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Topical Review

Acetylcholine Receptor Kinetics: Chemical Kinetics

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Introduction

The nicotinic acetylcholine receptor is one of more than 20 membrane proteins that are known to be involved in the facilitation and regulation of the transmission of signals between cells. The protein, located in the membrane of nerve or muscle cells, recognizes acetylcholine secreted by an adjacent cell as a specific chemical signal; this signal induces the formation of an ion-conducting transmembrane channel.

Many of the receptor proteins discovered in the brain (for instance, *see* Snyder, 1984) form transmembrane channels upon interacting with specific chemical signals, including the receptors activated by γ-aminobutyric acid, β-alanine, glycine (Barker, Dufy, Owen & Segal, 1983), and glutamate (Cull-Candy, 1983). The muscarinic acetylcholine receptor, however, is coupled to ion channels through GTP binding proteins; activation of the receptor leads to both GTPase activity (Haga et al., 1985) and the opening of ion channels (Pfaffinger et al., 1985).

In addition to neuronal receptors other proteins are known that are involved in the perception and processing of signals by cells. These proteins, which are of considerable current interest, include the rhodopsin photoreceptors in retinal rod cells (Stryer, 1983), in the unicellular eukaryote *Chlamydomonas* (Foster et al., 1984), and in bacteria (Spudich & Bogomolni, 1984), the chemoreceptors in bacteria (Adler, 1983; Koshland, 1983; Berg, 1984), and hormone receptors (Sutherland, 1972) in the hormone-sensitive adenylate cyclase system (Gilman, 1984). The initiating signal is different in each case and so is the response of the cell, but the basic principle is similar; a series of biochemical reactions is initiated and is involved in the perception, integration, and transformation of information (Adler, 1983). In such a series of events the receptor plays an important role in the processing of information and methods of modulation of receptor function may be common (Koshland, Russo & Gutterson, 1983). Studies of these proteins have relied on the approach generally used in investigations of protein-mediated reactions, an approach involving structure determination (Kendrew, 1956; Perutz, 1960), elucidation of reaction pathways (Krebs, 1923; Calvin & Bassham, 1962), and investigation of reaction mechanisms (Monod, Changeux & Jacob, 1963; Eigen, 1967; Hammes, 1978), and which now utilizes the methods of molecular biology (Fersht, 1980).

In contrast, investigations of the acetylcholine receptor protein have relied until recently, almost entirely on the development and application of electrophysiological techniques. This topic was reviewed in this journal by Adams in 1981. Considerable progress has been made since then using mainly biochemical approaches (reviewed in Conti-Tronconi & Raftery, 1982; Hess, Cash & Aoshima, 1983; Stroud, 1983; Changeux, Devillier-Theiry & Chemouilli, 1984; Maelicke, 1984; Merlie, 1984). We will first summarize the new structural information obtained by using methods and techniques that are by now well established. We will then describe the more recent approaches that allow chemical kinetic investigations of receptor function to be made and briefly summarize the results obtained so far. This review will emphasize investigations of the nicotinic acetylcholine receptor because this protein is by far the best characterized of receptor proteins and proves to be an ideal archetypal model for the function of neuronal receptors in general.

Key Words acetylcholine receptor · chemical kinetics · voltage-dependent regulation · carbamoylcholine · suberyldicholine

Structure of the Acetylcholine Receptor Protein

The 250-kD protein has been shown to consist of five peptide chains (α_2, β, δ, and γ) (Reynolds & Karlin, 1978; Lindstrom, Merlie & Yogeswaran, 1979). It was demonstrated that purified receptor can be incorporated in an active form into azolectin vesicles (Epstein & Racker, 1978) and that the purified reconstituted receptor has similar kinetic properties to the acetylcholine receptor in the native membrane (Wu, Moore & Raftery, 1981; Huganir & Racker, 1982; Tank, Huganir, Greengard & Webb, 1983). The protein sequences of the four subunits have been elucidated through sequencing of cDNA clones of *Torpedo* receptor (Noda et al., 1982, 1983*a*, 1983*b*; Sumikawa et al., 1982; Claudio, Ballivet, Patrick & Heinemann, 1983; Devillers-Thiery, Giraudet, Bentaboulet & Changeux, 1983).

The two α-subunits had previously been shown to be responsible for the binding of acetylcholine (Weill, McNamee & Karlin, 1974). The sequence studies allowed an identification of the acetylcholine binding site on the α-subunit (Kao et al., 1984) because it was possible to locate the disulfide bridge which, on the basis of biochemical evidence (Karlin, 1980), is in the vicinity of the site. That all four subunits were required and sufficient for receptor function was conclusively demonstrated by Mishina et al. (1984). These authors obtained the mRNAs specific for the four subunits by transcription of the corresponding cDNAs in (COS) monkey cells. The mRNAs corresponding to the four subunits were injected singly or in combination into *Xenopus* oocytes and expression was monitored by electrophysiological techniques, a technique that was developed by Barnard, Miledi and Sumikawa (1982).

Electron microscopy studies (Kistler et al., 1982; Zingsheim et al., 1982) have indicated that the receptor is funnel-shaped, with an overall length of 110 Å so that it extends beyond the membrane about 15 Å on the cytoplasmic side and 55 Å on the extracellular side. The subunits are arranged around the central ion channel, which traverses the entire length of the receptor. The ion channel is permeable to all monovalent and divalent cations below about 8 Å in diameter (Dwyer, Adams & Hille, 1980; Lewis & Stevens, 1983). More recently, correlation of the amino acid sequence to the electron microscopic studies has led to the proposal of structural models for the acetylcholine receptor structure (Finer-Moore & Stroud, 1984; Guy, 1984). It is exciting that Unwin has recently succeeded in crystallizing the receptor and the determination of its actual structure is well under way (Brisson & Unwin, 1985).

Investigations of the Dynamic Properties of the Acetylcholine Receptor

Introduction

Modulation of receptor function by changing the amount of acetylcholine released from an adjacent nerve cell has been ascertained in the sea snail, *Aplysia,* and shown to lead to altered behavior in response to environmental changes (Kandel & Schwartz, 1982). It is known that acetylcholine is released from the presynaptic cell within 0.5 to 1 msec of the arrival of an action potential; the concentration of the released acetylcholine at the postsynaptic membrane rises to about 300 μM (Kuffler & Yoshikami, 1975) within a few microseconds. The effect of acetylcholine concentration on receptor function has been uncertain. Ascertaining the differences between receptors in the peripheral and the central nervous systems, normal receptors and those of individuals suffering from diseases such as myasthenia gravis, or receptors altered by site-specific mutations (Mishina et al., 1985), and understanding the molecular basis of modulation of receptor function by acetylcholine, by phosphorylation (Gordon, Davis, Milfray & Diamond, 1977; Huganir & Greengard, 1983; Huganir, Miles & Greengard, 1984), and by many important pharmacological compounds (anesthetics, antidepressants, tranquilizers, barbiturates), are all outstanding problems of interest.

The relationship between acetylcholine concentration and the receptor-controlled electrical signals in muscle cells has been investigated since 1957, when Katz and Thesleff proposed (i) a cyclic equilibrium between active receptor forms that can form transmembrane channels (*R* in Fig. 3*c*) and inactive forms (*D* in Fig. 3*c*), and (ii) the requirement for the binding of more than one ligand molecule (*L*) to the receptor for changes in transmembrane voltage to occur. Katz and Thesleff (1957) clearly realized that classical physiological approaches could not differentiate between variations of the scheme in Fig. 3*c* and abandoned this approach. Nevertheless, countless variations of the experiments of Katz and Thesleff have been described in the last 25 years (for reviews *see* Adams, 1981; Colquhoun, 1981; Peper, Bradley & Dreyer, 1982). Many different constants have been estimated from such measurements and have been shown to depend on the experimental conditions used (Peper et al., 1982). How can one differentiate between apparent effects on receptor function due to experimental conditions and real effects caused by modulation of receptor function? The detection of the molecular steps involved in receptor functions that have been altered

depends on reliable measurements of the constants associated with receptor function. Quantitative studies in cells of transmembrane processes initiated by a chemical reaction have been hampered by the time resolution of the available techniques and by the uncertainty of determination and the limitation on variation of the concentrations of reactants. Excellent time resolution (<100 μsec) can now be obtained using the elegant techniques that exist for obtaining the conductance and lifetime of receptor channels in cells (Katz & Miledi, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1976). These techniques required data collection over considerable periods of time (min). Because the receptor desensitizes rapidly (msec) at high concentrations of receptor ligands (Hess, Cash & Aoshima, 1979; Sakmann, Patlak & Neher, 1980) to a receptor form evidently incapable of forming transmembrane channels (Cash & Hess, 1980) only very low concentrations of receptor ligands could be used. Measurements over a wide concentration range of acetylcholine receptor ligands have recently become possible. A few years ago we succeeded in the development of rapid mixing techniques that are suitable for making kinetic measurements of the receptor-controlled translocation of ions across vesicle membranes (Hess et al., 1979). This increased the time resolution by a factor of about 1000 over that obtainable by techniques previously employed for measuring transmembrane ion fluxes (Moore, Hartig, Wu & Raftery, 1979) and allows one to make measurements before receptor desensitization becomes significant. Many of the difficulties that are encountered in measuring the effect of acetylcholine concentrations on receptor function in cells using electrophysiological techniques are avoided. The concentration of both reactants, the receptor protein and acetylcholine or other ligands, are known and can be varied, and the solution compositions on both sides of the membrane are also known and can be varied. The time resolution of the rapid mixing techniques employed allows one to investigate separately the individual steps of the complex receptor-controlled translocation of ions: the rates of ion translocation mediated by the receptor before and after desensitization, receptor inactivation, and recovery of the desensitized receptor. Each of the reaction steps was shown to follow simple rate laws and could be investigated over a wide concentration range of ligands (from concentrations much lower than those required to saturate the receptor with ligand to concentrations much larger than those required for saturation) (Hess et al., 1979, 1983; Cash & Hess, 1980; Karpen et al., 1982; Pasquale et al., 1983; Takeyasu, Udgaonkar & Hess, 1983; Shiono et al., 1984). It has also become possible to carry out these investigations at different transmembrane voltages, which could be kept constant during the measurements (Takeyasu et al., 1983). Here we will describe the approach used to obtain information about the dissociation constants of the receptor : ligand complexes before and after receptor desensitization and the rate coefficients for desensitization and recovery from it. Although these measurements are restricted at present to receptor-containing vesicles, we will also describe the approaches being developed to make such measurements on cell surfaces. In measurements made with membrane vesicles we have employed some of the fast reaction techniques that were used previously for investigations of soluble regulatory enzymes (Gibson, 1966; Eigen, 1967; Hammes & Wu, 1974).

Methods

Although we shall use specific examples drawn from investigations of the acetylcholine receptor from the electric organ of *Electrophorus electricus* and *Torpedo* spp., it is anticipated that the techniques that we have developed and will describe briefly will be generally applicable to kinetic investigations of transmembrane processes involving a chemical reaction; examples include the many different receptors in diverse cells, and the proteins and enzymes involved in the transfer of molecules across membranes.

Membrane Vesicles

The methods for the preparation of receptor-rich membrane vesicles from the electroplax of *E. electricus* (Kasai & Changeux, 1971) or *Torpedo* spp. (Hazelbauer & Changeux, 1974) or for the incorporation of isolated receptors (Karlsson, Heilbronn & Widlund, 1972; Eldefrawi & Eldefrawi, 1973; Schmidt & Raftery, 1973) into membrane vesicles of known lipid composition (Epstein & Racker, 1978; Huganir, Schell & Racker, 1979), and the purification of receptor-containing membrane vesicles (Hess & Andrews, 1977) and their characterization (Sachs, Lenchitz, Noble & Hess, 1982) have been published.

Fast Reaction Techniques

A diagram of the rapid mixing device used in this laboratory is shown in Fig. 1. The apparatus represents a modification of the pulsed quenched-flow apparatus used by Fersht and Jakes to investigate the kinetics of the aminoacylation of tRNA

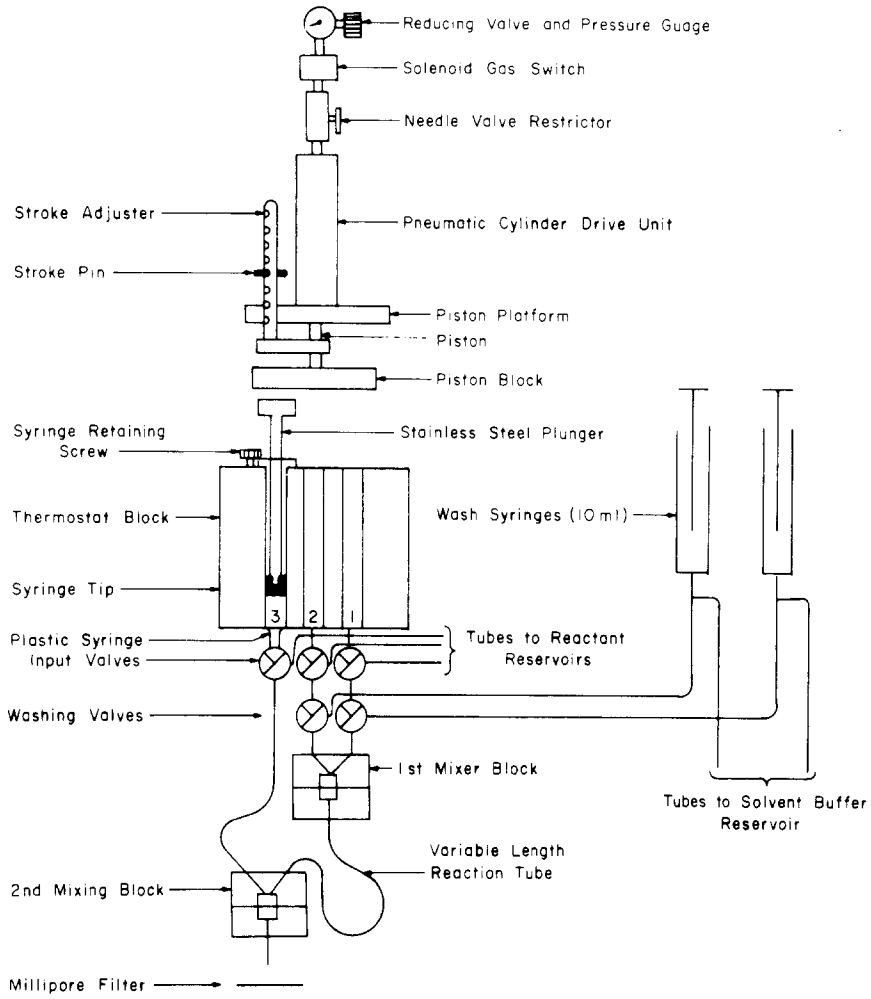


Fig. 1. The quenched flow apparatus. A partial schematic representation showing three syringes and the plumbing. The reaction time depends on the length of the reaction tube and the length of the time the solution is allowed to reside in this tube. The unit shown is mounted on one column. A machine capable of two consecutive reaction tubes has three such columns mounted on the base. (Reprinted with permission of Academic Press from Cash & Hess, 1981)

(Fersht & Jakes, 1975). In the apparatus shown in Fig. 1 the vesicles from one syringe and acetylcholine from another syringe pass into the first mixing block. After various periods of time in the reaction tube, the receptor-controlled influx of cations is quenched (stopped). This is accomplished by mixing the solution with *d*-tubocurarine, stored in a third syringe, and using the second mixing block (Fig. 1). The vesicle suspension is then pushed out of the apparatus on to a Millipore filter, and the vesicles are separated from the solution. The inorganic ion content of the vesicles on the filter can then be determined using a variety of techniques. The apparatus shown has been used with two consecutive reaction tubes and three mixers. Two consecutive reaction tubes are employed, for instance, when it is desirable to expose the receptor to a ligand for various periods of time before the measurement of receptor-controlled ion flux is initiated. Tracer ions and various concentrations of ligand can then be introduced in the second reaction tube for the measurement of the ion flux. This procedure was used to measure the rate of acetylcholine-induced desensitization of the receptor. The same mixing arrangements are also used when ion translocation is measured by optical means. The shortest possible reaction time is limited by the mixing of the solutions and is about one millisecond; in measurements of acetylcholine receptor-controlled ion flux in vesicles a reaction time of 5 msec can be obtained precisely (Cash & Hess, 1981).

Detection of Receptor-Controlled Cation Translocation

In the experiment in Fig. 2*a* a radioactive tracer ion, $^{86}Rb^{+}$, was used to measure receptor-controlled ion flux. In the experiment in Fig. 2*b* the inorganic ion content of the vesicles was measured using vidicon flame emission spectroscopy (Ramseyer, Morrison, Aoshima & Hess, 1981). With this technique it is possible to avoid the use of radioactive tracer ions and to demonstrate that the rates of receptor-con-

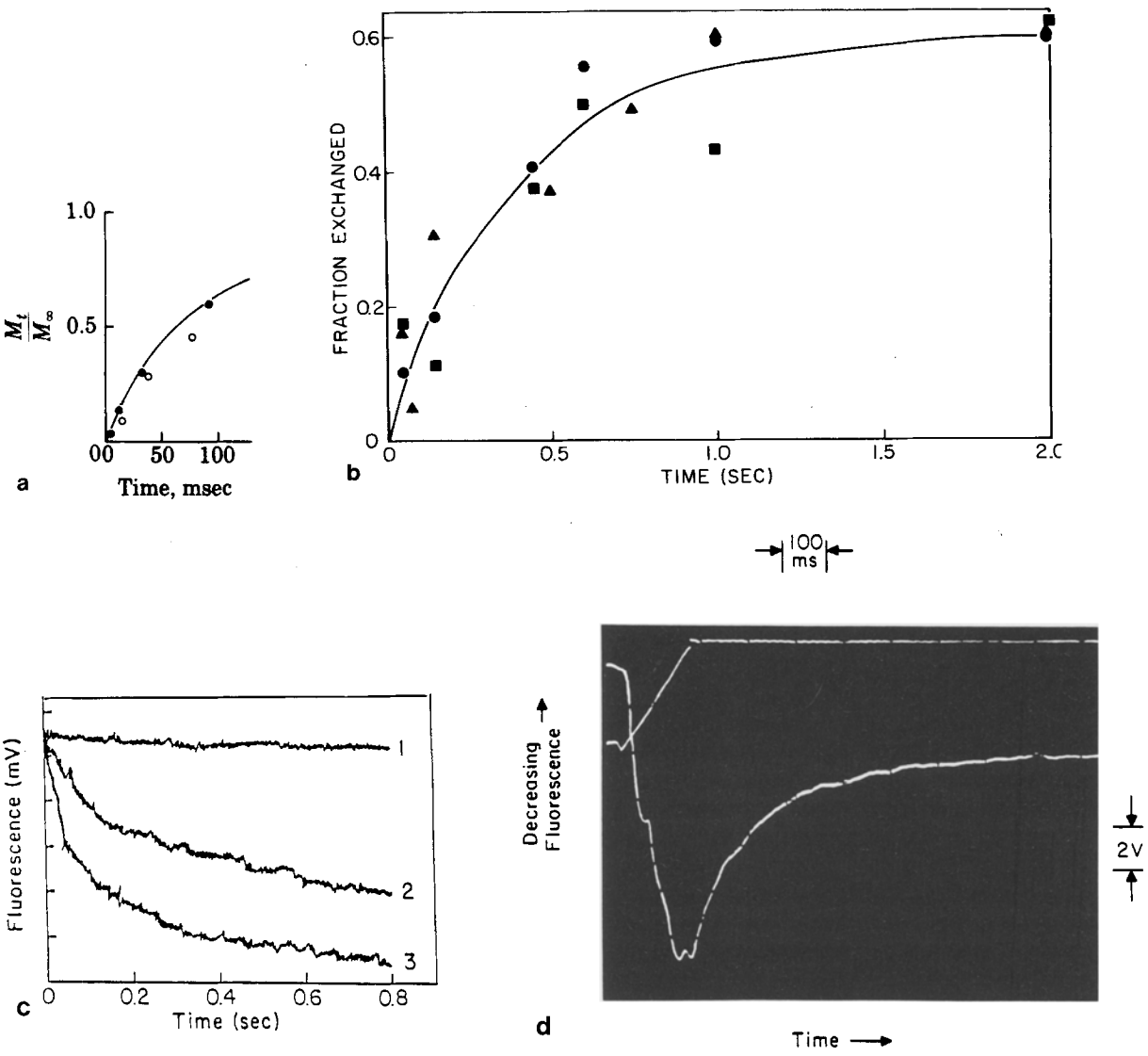


Fig. 2. Different techniques for measuring ion translocation processes in vesicles. (*a*) Quench-flow technique and tracer ions (Hess et al., 1979; Cash & Hess, 1981). Receptor-mediated flux of $^{86}Rb^+$ into vesicles in the presence of 300 μM acetylcholine in eel Ringer's solution. (M_t/M_∞) is the fraction of complete influx that has occurred at the time indicated. The closed and open circles represent data from two experiments. (Reprinted with permission from Cash, Aoshima, & Hess, 1981.) (*b*) Influx of Na^+ (●), Li^+ (▲), and K^+ (■) into vesicles in the presence of 1 mM carbamoylcholine measured by a quench-flow method and determined by vidicon flame emission spectroscopy. The solid curve was obtained from measurements of radioactive $^{86}Rb^+$ influx. (Reprinted with permission of Academic Press from Ramseyer et al. (1981).) (*c*) A comparison of Tl^+ and Cs^+ influx into *E. electricus* membrane vesicles loaded with anthracene 1,5-disulfonic acid. Curve 1, Cs^+ influx in the absence of acetylcholine; Curve 2, Tl^+ influx in the absence of acetycholine; Curve 3, Tl^+ influx in the presence of 1 mM acetylcholine. The experiment represented by curve 1 was performed as in *d*. In the experiments represented by curves 2 and 3, the chloride in eel Ringer's solution was replaced by NO_3^- and 35 mM Na^+ was replaced by 35 mM Tl^+. (Reprinted with permission of Academic Press from Karpen et al., 1983.) (*d*) Oscilloscope trace from a stopped-flow experiment in which acetylcholine-receptor-controlled influx of Cs^+ was measured. Fluorescence quenching of the fluorophore, anthracene-1,5-disulfonic acid (entrapped in *E. electricus* membrane vesicles), by Cs^+ was measured in the presence of 5 mM acetylcholine, eel Ringer's solution, in which NaCl was replaced by CsCl, pH 7.0, 1°C. The upper trace monitors the progress of the piston that drives the syringes. The ion flux profile (lower trace) begins where the piston stroke ends. (Reprinted with permission from *Biochemistry* **25**:1777–1785. Copyright 1986, American Chemical Society)

trolled translocation of $^{86}Rb^+$, Na^+, and K^+, are the same, within experimental error (Fig. 2*b*). A third technique that was developed to measure acetylcholine receptor-controlled ion flux is a spectroscopic method based on the fluorescence quenching by Tl^+ of a dye, 8-amino-1,2,6-naphthalenetrisulfonate (Moore & Raftery, 1980). The advantage of this compared to the quench flow technique is

that it allows a continuous recording of the time-dependent flux of ions into vesicles. One disadvantage of Tl^+ is that both *E. electricus* and *Torpedo* spp. membrane vesicles are very permeable to the cation in the absence of acetylcholine. As can be seen in Fig. 2*c*, the rate of Tl^+ flux into *E. electricus* vesicles is quite similar in the absence (curve 2) and the presence (curve 3) of saturating concentrations of acetylcholine (1 mM). Similarly, in gastric vesicles the permeability of Tl^+ was found to be 46 times larger than that of $^{86}Rb^+$ (Rabon & Sachs, 1981). Tl^+ is also known to have a toxic effect on certain membranes (Hille, 1975) and cannot be used in the presence of Cl^- because TlCl is only sparingly soluble in water. In experiments with *E. electricus* and *Torpedo* spp. all these disadvantages can be overcome by using Cs^+ as the fluorescence-quenching ion (Karpen et al., 1983). The membranes are essentially impermeable to Cs^+ in the absence of acetylcholine in the pertinent time region of the measurements (*see* curve 1, Fig. 2*c*, where the $t_{1/2}$ for the flux of Cs^+ into receptor-rich *E. electricus* vesicles is about 6 hr). In measurements of receptor-controlled Cs^+ flux using optical detection one obtains a signal-to-noise ratio that is equal to that obtained with Tl^+ (Fig. 2*d*). Furthermore, it has been possible to show that receptor-controlled flux of Cs^+ and $^{86}Rb^+$ are experimentally indistinguishable (Karpen et al., 1983).

Minimum Quantitative Mechanisms for Receptor-Controlled Transmembrane Ion Flux

Measurement of Receptor-Controlled Ion Translocation in Receptor-Containing Membrane Vesicles using Rapid Mixing Techniques

In experiments with *E. electricus* electroplax vesicles using quench flow techniques it has become possible to measure the rate and equilibrium constants pertaining to a model (Fig. 3*a*) that relates the receptor-controlled ion translocation rates to the ligand-binding steps, the rates of interconversion between active and inactive receptor states, and the equilibrium between the open- and closed-channel forms of the receptor saturated with ligand. An integrated rate equation (Eq. I, Fig. 3) pertaining to the model was derived and the value of the constants pertaining to the model were evaluated using a range of acetylcholine (5000-fold), carbamoylcholine (2000-fold) and suberyldicholine (10,000-fold) concentrations (Hess et al., 1983). The constants for acetylcholine are shown in Fig. 3*a*. Some of the features of the model in Fig. 3*a* (Cash & Hess, 1980) are: The receptor in the *E. electricus* electroplax can exist in two states, one an active (A) state that can form a transmembrane channel and one an inactive state (I) that cannot. Ligands bind to both states, but the affinity of the I state is about 100 times greater than that of the A state. We refer to the equilibrium mixture of active and inactive receptor states at any given ligand concentration as the desensitized receptor. The ligand-binding and channel-opening processes are fast, relative to the ion flux equilibration, and appear as equilibria. The interconversions between the active and inactive states are first order, with rates comparable with the ion flux equilibration and may be measured by this process. Channel opening occurs as a conformational change in receptor structure when two ligand molecules are bound to the active state. This allows ion flux through the channel, which is a process not involving depletion of receptor species (A, AL, etc.). Channel opening with one ligand molecule bound is negligible. In contrast, inactivation with one as well as with two ligand molecules bound is significant. The channel-opening process, characterized by the equilibrium constant Φ, perturbs the equilibrium between species A, AL and AL_2 and thereby affects the binding of ligand to the active state of the receptor.

The mechanism in Fig. 3*a* and the integrated rate equation, Eq. I, for ion translocation predict that, as the ligand concentration is increased, both the influx rate coefficient, J_A, and the rate coefficient for receptor inactivation, α, would increase to a limiting value when the receptor is saturated with ligand and thereafter remain constant. The results shown in Fig. 4 indicate that, while α depends on the ligand concentration as expected, J_A first increases and then decreases again when the ligand is suberyldicholine (Pasquale et al., 1983) or acetylcholine (Takeyasu et al., 1983). This decrease depends on the transmembrane voltage (Fig. 4) (Takeyasu et al., 1983).

The simplest mechanism that predicts the effect of high concentrations of suberyldicholine and acetylcholine on J_A, but not on α, is shown in Fig. 3*b*. Suberyldicholine and acetylcholine bind to a single regulatory site characterized by a dissociation constant, K_R, resulting in a decrease in the concentration of the open-channel form of the receptor $\overline{AL_2}$ and, therefore, in inhibition of the receptor (Pasquale et al., 1983; Takeyasu et al., 1983).

The blockage of the open receptor channel has recently been observed, by the patch clamp technique, in BC_3Hl cells at high concentrations of acetylcholine, carbamoylcholine, and suberyldicholine (Sine & Steinbach, 1984). The existence of inhibitory binding sites was previously proposed to explain the effects of local anesthetics (Adams, 1977; Heidmann & Changeux, 1978; Koblin & Lester, 1978; Neher & Steinbach, 1978; Neher, 1983; Changeux et al., 1984) and also the inhibition caused by

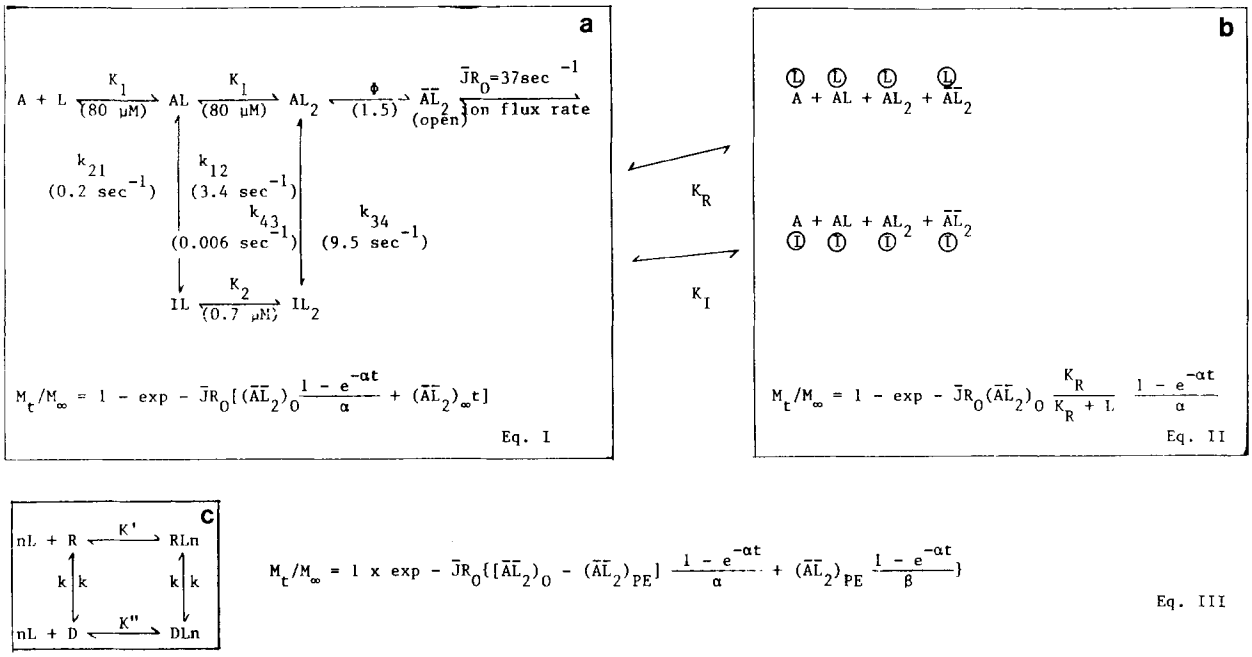


Fig. 3. Minimum Mechanisms. (*a*) The mechanism based on quench flow measurements with electroplax membrane vesicles. The active or *A* forms of the receptor can have two acetylcholine molecules bound to them to form species *AL*, AL_2, and $\overline{AL}_2$, where $\overline{AL}_2$ represents the open-channel form and *L*, acetylcholine. The forms that are incapable of forming transmembrane channels involve species *IL* and IL_2. The inactive form without bound ligand, *I*, is neither included nor excluded by the data. The microscopic dissociation constants of the active and inactive forms are represented by K_1 and K_2, respectively. $1/\Phi$ represents the channel-opening equilibrium constant and $\bar{J}R_O$ the rate coefficient for the ion translocation process. $\bar{J}$ is the specific reaction rate for the receptor-controlled ion translocation (Hess et al., 1981) and is directly related to the single-channel conductance (Hess et al., 1984). R_O represents moles of receptors per liter internal vesicle volume and is characteristic of the membrane preparation. k_{12} and k_{34} represent the rate constant for receptor inactivation, and k_{21} and k_{43} the rate constant for recovery of the receptor from the inactivation process. The values of the constants come from quench-flow measurements of receptor-controlled ion translocation with *E. electricus* membrane vesicles, 1°C, eel Ringer, pH 7.0, over a 5000-fold concentration range of acetylcholine. The rate coefficient for ion flux before receptor desensitization, after complete desensitization, for receptor desensitization, and recovery from desensitization, were all obtained from separate measurements under conditions where the rate laws were characterized by a single exponential. The integrated rate equation pertaining to the mechanism is given by Eq. I, in which $(\overline{AL}_2)_O$ and $(\overline{AL})_\infty$ represent the fraction of the receptor in the open-channel form before and after desensitization, respectively, and α is the rate coefficient for the desensitization process. (*b*) The extension of the minimum mechanism shown in *a* is based on quench-flow measurements with membrane vesicles prior to desensitization of the receptor. Ligand binding to a regulatory site is characterized by Ⓛ; K_R is the dissociation constant of this site (Pasquale et al., 1983; Takeyasu et al., 1983) and, in the case of acetylcholine, has been shown to depend on the transmembrane voltage (Takeyasu et al., 1983). The integrated rate equation which takes into account the regulatory site is given by Eq. II. K_I is the dissociation constant for noncompetitive inhibitors. The evidence that noncompetitive inhibitors such as the local anesthetic prociane, bind to an inhibitory site that is different from the regulatory site for acetylcholine is given in Fig. 5 (Shiono et al., 1984). Evidence that, in the case of procaine and cocaine, the value of K_I reflects the binding to a site present on both the closed- and open-channel form of the active form of the receptor has been given (Karpen, 1986). (*c*) The model proposed by Katz and Thesleff (1957) on the basis of electrophysiological measurements made with muscle cells, consisting of two forms of the receptor, *R* which can form open channels and *D* the desensitized form which cannot. *n* represents the number of ligand molecules involved in the reaction, with $n > 1$. *k* represents the rate constants for receptor inactivation and desensitization. The cyclic nature of the mechanism was proposed to allow the reactivation rate coefficient of the receptor to be independent of the inactivation rate coefficient. If we equate all the receptor species in *a* with *R* in the mechanism shown in this figure, and the slow desensitization process observed by Katz and Thesleff (1957) with the slower of the two desensitization processes observed in frog muscle (Sakmann et al., 1980) and *Torpedo* vesicles (Walker et al., 1981), one can account for both electrophysiological measurements with cells and quench-flow measurements with membrane vesicles. The integrated rate equation, Eq. III, which incorporates two consecutive desensitization processes has been derived (Hess et al., 1982). $(\overline{AL}_2)_{PE}$ represents the fraction of the receptor in the open-channel form at the end of the first desensitization process characterized by the rate coefficient. β is the rate coefficient for the second, slower desensitization process. The limited experimental conditions for which Eq. III is valid have been given (Hess et al., 1982).

high concentrations of decamethonium (Sakmann & Adams, 1978) and of dansylcholine (Cohen & Changeux, 1973). Inhibition of receptor function is believed to occur by the blockage of the open channel by these positively charged compounds (Neher & Steinbach, 1978; Oswald, Heidmann & Changeux, 1983; Cox et al., 1984). It has recently been shown (Shiono et al., 1984) that acetylcholine and the anesthetic procaine bind to two different inhibitory sites (Fig. 5), and that both procaine and cocaine bind to

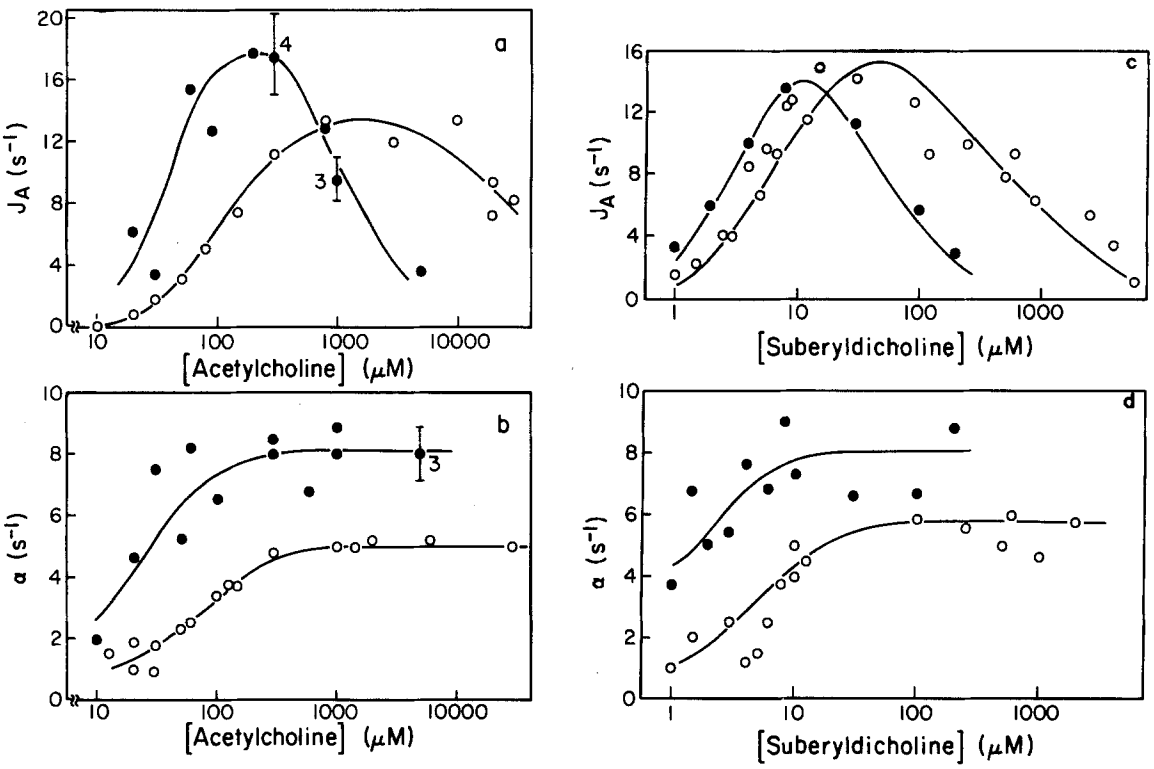


Fig. 4. The effect of acetylcholine concentration and suberyldicholine concentration on the rate coefficient for ion translocation, J_A (*a* and *c*), and on the inactivation rate coefficient, α (*b* and *d*). Experiments using the quench flow technique, *E. electricus* electroplax membrane vesicles, 1°C, eel Ringer's solution, pH 7.0. The method for evaluating the rate coefficients J_A and α have been described (Cash & Hess, 1980; Aoshima et al., 1981). The solid lines are calculated lines and are based on the evaluation of all the constants pertaining to the mechanism (Hess et al., 1983). The method for making quench flow measurements in vesicles using a voltage clamp (constant voltage) has been described (Takeyasu et al., 1983). (*a*) Effect of acetylcholine concentration on J_A when V_m = 0 mV (○) and V_m = −45 mV (●). The data obtained at −45 mV were normalized to those obtained at V_m = 0 mV as described previously (Takeyasu et al., 1983). The solid lines were calculated from the relationship between J_A and the fraction of receptor sites in the open-channel form (Cash & Hess, 1980; Pasquale et al., 1983), using a K_I value of 80 μM (V_m = 0 mV) and 75 μM (V_m = −45 mV) and a K_R value (V_m = −45 mV) of 800 μM and 50 mM (V_m = 0 mV). For a definition of the symbols *see* Fig. 3. (*b*) The α values were obtained at transmembrane voltages (V_m) of 0 mV (○) and −45 mV (●). (*c*) Effect of suberyldicholine concentration on J_A at V_m = 0 mV (○) and V_m = −45 mV (●). The solid lines were calculated from the relationship between J_A and the fraction of receptor sites in the open-channel form (Cash & Hess, 1980; Pasquale et al., 1983) using a K_I value of 4.5 μM and a K_R value of 500 μM at V_m = 0 mV and a K_I value of 4.5 μM and a K_R value of 25 μM at V_m = −45 mV. For a definition of the symbols *see* Fig. 3. (*d*) The α values were obatined at transmembrane voltages of 0 mV (○) and −45 mV (●)

all forms of the active receptor, not just to the open-channel form (Karpen & Hess, 1986).

Comparison Between Measurements with Receptor-Containing Membrane Vesicles and Measurements with Cells

Electrophysiological Measurements in which the Effect of Ligand Concentration on Receptor Function on Cells is Measured by Electrical Recordings from a Large Number of Receptors

The investigations were initiated when Katz and Thesleff (1957) proposed (i) a cyclic equilibrium between active receptor forms (which can form transmembrane channels (*R* in Fig. 3*c*)) and inactive forms (*D* in Fig. 3*c*), and (ii) the requirement for the binding of more than one ligand molecule (*L*) to the receptor for changes in transmembrane voltage to occur. The number of ligand molecules binding to the receptor are represented by *n* (Fig. 3*c*). *K'* and *K''* represent the dissociation constants of the ligand for the *R* and *D* receptor forms, respectively, with $K' \approx 20\,K''$. The desensitization of the receptor (the interconversion between the *R* and *D* forms of the receptor, Fig. 3*c*) was observed to occur in seconds.

Many variations of the experiments of Katz and Thesleff have been described in the last 25 years (for reviews *see* Steinbach, 1980; Adams, 1981; Peper et al., 1982), but many questions remain to be

answered. In chemical kinetic measurements made with membrane vesicles, the dissociation constants of the receptor and acetylcholine as well as the rate coefficients for receptor desensitization (inactivation) have much larger values than those observed in electrophysiological measurements made with cells. An explanation for this apparent disagreement emerged when it was discovered in quench-flow measurements with vesicles, in which the receptor is exposed to ligand for only a few msec, that, initially, receptor desensitization proceeds at almost 100 times the rate estimated from electrophysiological measurements (Hess et al., 1979). A rapid desensitization process was also observed in frog muscle cells using the single-channel current recording technique (Sakmann et al., 1980). Frog muscle and *Torpedo californica* receptors exhibit an additional desensitization process that occurs in the second time region (Sakmann et al., 1980; Walker et al., 1981; Feltz & Trautmann, 1982; Hess, Pasquale, Walker & McNamee, 1982), the same time region that is usually reported in electrophysiological measurements. A second slow desensitization process, which occurs in the hour time region, has recently been discovered in the *E. electricus* electroplax receptor (Aoshima, 1984). Measurements made in the msec time region, prior to receptor inactivation (desensitization), indicate that the *E. electricus* receptor binds acetylcholine with a dissociation constant K_1 of 80 μM (Fig. 3*a*) (Aoshima, Cash & Hess, 1981; Cash, Aoshima & Hess, 1981). The rapidly desensitized receptor binds acetylcholine almost 100 times better, K_2 = 0.7 μM. In equilibrium measurements the completely desensitized receptor has a dissociation constant for acetylcholine of 0.01 μM (Weber & Changeux, 1974). The time-dependent increase in the binding affinity of the acetylcholine receptor on exposure to labeled acetylcholine or fluorescent dansyl-6-choline is well documented (Neubig & Cohen, 1980; Neubig, Boyd & Cohen, 1982; Heidmann, Bernhardt, Neumann & Changeux, 1983), each time zone being characterized by a different and lower (but higher-affinity) receptor:ligand dissociation constant. When we consider the receptor species in Fig. 3*a* to be equivalent to *R* in the mechanism of Katz and Thesleff (1957) (Fig. 3*c*), we can account for the electrophysiological measurements. (i) The desensitized form of the model based on the quench-flow measurements (species *IL* and IL_2, Fig. 3*a*) corresponds to the low-affinity ligand-binding form of the model based on electrophysiological measurements ((species *R* and *RLn*, Fig. 3*c*). (ii) The second slow inactivation process observed in frog muscle and *T. californica* receptor, with a time constant of about a second (Sakmann et al., 1980; Walker et al., 1981; Feltz & Trautmann, 1982), cor-

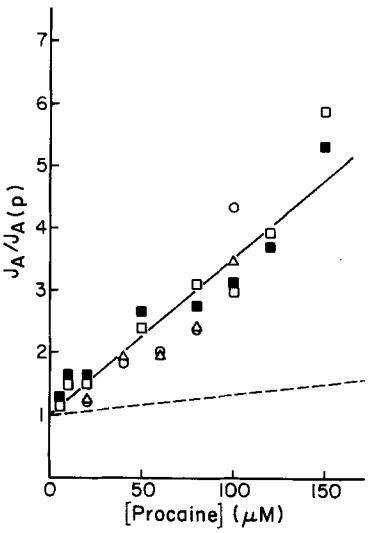


Fig. 5. Effect of procaine on the rate of ion translocation in membrane vesicles. The ratio of the rate constant for ion flux initiated by a constant concentration of acetylcholine, J_A, to the rate constant obtained in the presence of procaine, $J_{A(P)}$, is plotted *vs.* procaine concentration according to Eqs. IV, V(a) or V(b). The ratio $J_A/J_{A(P)}$ was obtained from quench-flow measurements using $^{86}Rb^+$ as the tracer ions and *T. californica* electroplax membrane vesicles, pH 7.0, 1°C, V_m = −25 mV (Shiono et al., 1984). In Eqs. IV and V, P_O represents procaine concentrations, L_O acetylcholine concentrations, and K_P and K_R the dissociation constants of the inhibitory sites of the receptor with procaine and acetylcholine, respectively. The measurements at different but constant acetylcholine concentrations were made as a function of the procaine concentrations indicated on the abscissa of the graph. Acetylcholine concentrations used: (△) 10 μM; (□) 25 μM; (○) 60 μM; (■) 1000 μM. The coordinates of the solid line were calculated by using all the measurements and a K_P value of 40 μM. The dashed line was drawn on the assumption of a common inhibitory site by using Eq. Vb, a K_P value of 40 μM and a K_R value of 160 μM determined in a separate experiment (Shiono et al., 1984). With 1000 μM acetylcholine the slope of the dashed line gives an apparent K_P value of 280 μM which is sevenfold larger than the observed value, indicating the existence of separate inhibitory sites, one for acetylcholine and another for procaine.

Regulatory site not occupied by acetylcholine.

$$J_A/J_{A(P)} = 1 + P_O/K_P. \qquad \text{Eq. IV}$$

Regulatory site occupied by acetylcholine, $L_O \gg K_R$: Eq. V

(a) Separate sites for acetylcholine and procaine

$$J_A/J_{A(P)} = 1 + P_O/K_P. \qquad \text{Eq. Va}$$

(b) Common sites for acetylcholine and procaine

$$J_A/J_{A(P)} = 1 + (P_O/K_P)[K_R/(K_R + L_O)] \qquad \text{Eq. Vb}$$

responds to the desensitization process observed in electrophysiological measurements. From the published experiments it is not possible to decide whether the second desensitization process proceeds from species A or I of the mechanism in Fig. 3*a*. The great variability of the constants obtained in electrophysiological experiments, and the report that the constants evaluated depend on the experimental conditions (particularly on the distance between the pipette from which the ligand is applied and the cell surface) (Dreyer & Peper, 1975), suggests that diffusion of applied acetylcholine to the cell surface receptors, which can be over 10 μm from the point of application of the ligand, is slow (~1 sec) compared to the rate of desensitization. The measured electrical signals in electrophysiological measurements would then reflect receptors in various states of desensitization and exposed to different concentrations of ligand (Del Castillo & Katz, 1957).

Single-Channel Current Measurements with Electroplax Cells of *E. electricus*

It is possible to compare the results obtained in chemical kinetic measurements, made with vesicles, with those obtained in single-channel current measurements, made with the cells from which the vesicles were prepared. The relationship between $\bar{J}$, the specific reaction rate obtained from chemical kinetic measurements (Hess, Aoshima, Cash & Lenchitz, 1981), and γ, the single-channel current conductance, has recently been derived (Hess et al., 1984).

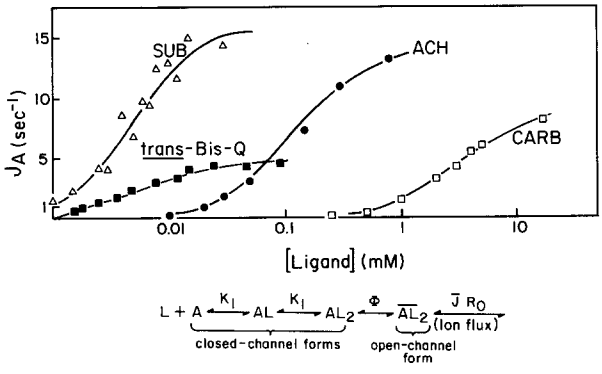


Fig. 6. Effect of the concentration of activating ligand, shown for four different ligands, on the rate coefficient for ion translocation, J_A, at pH 7.0 and 1°C. The measurements shown have been corrected for the observed inhibition of the ion translocation rates by high concentrations of the ligand (Pasquale et al., 1983; Takeyasu et al., 1983). (Reprinted from Molecular Basis of Nerve Activity; Changeux, Hucho, Maelicke and Neumann, editors; Walter de Gruyter, Berlin–New York 1985)

$$\bar{J} = \frac{RT}{F^2} \frac{\gamma}{[M]} N_A$$

were R, T, and F represent the molar gas constant, absolute temperature, and Faraday constant, respectively. γ, N_A and $[M]$ represent the single-channel conductance, Avogadro's Number, and the molar concentration of inorganic ions that can pass through the channel, respectively. The evaluation of γ has been described in detail (Neher & Sakmann, 1976; Del Castillo & Webb, 1977). The evaluation of $\bar{J}$ is based on measurements of the effect of ligand concentration on J_A, the rate coefficient for the receptor-controlled ion translocation in the absence of inactivation (Hess et al., 1981). The dependence of J_A on ligand concentration for four activating ligands is shown in Fig. 6. At least a 2000-fold concentration range was used with each ligand. It can be seen that at saturating concentrations of the activating ligand, the maximum J_A value, $J_{A(max)}$(obs), observed for suberyldicholine is about twice that for carbamoylcholine and three times larger than that for *trans*-Bis Q (Delcour & Hess, 1985). It is well known that equilibria between protein conformations can be strongly influenced by the ligand molecules that are bound. We suggested (Cash & Hess, 1980), therefore, that the reasons for these differences in $J_{A(max)}$(obs) values are due to the equilibrium between the fully liganded receptor in the closed- and open-channel states and that the number of ions that pass through the open receptor channel is independent of the ligand used. This assumption is in agreement with other results (Gardner, Ogden & Colquhoun, 1984) that have demonstrated that the single-channel conductance of the receptor in embryonic rat muscle is the same for 10 different ligands.

The specific reaction rate, $\bar{J}$, of the receptor-controlled ion translocation process can be calculated from the $J_{A(max)}$ value and a knowledge of R_O, the moles of receptor sites per liter internal vesicle volume (Hess et al., 1981). Our ability to separate vesicles with functional receptors from those without (Hess & Andrews, 1977) and to obtain vesicle

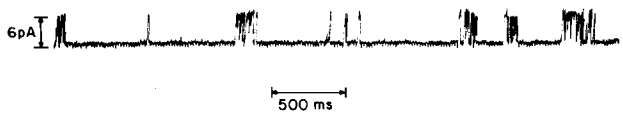


Fig. 7. A single-channel recording from an *E. electricus* electroplax showing typical bursts of openings of the acetylcholine receptor channel activated by 0.5 mM carbamoylcholine at −120 mV, 12°C. Outside eel Ringer's solution, pH 7.0, was on either side of the excised patch. The conductance of the receptor channels was 50 pS at 12°C (Pasquale, Udgaonkar & Hess, 1986)

preparations with uniform distributions of vesicle size and receptor concentration (Sachs et al., 1982) allowed us to determine R_O and, therefore, $\bar{J}$ (Hess et al., 1981), which has a value of 3×10^7 M^{-1} sec^{-1} at pH 7.0 and 1°C.

In Fig. 7 is a recording from acetylcholine receptor channels in an electroplax cell in eel Ringer's solution, pH 7.0. The acetylcholine receptor from this cell was also used in the chemical kinetic measurements with vesicles. The deviations from the baseline in Fig. 7 represent the currents passing through a single receptor-formed channel. Short bursts of channel activity are observed and those bursts are separated by much longer intervals of no channel activity. These periods of channel activity and the intervals between them have been interpreted as representing the lifetimes of two states of the acetylcholine receptor: an active state that can give rise to transmembrane channels and an inactive state than cannot (Sakmann et al., 1980). The relationship between the mean lifetime of the burst of channel activity, τ (burst), determined in single-channel current measurements, and the rate coefficient for receptor inactivation, α, determined in chemical kinetic measurements with vesicles (Aoshima et al., 1981), has been derived (Hess et al., 1984): τ^{-1} (burst) = α, at high ligand concentrations. The distribution of the frequency of occurrence of the bursts of channel activity is given in Fig. 8. τ^{-1} has a value of 0.1 sec corresponding to an α value of 10 sec^{-1}.

A comparison of the values of $\bar{J}$ and α obtained from chemical kinetic measurements made with vesicles and from single-channel current measurements made with the electroplax cell from which the vesicles were prepared is given in Table 1. The agreement between the two types of measurements is good. This agreement is important, not only because we are comparing measurements with vesicles and with the cells from which the vesicles were prepared, but also because the techniques are complementary and the assumptions made are quite different. The interpretation of the specific reaction rate, $\bar{J}$, and the rate coefficient for receptor inactivation, α, depends on the model (Hess et al., 1983).

A Minimum Model

The model in Fig. 3*a* and *b* is a minimum one in that it contains only the constants and intermediates required by the measurements. For instance, the absence of free *I* in the Scheme (Fig. 3*a*) implies that its contribution is not required to account for the measurements rather than that it cannot be formed. The difference in the ligand dependencies of ion flux

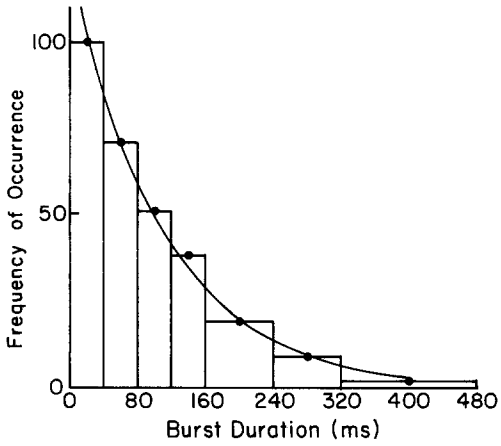


Fig. 8. A distribution of burst durations of single-channel currents through the acetylcholine receptor of the *E. electricus* electroplax activated by 2 mM carbamoylcholine at −80 mV, 12°C. The data was fitted with a single exponential, and a mean burst duration of 100 msec was obtained. Periods of activity were accepted as bursts if they were preceded and followed by inactive periods whose durations were more than five times the mean gap duration measured between bursts. Periods of activity were not accepted for analysis if they had higher than unit conductance levels. The mean interval between bursts defined in this way was about 200 msec (Pasquale et al., 1986)

Table 1. Comparison of chemical kinetic measurements and single-channel current measurements, using eel electroplax vesicles or eel electroplax cells, eel Ringer's solution, pH 7.0

Ligands	Values calculated from	
	Chemical kinetics (vesicles)	Single-channel current (cell)
Acetylcholine, carbamoylcholine, suberyldicholine	$\bar{J} = 3 \times 10^7$ M^{-1} sec^{-1}[a]	$\bar{J} = 5 \times 10^7$ M^{-1} sec^{-1}[b]
Carbamoylcholine	$\alpha = 14$ sec^{-1}[b]	$\alpha = 10$ sec^{-1}[b]

[a] 1°C.
[b] 12°C.

and receptor desensitization (inactivation) can be explained by different sites being responsible for receptor desensitization and ion flux (Dunn & Raftery, 1982; Walker, Takeyasu & McNamee, 1982; Dunn, Conti-Tronconi & Raftery, 1983). The difference in ligand dependencies, however, also can be explained, more simply, by channel opening with one ligand molecule bound being negligible (Walker, Richardson & McNamee, 1984) while desensitization (inactivation) with one as well as two ligand molecules bound is significant (Cash & Hess, 1980). This latter explanation was found to be adequate to account for measurements made with *E. electricus* vesicles over a wide concentration range of the three activating ligands (acetylcholine, carbamoylcholine and suberyldicholine) which were investigated (Hess et al., 1983).

The channel-opening equilibrium constant, Φ^{-1}, is explicitly introduced into the mechanism to account for the observed cooperativity in the effect of ligand concentration on the influx rate coefficient, J_A, and the differences in the $J_{A(\max)}$ values obtained with different ligands (Fig. 6). If channel opening with one ligand molecule bound is negligible, then the value of $(\Phi^{-1})_L$, associated with the monoliganded receptor, is much smaller than the value of $(\Phi^{-1})_{L2}$, associated with the biliganded receptor form. From the principle of detailed balance (for example, *see* Hammes, 1978), $(\Phi^{-1})_L/(\Phi^{-1})_{L2} = K_1'/K_1$, where K_1' and K_1 represent the ligand dissociation constants of the open- and closed-channel forms of the receptor, respectively. Consequently, ligand is more tightly bound to the open-channel form of the receptor than to the closed-channel form.

We can see that the minimum model is complex because of the different binding affinities of the various receptor forms, the different rate coefficients for receptor desensitization, and the voltage- and concentration-dependent inhibition of receptor function by acetylcholine and its analogs. The investigations of receptor-controlled ion translocation presented demonstrate the power of chemical kinetics using rapid reaction techniques, which allow investigations over wide ranges of ligand concentration and permit one to observe individual steps of a complex mechanism in different time regions, thereby facilitating analysis of the process.

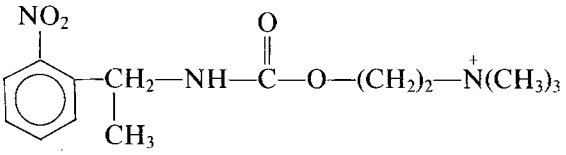
Chemical Kinetic Measurements of Receptor-Controlled Ion Translocation on Cell Surfaces

How can the chemical kinetic approach for investigation of receptor function be made generally useful? Single-channel current measurements allow one to obtain information about the single-channel conductance and the lifetimes of receptor states in a great variety of cells but at the moment, because of receptor desensitization, are restricted to low concentrations of ligands. The wide range of ligand concentrations and the time region available in chemical kinetic measurements allow one to dissect a complex process into individual steps, but at the moment the approach is restricted to receptor-rich vesicles prepared from the electric organ of electric fish. The recently developed loose patch-clamp technique (Stühmer, Roberts & Almers, 1983) allows one to measure, locally, the currents from many channels of a cell with a high signal-to-noise ratio. So far measurements utilizing this technique have been made only with sodium channels in frog muscle that open in response to an appropriate change in the transmembrane voltage of the cell.

The technique requires that a number of control measurements are made before the channel currents are measured. Exposing the receptor to acetylcholine during this time would inactivate the receptor and drastically reduce the signal. In order to overcome this problem, compounds are needed that can be converted rapidly from an inactive precursor to an active receptor ligand. In principle, the photoisomerizable agonist, Bis Q (Deal, Erlanger & Nachmansohn, 1969), appeared to be ideal for such investigations. The photoinduced isomerization of *cis* Bis Q to the *trans* form produces an active receptor-ligand (for a review *see* Lester & Nerbonne, 1982). After separating *cis* and *trans* Bis Q using HPLC (Delcour, Cash, Erlanger & Hess, 1982), it was shown (Delcour & Hess, 1985) that in *E. electricus* vesicles *cis* Bis Q itself inactivates (desensitizes) the receptor, and that the *trans* form becomes an inhibitor of receptor function at progressively lower concentrations as the transmembrane voltage is decreased to more negative values. In *T. californica* electroplax *trans* Bis Q is not an agonist (Delcour & Hess, 1985).

To avoid the problems inherent in using Bis Q in kinetic measurements, other approaches are desirable. One consists of the preparation of a photolabile protecting group for amino groups, a functional group of carbamoylcholine and many other well-known neurotransmitters. The *ortho*-nitrobenzyl moiety has been used extensively as a photosensitive protecting group for carboxylate, phosphate, hydroxyl, and amine residues in organic synthesis (Morrison, 1969; Pillai, 1980). Recently, it has been used to derivatize biological substrates that can be released rapidly by photolytic irradiation *in situ*. The development and application of *ortho*-nitrobenzyl photochemistry to achieve rapid changes in con-

centration of ATP (Kaplan, Forbush & Hoffman, 1978; McCray, Herbette, Kohave & Trentham, 1980; Goldman, Hibberd, McCray & Trentham, 1982; Goldman, Hibberd & Trentham, 1984) and cyclic nucleotides (Engels & Schlaeger, 1977; Nerbonne, Richard, Nargeot & Lester, 1984) is well documented. An extension of these compounds to the acetylcholine receptor has suggested the following compound:



which is photolyzed to 2-nitrosoacetophenone and carbamoylcholine in the microsecond time region (Walker, McCray & Hess, 1986).

Figure 9 and Table 2 indicate the wealth of information that can be obtained by a combination of photolabile protecting groups and the current integration technique. This combination is expected to have sufficient time resolution to allow one to observe and measure four phases of the reaction independently (Fig. 9 and Table 2): a rise time of the current (time zone *A*), a plateau corresponding to the maximum observable current (time zone *B*), a decay of the current (time zone *C*), and a small residual current (time zone *D*), which is due to the few channels that remain open after the receptor is completely desensitized. Variation in the ligand concentration gives a further resolution of the current rise time and the plateau of the curve in Fig. 9. At low ligand concentrations the rate constants for the ligand-binding process are obtainable from time zone *A* (Eq. VI, Table 2) and the dissociation constant of the receptor:ligand complex from time zone *B* (Eq. VIII, Table 2). At high ligand concentrations the rate constants for the channel-opening process are obtainable from time zone *A* (Eq. VII, Table 2) and the dissociation constant of the regulatory site from time zone *B* (Eq. IX).

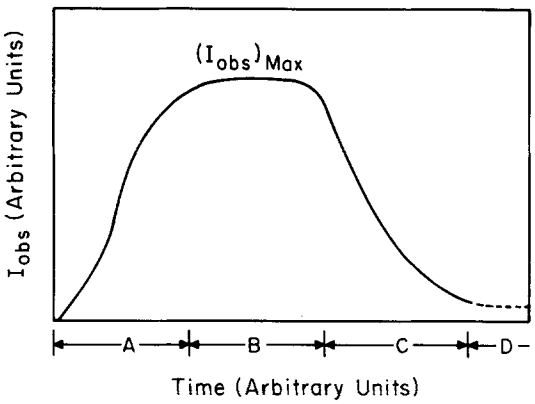


Fig. 9. Integrated current measurements with cells. The schematic drawing illustrates how steps of a complex process can be spread along the time axis, which helps one to resolve the individual terms experimentally. For the purpose of Table 2, the time zone is divided into individual segments

Conclusions

The last review on acetylcholine receptors that appeared in this journal was devoted almost entirely to measurements made with cells using electrophysiological techniques (Adams, 1981). The advantage of those techniques is that they can be used with a variety of cells. The disadvantages are uncertainty in the concentration of ligand that is present at the receptor sites (Del Castillo & Katz, 1957) and that the population of receptors that gives rise to the measurements is likely to contain desensitized receptors.

The general concept that the only function of acetylcholine receptors is to relay signals from cell to cell is based on electrophysiological measurements, which indicated that the concentration of acetylcholine that appears between cells during the signal transmission is at least 100-fold higher than the concentration necessary to saturate the receptor, and that receptor inactivation is slow compared to the known frequency of signal transmission between cells. We now know that the structure of the acetylcholine receptor molecule is considerably more complicated than the structures of proteins that are known to carry out much more complex functions than those ascribed to the acetylcholine receptor. Bacteriorhodopsin and the (Na^+,K^+)-ATPase are examples of comparatively simpler structures which carry out the more complex task of translocating ions across the cell membrane against a concentration gradient (Stoeckenius & Bogomolni, 1982; Skou & Norby, 1979).

New and powerful methods have been developed (Katz & Miledi, 1972; Neher & Sakmann, 1976), which allow one to measure the conductance of the single receptor channel, the lifetime of the open channel, and in some cases the lifetime of the active and desensitized receptor states (Sakmann et al., 1980). These methods can be used with a variety of cells (Sakmann & Neher, 1984), but the measurements, which have to be made over a time span that is long enough for the receptors to desensitize, are restricted to low concentrations of ligand so that some of the receptors remain in the active nondesensitized state. The method does not allow one to distinguish between more than two

Table 2. Chemical kinetic measurements with cells[a]

I. Rise-time (time zone A, Fig. 9)[b]

$$A \underset{k_{21}}{\overset{k_{12}L}{\rightleftharpoons}} AL \underset{k_{32}}{\overset{k_{23}L}{\rightleftharpoons}} AL_2 \underset{k_{closed}}{\overset{k_{open}}{\rightleftharpoons}} \overline{AL}_2 \text{ (open channel)}$$

(i) If $AL_2 \rightleftharpoons \overline{AL}_2$ is the fast step ($L \leq K_1$) and $k_{12} = k_{23}$, $k_{21} = k_{32}$

$$k_{obs} = 2(k_{12}L + k_{21})^{c} \qquad \text{Eq. VI}$$

(ii) If $AL_2 \rightleftharpoons \overline{AL}_2$ is the slow step ($L \gg K_1$)

$$k_{obs} = k_{closed} + k_{open}\left(\frac{L}{L + K_1}\right)^2 \qquad \text{Eq. VII}$$

II. $(I_{obs})_{max}$ (time zone *B*, Fig. 9)

$$\text{when } L \ll K_R \quad (I_{obs})_{max} = I_{max}\frac{L^2}{L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi}\ ^{*} \qquad \text{Eq. VIII}$$

$$\text{when } L \gg K_R,\ (I_{obs})^{R}_{max} = (I_{obs})_{max}\frac{K_R}{K_R + [R]}\ ^{**} \qquad \text{Eq. IX}$$

$$\frac{(I_{obs})_{max}}{(I_{obs})^{R}_{max}} = 1 + \frac{[R]}{K_R} \qquad \text{Eq. X}$$

III. Decay time (time zone *C*, Fig. 9)

$$(I_{obs})_t - (I_{obs})_{t=\infty} = [(I_{obs})_{t=\text{initial}} - (I_{obs})_{t=\infty}]e^{-\alpha t} \qquad \text{Eq. XI}$$

IV. Time zone D, Fig. 9, represents observed current due to open channels after the desensitization process has gone to completion.

[a] The relationship between integrated current measurements and chemical kinetic constants. The Table illustrates that, in addition to spreading a complex process along a time axis, the ability to vary a ligand concentration over a wide range further simplifies the analysis of complex processes.

[b] The symbols A, AL, etc. have been defined in Fig. 3*a* and its legend.

[c] Observed rate coefficient for rise-time. The term of 2 arises because there are two pathways to form AL. The simplifying assumptions $k_{12} = k_{23}$ and $k_{21} = k_{32}$ are made because chemical kinetic measurements indicate (Cash & Hess, 1980) that one microscopic dissociation constant K_1 (*see* Fig. 3*a*) is sufficient to account for the formation of AL and AL_2. The rate equation for the rise-time can be integrated in closed form, even if the rate constants are not equal.

* The fraction represents the fraction of the receptor in the open-channel form ($\overline{AL}_{2O}$) in terms of the minimum mechanism in Fig. 3*a* (Cash & Hess, 1980). L represents the molar concentrations of ligand, $\Phi = AL_2/\overline{AL}_2$ (*see* Fig. 3*a*), and K_1 has been defined (Fig. 3*a*).

** $I_{obs(max)}$ and $I^{R}_{obs(max)}$ represent the maximum current observed in the absence and or presence of a noncompetitive inhibitor, R the molar concentration of the inhibitor, and K_R its dissociation constant.

states of the channel—an open state and a closed state (Colquhoun & Hawkes, 1981, 1982), although any plausible mechanism must postulate at least several nonconducting states. It is also not always possible to determine whether successive openings originate from the same receptor channel and, consequently, gaps between openings become difficult to interpret. Hence much kinetic information is lost. Additional important information can, however, be obtained using chemical kinetic measurements.

The agreement which we demonstrate between single-channel current measurements with cells and chemical kinetic measurements with membrane vesicles obtained from the plasma membranes of those cells establishes, for the first time, that complementary information can be obtained by using both approaches. So far chemical kinetic investigations of receptor function, in which large variations of ligand concentrations can be used and a 5-msec time resolution can be achieved, have been possible

only with receptor-containing membrane vesicles using quench-flow and stopped-flow techniques. It has been possible to resolve the complex ion translocation process, mediated by the acetylcholine receptor, into individual steps which could be investigated over a wide range of acetylcholine concentrations. This has led to a self-consistent model in which the dissociation constants of the active and the inactive forms of the receptor, the channel-opening equilibrium constant, the specific reaction rate, and the rate coefficient for receptor inactivation and reactivation can be determined (Hess et al., 1983). It was possible to demonstrate that the inactivation rate coefficient and the specific reaction rate of receptor-controlled ion translocation, obtained with kinetic measurements made with vesicles, were in good agreement with the corresponding parameters that were obtained from single-channel measurements made with the cells from which the vesicles were prepared (Hess et al., 1984; Pasquale et al., 1986). Chemical kinetic measurements have demonstrated that the affinity of the active form of the receptor, for acetylcholine, is significantly lower than determined by electrophysiological measurements so that variation in the concentration of acetylcholine between cells during the transmission process can have an effect on signal transmission. These investigations have also led to the discovery of a voltage-dependent regulatory site for acetylcholine. Depending on the amount of acetylcholine released from the nerve terminal and on the resting transmembrane potential of the cell, a slight change in transmembrane potential (5 mV) can have a dramatic effect on the number of receptor molecules that can form transmembrane channels and, therefore, on the transmission of signals between cells. Variations in acetylcholine concentration have been suggested to be important in adaptation processes in *Aplysia* (Kandel & Schwartz, 1982).

Chemical kinetic measurements have also indicated that inactivation of the receptor in the msec time region occurs almost 100 times faster than was deduced from electrophysiological experiments. Reactivation occurs in the 200-msec time region. The inactivation-reactivation of the receptor could have an important physiological function, namely, the storage of information for short periods of time.

Feedback control, and programming at the molecular level, involving the interaction of chemical signals with regulatory enzymes, appears to be an important way to regulate the synthetic and degradative pathways within a cell. Are acetylcholine receptors similarly important in the regulation of signal transmission and is such a role reflected in the relatively complex molecular architecture that allows them to be responsive to changes in both ligand concentration and transmembrane voltage? Almost no pertinent information is available, except in the case of acetylcholine receptors in the electric organs of *E. electricus* and *Torpedo* spp. Modern chemical kinetics can, however, contribute to answering these important questions.

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