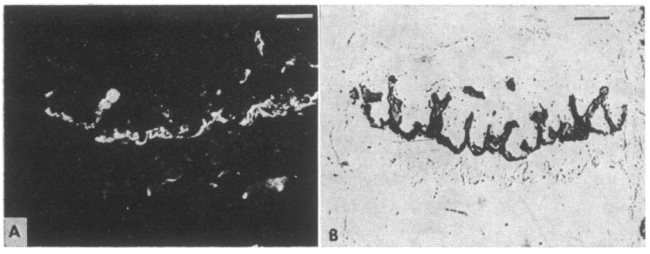

FIG. 3. Electron micrographs showing the localization of AcCh-receptor and -esterase in the excitable membrane of eel electroplax. (*A*) Immunochemical localization of α-toxin of *Naja nigricollis* (reacting with the receptor) bound to a section of an electroplax. (5 μg/ml of α-toxin, 0.2% formaldehyde, 100-fold dilution of rabbit and sheep serum.) The length of the bar is 100 μm. (*B*) Localization of acetylcholinesterase on a section of electroplax by Koelle's reaction. Orientation of sections in *A* and *B* are identical. (From Bourgeois, Tsuji, Boquet, Pillot, Ryter, and Changeux, ref. 33.)

It may also be mentioned that a preparation has been recently developed that permits studies of the proteins on isolated fragments of the excitable membrane of the electroplax. By homogenization and sonication of electric eel tissue in the presence of saccharose, followed by differential centrifugation in discontinuous saccharose gradients, a particulate fraction is obtained in which fragments of excitable membranes, free of cytoplasm, form closed microsacs (or "vesicles," ref. 28). After the incubation of these microsacs in ^{22}Na, the efflux of ^{22}Na and the effects of cholinergic compounds on the rate of the efflux were tested. A striking parallelism was found when the effects on the Na^+ *efflux* were compared with the effects of the same compounds on the membrane potentials of the intact electroplax. Even the sigmoid shape of the dose-response curve is essentially similar in the experiments with the microsacs and with the intact cell (40). Thus, the preparation opens the possibility for the comparison of the properties and behavior of the proteins in the isolated substructure with those in the intact cell and in solution. The integration of the results obtained under these three conditions should provide a more satisfactory picture of the properties and function of the proteins associated with excitation than the analysis of any one of them.

SIMILARITY OF CHEMICAL EVENTS IN CONDUCTION AND SYNAPTIC TRANSMISSION

The fundamental similarity of the mechanism of propagation of nerve impulses along axon and across synaptic junctions has been postulated by many prominent neurobiologists (e.g., ref. 41). The biochemical data accumulated lend strong support to this view. Many observations on which the neurohumoral-transmitter theory was based have found new interpretations in agreement with the unified concept and their number is still increasing. The evidence, more fully discussed in preceding papers, may be briefly summarized. (*a*) The limitation of the action of AcCh and curare to synaptic junctions has been shown, by the use of a great variety of experimental devices and different preparations, to be due to structural barriers that surround conducting membranes and prevent lipid and insoluble quaternary ammonium derivatives from reaching and reacting with the receptor, except at the junction where the membranes are less, or not at all, protected. The tertiary analogues of these compounds penetrate the barrier and act on the excitable membrane, affecting electrical activity as predicted by theory. Even on so-called unmyelinated fibers, e.g., the squid giant-axon, where the surrounding Schwann cell is only 400 nm (4000 Å) thick, AcCh and curare do not act. When, however, these axons are exposed for a short period of time to a few micrograms of snake venom (the active principle is phospholipase A (EC 3.1.1.4.), both AcCh and curare reach the excitable membrane and affect the electrical activity; whereas before exposure to the venom the quaternary compounds fail to penetrate into the axon interior, they are found there after the exposure. (*b*) The appearance of AcCh in the perfusion fluid of synaptic junctions after nerve stimulation is an artifact due to the presence of physostigmine, a potent competitive inhibitor of AcCh-esterase, which prevents the rapid hydrolysis of AcCh under physiological condition and permits, therefore, its appearance outside the membrane. According to the recent data, the amounts of AcCh released per nerve terminal are of the order of 10^{-22} mol, i.e., a few thousand molecules. Not a trace of AcCh is found in the perfusion fluid in the absence of the inhibitors. Loewi's well-known experiments on the frog heart (41*a*) are not reproducible. (*c*) Electron micrographs have shown the presence of AcCh-esterase in the pre- and postsynaptic membranes; both membranes have been shown to react to AcCh, curare, etc., indicating that both receptor and enzyme are present in both membranes and are functional. (*d*) The speculations of a quantal release of AcCh from synaptic vesicles are difficult to reconcile with the results of recent chemical investigations (for a detailed discussion, see refs. 20, 23).

An alternative explanation offered for the role of AcCh in synaptic transmission is presented in Fig. 4. As in axonal membranes, AcCh is released during activity from the storage form and acts *within* the terminal membrane, triggering the series of reactions that increase the ion permeability of that membrane. A few thousand molecules released and acting inside of a well-organized membrane structure may be extremely potent and efficient. The signal does not have to find its target protein in a second cell; there is no problem of how it may cross a membrane containing a high concentration of the enzyme that has the specific function of inactivating

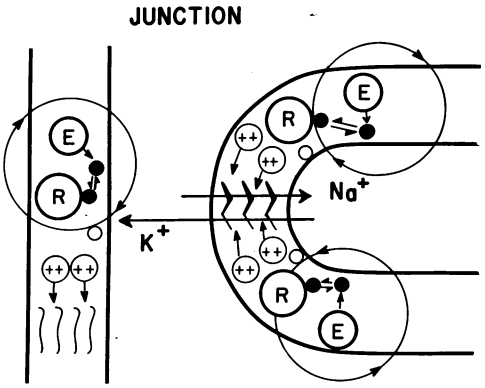

FIG. 4. Schematic presentation of the role of AcCh within junctional membranes, postulated to be similar to that within conducting membranes (see Fig. 1; same symbols). AcCh is released by the electrical currents arriving at the terminal and acts as a signal, which is amplified in the same way as in the conducting membrane and leads to increased ion permeability of the terminal membrane. Per 1000 molecules of AcCh released within the terminal membrane, millions of K^+ ions flow out into the nonconducting gap. These events lead to a release of AcCh within the postsynaptic membrane, whereby the same series of reactions is initiated there.

it. For 1000 molecules of AcCh released and acting within the membrane, millions of Na^+ ions will enter and an equivalent amount of K^+ ions will flow out, due to a similar amplification process as described for the axonal membrane. The K^+ ions will cross the gap. These processes will lead to release of AcCh in the postsynaptic membrane and the same sequence of events will take place there, initiating the excitation of the next cell unit, nerve or muscle. When, in 1934, the efflux of K^+ ions was demonstrated from axons (42), a similar efflux was demonstrated at nerve endings (43). Eccles (44) suggested K^+ ions to be the transmitters, whereas Feldberg attributed this role to AcCh. But, at that time, the events in the membranes were not understood on a molecular level. The connection between the action of AcCh as being essential for the efflux of the K^+ ions in the terminal membrane and its subsequent release in the postsynaptic membrane could not possibly be realized. For a long time no flow of current could be detected from nerve terminals. This failure was assumed to be a support of the chemical transmitter theory and a contradiction to the theory presented here. The failure seemed most conspicuous in experiments with the giant synapse of squid (45). Recent observations have removed this objection by demonstrating the flow of current from the nerve terminal (46); in the case of the giant synapse, the flow of current was found in both directions (47).

The chemical theory proposed integrates the vast amount of biochemical data with the views of those neurobiologists who assumed a basic similarity of the properties of axonal and synaptic membranes, and with the observations that formed the basis of the neurohumoral-transmitter theory, by providing for them new interpretations on a molecular level. The mechanism of nerve impulse conduction, a central problem of nuerosciences, has been opened to the analysis on a cellular, subcellular, and molecular level, and to the use of the many new methods and techniques that are available or

are being developed in the field of molecular biology. Moreover, of great importance for this development may become the introduction of new preparations that are more suitable for the study of special aspects of the problem than the electroplax, such as, e.g., that of the mouse neuroblastoma by Nirenberg and his associates, a cell line that may permit the application of genetics to the problem of the properties and function of the proteins that are essential in bioelectricity (e.g., refs. 48, 49).

Addendum in proof: In the recent issue of *J. Membrane Biol.* (1971, 6, 1–88) Changeux and his associates compare various parameters of Na^+ and K^+ fluxes from the microsacs, as a result of chemical stimulation, with those across the axonal membranes by electrical stimulation of squid giant-axons. Calculated on the basis of the Hodgkin–Huxley independent principle, the fluxes (ϕ Na and K, mol per cm^2 per sec), the permeabilities (p Na and K, cm^2/sec), and the conductances (g Na and K, mho/cm^2) reveal a striking similarity between the response to chemical stimulation of the isolated and purified membranes, with no EMF involved, and the response to electrical stimulation of axonal membranes of the axons. Since in both membranes the same protein assemblies are present and respond to specific ligands reacting with these protein in a similar way, the similarity of the parameters represents one of the most striking supports of my theory, i.e., that the same signal activates the same protein assemblies that are in control of ion permeability in synaptic and conducting membranes.

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