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# Determination of the Chemical Mechanism of Neurotransmitter Receptor-Mediated Reactions by Rapid Chemical Kinetic Techniques†

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ABSTRACT: Rapid chemical reaction techniques play an important role in unraveling the mechanism of reactions mediated by soluble proteins, including enzymes involved in the regulation of intracellular processes and the biosynthesis of proteins and nucleic acids. Regulatory proteins change conformation rapidly and must, therefore, be studied in the microsecond-to-millisecond time region. Similar techniques for investigating reactions mediated by membrane-bound neurotransmitter receptors were not available, and the mechanisms of the reactions are poorly understood. These proteins must be studied in a membrane-bound form in cells or vesicles, over a wide range of reactant concentrations, and in the microsecond-to-millisecond time region. Four rapid-mixing techniques for making kinetic measurements in the microsecond and millisecond time regions have now been developed for investigating neurotransmitter receptors in the membranes of neurons and muscle cells, thus extending chemical kinetic approaches to membrane-bound proteins and intercellular processes. Neurotransmitter receptors regulate transmission of signals between neurons (approximately $10^{12}$ in the human nervous system), thereby allowing perception of stimuli, integration and storage of information, and reaction to the environment. Six structurally related neurotransmitter receptors, and many isoforms, have been identified by use of recombinant DNA technology. Modern electrophysiological techniques show that these different proteins, upon binding a specific chemical signal (neurotransmitter), transiently open transmembrane channels, which are characterized by their ion selectivity, conductance, and lifetime. To be able to account for the receptor-mediated voltage changes that trigger signal transmission between cells, we still need to know the concentration of open receptor–channels. This concentration changes with time and is affected by the concentration of neurotransmitter. Rapid reaction techniques are particularly suitable for determining the relationship between neurotransmitter concentration and the time-dependent concentration of the open receptor-channels. The four rapid reaction techniques adapted or developed for studying receptor mechanisms are quench- and stopped-flow, adapted for use with vesicles, and cell-flow and laser-pulse photolysis for use with single cells. The approach was initiated when it was found that the neurotransmitter receptors desensitize (become transiently inactive) faster, by almost two orders of magnitude, than was believed. Before fast reaction techniques were used, the chemical properties of only desensitized forms were investigated, although this was not recognized. So far, the chemical mechanism(s) of the excitatory (cation-specific) acetylcholine receptor in membrane vesicles, electroplax cells, and single clonal cells, and the inhibitory (anion-specific) γ-aminobutyric acid (GABA) receptor in primary cerebral cortical cells have been investigated with the new techniques. A minimum mechanism and its constants for the acetylcholine receptor from the *Electrophorus electricus* electroplax have been determined. Together, they account for the concentration of open channels and desensitization rates over a 5000-fold range of acetylcholine concentration. The mechanism appears also to account for the muscle and neuronal acetylcholine receptor, and for the structurally related GABA receptor. When comparison has been possible, the results obtained by chemical kinetic techniques also accounted for results obtained by the single-channel current recording technique. Measurements of the conductance and lifetime of receptor-formed transmembrane channels enhanced our understanding of (1) differences between receptor isoforms in different cells, (2) changes in receptor mechanism during development, or produced by protein engineering, (3) changes in receptor mechanism relevant to diseases of the nervous system, and (4) the effects of therapeutic drugs and abused compounds. The new techniques can be used in all these areas to measure the effect of neurotransmitter concentration on channel opening and on receptor desensitization and resensitization, and to determine and combine constants of individual steps of the reaction pathway into an overall chemical mechanism. Thus, it is now becoming possible to relate the receptor-mediated reaction, and factors that affect it, to changes in the transmembrane voltage of the cell, and therefore to the transmission of signals between cells of the nervous system.

Neurotransmitter receptors in the membranes of neurons (approximately $10^{12}$ in humans) enable and control transmission of signals between the cells, thereby allowing perception of a stimulus, integration and storage of information,and reaction to stimuli. A critical change in transmembrane voltage in any one of the cells, brought about by receptor-

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mediated reactions, triggers an all-or-none phenomenon, the propagation of an electrical signal along a process (axon) of the cell to a terminal at a junction (synapse) with another cell, where the secretion of a chemical signal (neurotransmitter) occurs (Kandel et al., 1991). The resulting change in the transmembrane voltage of the postsynaptic cell is initiated by the binding of a neurotransmitter to its specific receptor protein. The binding reaction leads to the transient ($\sim$1 ms) formation of cation- or anion-conducting (depending on the receptor) transmembrane channels and to the formation of desensitized (transiently inactive) forms of the protein within 3–200 ms [Hess et al., 1979; Sakmann et al., 1980; Cash & Subbarao, 1987; Trussel & Fischbach, 1989; reviewed by Ochoa et al. (1989) and Changeux (1990)]. To understand the receptor-mediated reaction that determines the rate at which inorganic ions cross the cell membrane and, therefore, the change in voltage and consequent signal transmission to another cell, the chemical mechanism of the reaction must be known. This involves determination of (i) the reaction pathway leading to the formation of open-channel and desensitized receptor forms, (ii) the concentration of open channels that changes with time and is affected by the concentration of neurotransmitter, (iii) the rate and equilibrium constants of individual reaction steps, and (iv) their combination in the overall mechanism.

## IN SEARCH OF THE NONDESENSITIZED ACETYLCHOLINE RECEPTOR

Katz and Thesleff (1957), in their studies of the nicotinic acetylcholine receptor in frog muscle, realized the necessity of elucidating the chemical mechanism of these reactions, but techniques to measure the reactions of membrane-bound proteins in the millisecond time region, in which both channel opening and receptor desensitization occur, were not available. Classical electrophysiological approaches, ligand-binding experiments, and measurements of receptor-mediated flux of inorganic ions across the membrane of vesicles in the second time region give information about equilibrium mixtures of receptor forms, but they do not elucidate the reaction that leads to the formation of open receptor–channels and signal transmission [reviewed by Hess et al. (1983), Ochoa et al. (1989), and Changeux (1990)]. Recording the current passing through single channels (Neher & Sakmann, 1976) is a powerful tool for measuring the lifetime and conductance of a channel, but it has not given reliable information about the chemical mechanism of receptor function [reviewed by Madsen and Edeson (1988)].

Rapid chemical kinetic techniques for studying proteins in solution are used to elucidate chemical mechanisms of reactions involving fast ligand-binding steps and interconversions between protein conformations with different ligand-binding and functional properties. The time resolutions of the techniques allow one to study a reaction over a wide range of reactant concentrations and in different time regions, thus making it possible to separate individual, sequential steps of a reaction along the time axis. Simple rate laws, and analytical expressions for the concentration-dependence of the rates of each of the steps, are often applicable (Eigen & Hammes, 1963; Eigen, 1967; Hammes, 1982; Fersht, 1985; Johnson, 1992). Reaction intermediates that may not be observed at low ligand concentrations, which must be used when the time resolution of the technique employed is inadequate, may be detected at high ligand concentrations (Eigen & Hammes, 1963; Eigen, 1967; Hammes, 1982; Fersht, 1985; Johnson, 1992). Similar methods for studying processes mediated by proteins such as neurotransmitter receptors, which must be investigated embedded in a membrane separating two aqueous solutions, were not available. New approaches were needed. We began the development of new approaches with the nicotinic acetylcholine receptor because it is abundant in the electric organ (electroplax) of *Electrophorus electricus* and *Torpedo* spp [reviewed by Nachmansohn (1959)].

Kasai and Changeux (1971) had prepared membrane vesicles from *E. electricus* electroplax and measured the receptor-controlled transmembrane flux of inorganic tracer ions with a time resolution of about 2 s. An approximately 7-fold purification of their preparation yielded a population of vesicles (Hess & Andrews, 1977; Kim & Hess, 1981; Sachs et al., 1982) in which ion flux into or out of the vesicles in the absence of an activating ligand was negligible. When the purified vesicle preparation was used in similar measurements, also with a low time resolution, (Figure 1A) (Hess et al., 1978), the receptor-controlled flux induced by 1 mM carbamoylcholine (a stable analogue of acetylcholine) was seen to be biphasic: an initial phase too rapid to be measured by the techniques then available followed by a much slower phase. This raised the interesting possibility that the conversion of active to inactive receptor forms was considerably more rapid than was believed, and gave the first indication that in previous experiments [reviewed by Colquhoun (1979) and Karlin (1980)] many properties of the *active* form of the receptor (responsible for signal transmission) had not been measured. Measurements in the millisecond domain indicate that what had been measured were the chemical properties of equilibrium mixtures of active and desensitized receptor forms. The possibility of measuring the properties of the active receptor form responsible for signal transmission was the impetus for developing rapid reaction techniques suitable for elucidating the chemical mechanism of membrane-bound neurotransmitter receptors. To meet this need, rapid-mixing techniques for use with vesicles (Hess et al., 1979; Cash & Hess, 1981; Karpen et al., 1983), rapid perfusion of single cells (Krishtal et al., 1983; Clapham & Neher, 1984; Brett et al., 1986), a cell-flow technique (Udgaonkar & Hess, 1987a; Hess et al., 1987), and laser-pulse photolysis of photolabile inactive precursors of neurotransmitters (Walker et al., 1986; Milburn et al., 1989; Wilcox et al., 1990; Billington et al., 1992a,b; Matsubara et al., 1992) were developed.

## EVOLUTION OF RAPID REACTION TECHNIQUES FOR STUDYING NEUROTRANSMITTER RECEPTOR FUNCTION IN THE MICROSECOND-TO-MILLISECOND TIME REGION

Our first measurements were made using the purified vesicles prepared from *E. electricus*. Existing flow techniques, originally designed for measuring reactions in solution, were adapted for use with vesicles containing acetylcholine receptors (Hess et al., 1979; Cash & Hess, 1981).

*Pulsed Quench-Flow Technique with Millisecond Time Resolution.* A pulsed quench-flow technique (Fersht & Jakes, 1975) was adapted so that the tracer ion content could be measured (Hess et al., 1979) within 5 ms of mixing vesicles with carbamoylcholine and radioactive ions, a 200-fold improvement in time resolution compared with the previously available techniques. The time course of the receptor-mediated flux of inorganic ions, and the rate of desensitization, was measured over a 5000-fold range of acetylcholine concentration and over wide concentration ranges of other compounds that activate or inhibit the receptor (Hess et al., 1983; Karpen et al., 1986a,b; Takeyasu et al., 1986). Specific phosphorylation of the receptor was also found to affect desensitization (Huganir et al., 1986). Both desensitization induced by

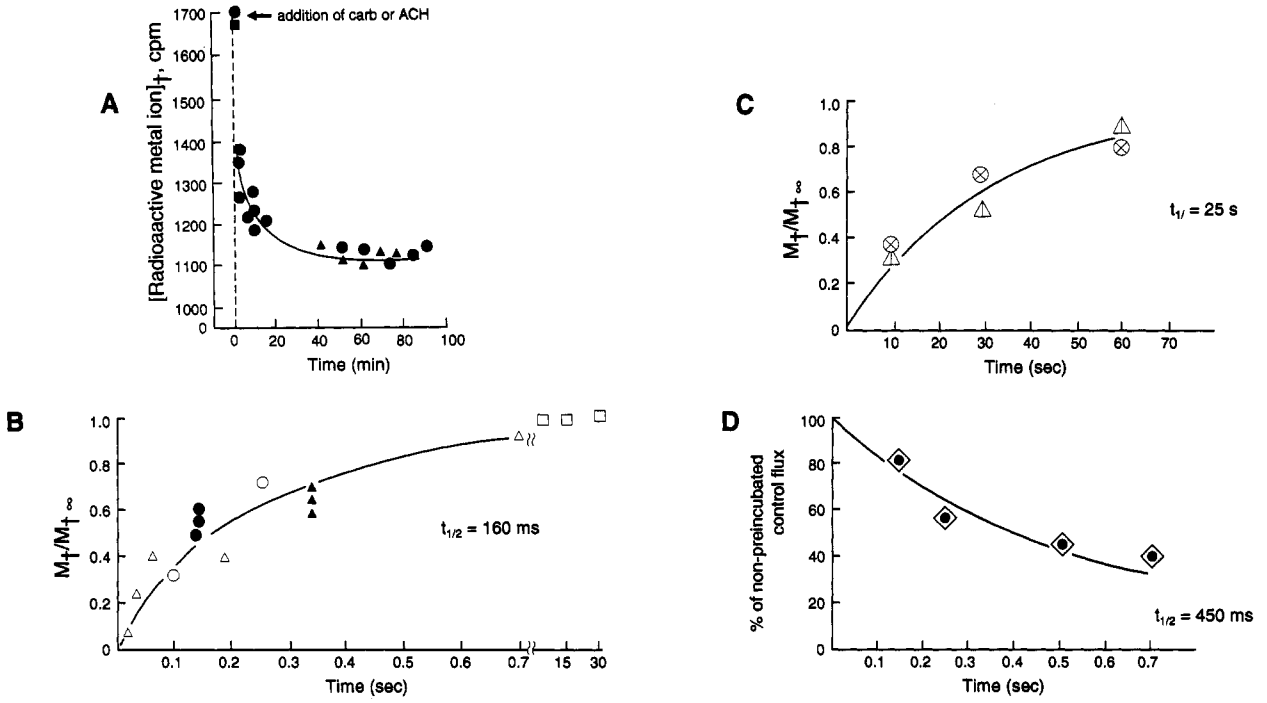


FIGURE 1: Examples of slow (20 s) and rapid (20 ms) mixing experiments with vesicles containing acetylcholine receptors and prepared from the *E. electricus* electroplax. The vesicles were prepared in *E. electricus* Ringer's solution (169 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM phosphate buffer, pH 7.0). (A) 1 mM carbamoylcholine, 4 °C; (B–D) 1 °C. (A) Low-time resolution measurements of $^{86}Rb^+$ efflux. Vesicles were equilibrated with $^{86}Rb^+$; activating ligand (●, 1 mM carbamoylcholine (carb); ▲, 10 μM acetylcholine (ACH)) was added to induce efflux of $^{86}Rb^+$; samples were removed by filtration at various times, and the radioactive tracer ion content of the vesicles was determined. (B–D) Vesicles were rapidly mixed with $^{86}Rb^+$ and carbamoylcholine in a quench-flow apparatus. The influx of ions was quenched after various periods of time, and the radioactive tracer ion content of the vesicles was determined. *M* is proportional to the concentration of $^{86}Rb^+$ in the vesicles. The subscript indicates the time of measurement. (B) Quench-flow measurements of $^{86}Rb^+$ influx induced by 1 mM carbamoylcholine, mediated by the receptor before desensitization (△, ▲, ○, ●). Influx after desensitization (□) is shown on a different time scale. (C) Quench-flow measurements of $^{86}Rb^+$ influx after the receptor has desensitized. Vesicles were preincubated with 1 mM carbamoylcholine for 10 s before the addition of $^{86}Rb^+$ and subsequent influx measurements. (D) The desensitization rate was measured by exposing vesicles for various periods of time to 1 mM carbamoylcholine before the addition of $^{86}Rb^+$. The same concentration of carbamoylcholine and $^{86}Rb^+$ was then added, and influx was measured for a constant period of time, 6 s. The ordinate gives the results as a percentage of the influx measured when carbamoylcholine was absent during the preincubation. [Figure 1A was reprinted and modified from Hess et al. (1978) and Figure 1B–D from Hess et al. (1979) with permission.]

specific activating ligands (acetylcholine, carbamoylcholine, and suberyldicholine) and recovery after dilution of the ligand were investigated (Hess et al., 1983) by means of double incubations and concentration jumps in a quench-flow instrument that we constructed; vesicle breakage was held to a minimum by using relatively low applied pressures to mix solutions, and appropriate mixers and tubings through which the vesicle suspensions flowed (Cash & Hess, 1981).

The quench-flow approach made it possible to obtain the new results shown in Figure 1B–D. The conditions in the experiments illustrated in Figure 1B,C were the same as those in Figure 1A except that the receptor-controlled influx of tracer ions was measured by quench-flow rather than by a technique with a low time resolution. In Figure 1B one sees a rapid influx of radioactive ions, governed by a single-exponential rate law and characterized by a $t_{1/2}$ value of 160 ms, followed by a slow influx phase with a $t_{1/2}$ value of 25 s, about 150 times slower than the initial fast phase. The slow phase in Figure 1B can be studied separately, by preincubating the receptor with carbamoylcholine before mixing the vesicles with carbamoylcholine and tracer ions (Figure 1C). The $t_{1/2}$ value for tracer ion flux in this experiment is similar to the $t_{1/2}$ value of the slow phase when tracer ion flux is measured by a technique with low time resolution (Figure 1A). Direct measurement of the rate of desensitization ($t_{1/2}$ = 450 ms) (Figure 1D) indicated that, at the concentration of carbamoylcholine used, the receptor desensitized completely within 1 s. Thus, in the experiment in Figure 1A and in previous experiments done with a low time resolution [reviewed by Colquhoun (1979) and Karlin (1980)], the properties of *mixtures* of active and desensitized receptor forms already at or near equilibrium had been measured, although this was not recognized. The chemical properties of the active receptor form primarily involved in signal transmission cannot be measured using techniques with low time resolutions, as can be seen in Figure 1A,C.

Since the development of a quench-flow method for use with vesicles prepared from *E. electricus*, quench- and stopped-flow methods have also been used with vesicles prepared from the *Torpedo* sp. electroplax, which is considerably richer in nicotinic acetylcholine receptors [Neubig & Cohen, 1980; Moore & Raftery, 1980; Walker et al., 1981; Hess et al., 1982; Okonyo et al., 1991; reviewed by Ochoa et al. (1989) and Changeux (1990)]. More recently, the quench-flow technique was used to study the γ-aminobutyric acid (GABA) receptor in vesicles prepared from rat brains (Cash & Subbarao, 1987a,b, 1988). For all practical purposes, however, flow techniques in which the concentration of inorganic ions inside vesicles is measured can be used only when a tissue exceptionally rich in the receptor protein of interest is available. To extend the chemical kinetic approach to other tissues and other neurotransmitter receptors, we developed a cell-flow method for use with single cells.

*Cell-Flow Technique with a 5-ms Time Resolution (Udgaonkar & Hess, 1987).* The concentration of open receptor–channels can be determined by measuring the current passing through the channels at a constant transmembrane voltage, using the whole-cell current-recording technique (Hamill et

al., 1981; Mary & Neher, 1983). The approach is analogous to using a pH electrode to measure hydrogen ion release at constant pH in chemical reactions in which the product formed releases or takes up hydrogen ions from a solution. We use a simple device of the kind pioneered by Krishtal and Pidoplichko (1980) to flow neurotransmitter solutions over a single cell suspended in the center of the stream by a recording electrode. The cell is at 180° to the porthole of the flow device from which the solution emerges. Many different flow methods have been employed to equilibrate receptors rapidly with neurotransmitters (Krishtal & Pidoplichko, 1980; Fenwick et al., 1982; Krishtal et al., 1983; Clapham & Neher, 1984; Brett et al., 1986); to obtain a high time resolution, the neurotransmitter solution was made to flow *rapidly* over a cell or small (1–2 $\mu$m) membrane patch. However, the force exerted by a rapidly flowing solution makes the seal between the recording electrode and the membrane unstable. And when a membrane patch is used the number of receptors may be several orders of magnitude less than in a whole cell. These disadvantages require that many measurements of the same process be made with many cells or patches, which differ in the number of receptor molecules present. The problems can be overcome by using low flow rates, which allow one to make many measurements with the same cell (Udgaonkar & Hess, 1987a; Hess et al., 1987), and then correcting the observed current for desensitization occurring as the current rises. With the flow rates we use (a few centimeters per second), receptors on the cell surface facing the flow device are in contact with neurotransmitter before the solution reaches the opposite side of the cell and, therefore, desensitize first. The available hydrodynamic theory (Landau & Lifshitz, 1959; Levich, 1962) allows one to correct the observed current for desensitization that occurs while some receptors on the cell surface farthest from the flow device are still equilibrating with the neurotransmitter (see the legend to Figure 2A) (Hess et al., 1987). The theory is only applicable if the cell is nearly spherical. It is possible to obtain approximately spherical cells, even from cells with many processes (Geetha & Hess, 1992; Matsubara & Hess, 1992). The time resolution of the cell-flow technique, the maximum time it takes for the neurotransmitter concentration at the cell surface to reach that of the solution flowing from the flow device, depends on the flow rates and the diameter of the cell (Hess et al., 1987). Time resolutions in the range of 2–10 ms are obtained (Figure 2A,B). Double-mixing experiments can be done, and the same information can be obtained as with the quench-flow technique; but the cell-flow method has the advantage of allowing one to make measurements with specific, single cells from the mammalian central nervous system.

The result of a typical experiment, in which a solution of acetylcholine flowed over a clonal muscle cell containing acetylcholine receptors, is shown in Figure 2A. The current rises as the receptors on the cell surface equilibrate with the flowing solution and then decreases as they desensitize. In this case the current rise time, the time it takes for the current to reach its maximum value, is 80 ms. The line parallel to the abscissa is the current that would be obtained if the receptors did not desensitize and is denoted as $I_A$, where A represents the active, nondesensitized receptor form. $I_A$ is then a measure of the concentration of open receptor–channels in the absence of desensitization. In experiments with the glutamate receptor, which desensitizes very rapidly (Trussel & Fischbach, 1989), higher flow rates must be used to obtain current rise times of $\sim$2 ms in cell-flow experiments (Figure 2B). As can be seen (Figure 2B), even when the current rise time, a measure of the time needed for glutamate to equilibrate with the receptors, is only 2.5 ms desensitization is considerable, and theory must be used to correct the observed current for desensitization. The upper line parallel to the abscissa gives the current after correction for desensitization.

The importance of a time resolution that allows one to measure the properties of *non*desensitized receptors is also demonstrated in Figure 2C, in which a primary cortical cell containing GABA receptors was used. 500 $\mu$M GABA (solid line) or 500 $\mu$M GABA and 5 $\mu$M picrotoxin (dashed line) flowed over a cell suspended in the solution stream and the resulting whole-cell current was recorded. Picrotoxin is a well-characterized and specific inhibitor of the GABA receptor (Engblom et al., 1989). The time resolution is sufficient to resolve the individual, sequential steps of the reaction. The rising (part a) and decaying (parts b and c) phases of the current occur in three time regions. The rising phase reflects mainly equilibration of the receptors with GABA; the decaying phases give information about the concentrations of the two receptor forms shown to be present, and about their rate coefficients for desensitization. About 60% of the receptors are associated with a desensitization rate coefficient $\alpha$ of 4.4 $s^{-1}$ and 40% with a rate coefficient $\beta$ of 0.6 $s^{-1}$. The GABA–receptor dissociation constant of the rapidly desensitizing receptor form is 40 $\mu$M and that of the slowly desensitizing form is 320 $\mu$M (Geetha & Hess, 1992). When a method with a low time resolution is used (i.e., when the progress of the reaction is first measured 500 ms after its initiation), 5 $\mu$M picrotoxin appears to inhibit the receptor completely (panel c), in agreement with previous reports [reviewed by Ticku (1986)]. However, when a method with a 10-ms time resolution (dashed line, panels a and b) is used, the current due to the rapidly desensitizing receptor form is the same, $\sim$1 nA, whether or not 5 $\mu$M picrotoxin is present (Geetha & Hess, 1992). Thus, the effect of picrotoxin on the rapidly desensitizing form cannot be observed when a low time resolution is used because this form is desensitized within 500 ms, before the first measurement is made.

Although we could measure the properties of the receptor before desensitization with the cell-flow technique, it was still not possible to measure the much faster rates at which receptor–channels open and close using this technique; a new method with a higher time resolution was needed. Accordingly, photolabile precursors of neurotransmitters were synthesized, and laser-pulse photolysis was used to release the neurotransmitter, or an analogue, in the microsecond time region.

*Laser-Pulse Photolysis Technique with a 100-$\mu$s Time Resolution (Billington et al., 1992a; Matsubara et al., 1992).* Advantage was taken of the pioneering work of Kaplan and Trentham [Kaplan et al., 1978; reviewed by McCray and Trentham (1989)] in the synthesis of caged phosphates. Most neurotransmitters, and carbamoylcholine, contain amino groups, and so a photolabile protecting group that can be used with any neurotransmitter containing an amino group was designed. Several photolabile precursors of carbamoylcholine (acetylcholine does not contain an amino group) were synthesized (Walker et al., 1986; Milburn et al., 1989). The effects of substituents on the photolabile group on the photolysis rate were assessed. The effects, if any, of the photolabile precursors and their photolysis products on the receptors and/or the cells were also determined. The most useful precursor of carbamoylcholine synthesized so far is *N*-($\alpha$-carboxy-2-nitrobenzyl)carbamoylcholine (Figure 3A). It is water-soluble and is photolyzed to carbamoylcholine and 2-nitroso-$\alpha$-ketocarboxylic acid within about 120 $\mu$s, with a quantum yield of 0.8 (Milburn et al., 1989). The instrumentation we use for

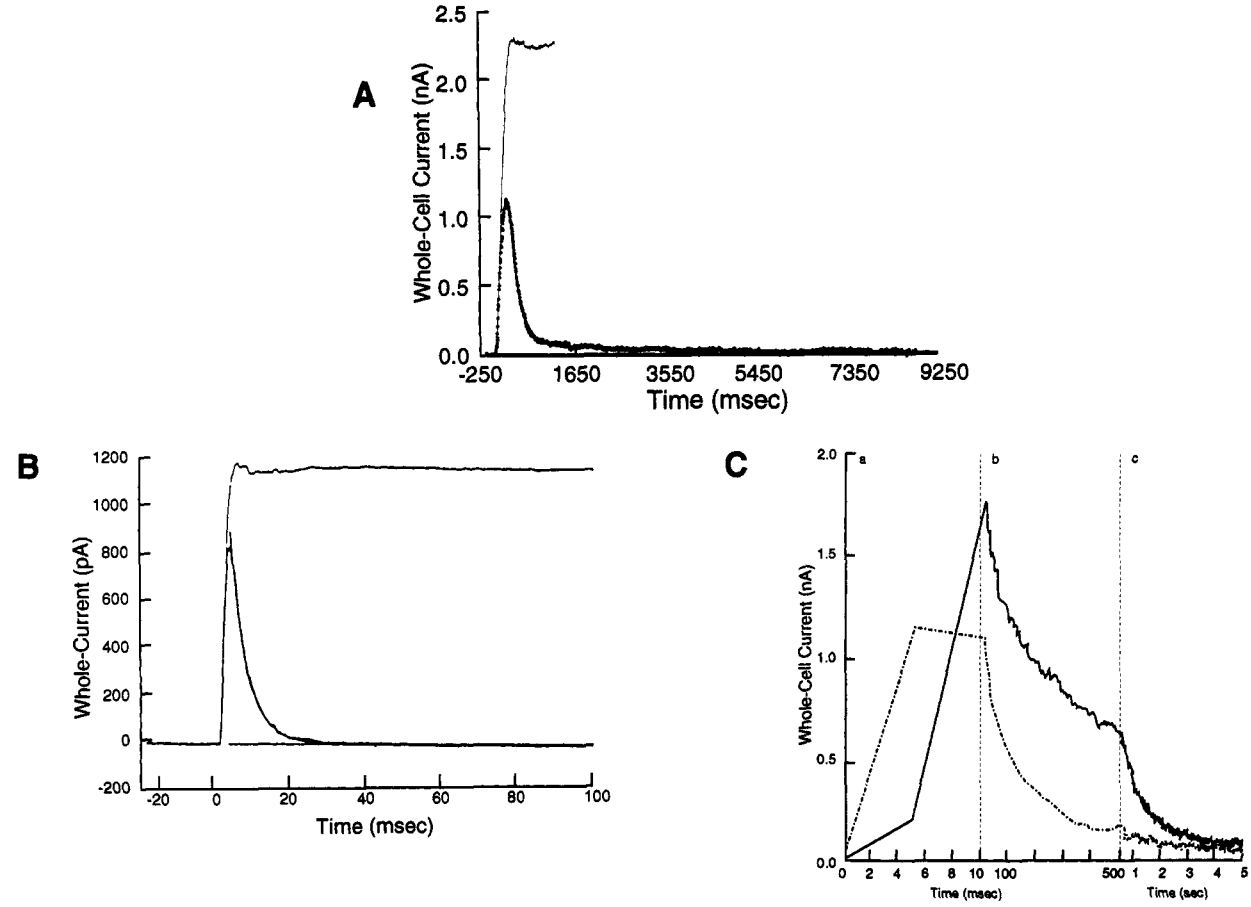


FIGURE 2: Cell-flow measurements in the millisecond time region. (A) Acetylcholine-induced ionic current in a $BC_3H1$ cell. The cell was placed under whole-cell voltage clamp at a membrane potential of –60 mV, pH 7.4, 21–23 °C. A solution of 200 μM acetylcholine flowed around the cell. The thin line parallel to the abscissa was calculated from eq A[1] and represents the current that would be obtained in the absence of desensitization. To obtain the value of the current corrected for desensitization, $I_A$, the rising phase of the observed current is divided into constant time intervals (5 ms in the experiment shown) to take into account the uneven rate at which the ligand solution flows over the cell surface (Landau & Lifshitz, 1959). The observed current during each time interval is then corrected for desensitization occurring during that interval. After the current $(I_{obs})_{\Delta t_i}$ is measured for each of $n$ constant time intervals ($n\Delta t = t_n$), the corrected current is given by eq A[1] (Udgaonkar & Hess, 1987), where $(I_{obs})_{\Delta t_i}$ is the observed current during the $i$th time interval. $(I_A)_{t_n}$ is equal to $I_A$ when the value of $t_n$ is equal to or greater than the current rise time, $t_r$. $\alpha$ is the rate coefficient for desensitization. (B) Glutamate (2 mM)-induced ionic current in a primary embryonic spinal cord cell at –60 mV, pH 7.4, 21–23 °C. The receptor desensitizes with a $t_{1/2}$ value of 3 ms. During the time the current reaches its maximal value, 2.5 ms, ~40% of the receptor has been converted to its desensitized form. The thin line parallel to the abscissa was calculated from eq A and represents the current that would be obtained in the absence of desensitization. The lower line parallel to the abscissa is the baseline. (C) GABA-induced ionic current in an embryonic mouse cerebral cortical cell, at pH 7.2, 21–23 °C, –70 mV. The solid line represents the current obtained in presence of 500 μM GABA in three different time zones: (panel a). The rising phase of the current lasts 10 ms. About 60% of the current decay, considered to be due to desensitization, is characterized by a rate coefficient of 4.4 $s^{-1}$ (panel b) and 40% by a rate coefficient of 0.6 $s^{-1}$ (panel c). The dissociation constant of GABA from the site controlling channel opening of the rapidly desensitizing receptor form is 40 μM and for the slowly desensitizing form is 320 μM. The dashed trace was obtained under the same experimental conditions except for the presence of 5 μM picrotoxin (Geetha & Hess, 1992). [Figure 2 panels A and C were reprinted from Hess et al. (1987) and Geetha and Hess (1992), respectively, with permission. Figure 2B is from Ying Chen, unpublished results].

making kinetic measurements with cells is illustrated in Figure 3B. A receptor-containing cell (in this case the acetylcholine receptor) is attached to an electrode so that the current from the whole cell can be recorded. The solution surrounding the cell contains a photolabile precursor of carbamoylcholine that has no detectable effect on the receptor, thus allowing one to equilibrate the compound with the receptors on the cell surface. A pulse of laser light removes the protecting group, and the caged compound is completely photolyzed within about 120 μs. The carbamoylcholine generated binds to the receptors, which form channels, causing a measurable current to flow. A cell-flow device is included in the equipment so that we can flow solutions of known carbamoylcholine concentration over the cell before and after each laser pulse; the amplitude of the current in the cell-flow measurement, after it has been corrected for desensitization, is used to calibrate the amount of carbamoylcholine liberated in the photolysis experiment and to check that the receptors have not been damaged by the laser light. A current trace from such an experiment is shown in Figure 3C (Matsubara et al., 1992). Caged carbamoylcholine (400 μM) was allowed to equilibrate with receptors on the surface of a $BC_3H1$ cell before photolysis. The precursor was photolyzed within ~120 μs, whereas the current reaches its maximum value in milliseconds (<2 ms). The desensitization reaction is shown on a different time scale; most of the receptor channels close within ~100 ms.

Previously, the rate constants for channel opening and closing, $k_{op}$ and $k_{cl}$, were determined by recording single-channel currents in a reaction that had reached a *quasi*equilibrium before the measurements could be made [reviewed by Madsen and Edeson (1988), Colquhoun and Sakmann (1985),

[1] Equation A (Udgaonkar & Hess, 1987):

$$(I_A)_{t_n} = (e^{\alpha\Delta t} - 1)\sum_{i=1}^{n}(I_{obs})_{\Delta t_n} + (I_{obs})_{\Delta t_n} \qquad (A)$$

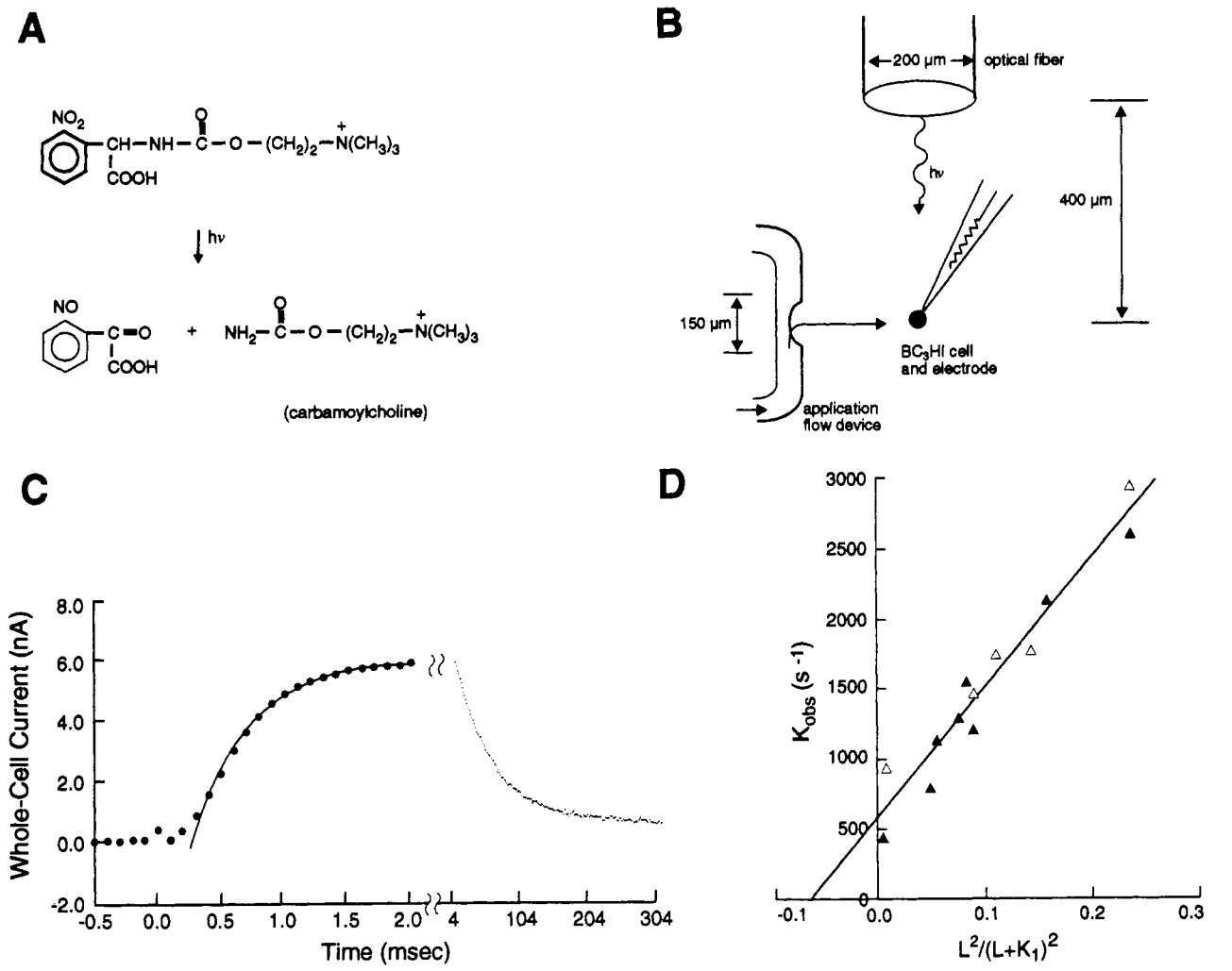


FIGURE 3: Laser-pulse photolysis. (A) Caged carbamoylcholine [*N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine] is photolyzed to 2-nitroso-α-ketocarboxylic acid and carbamoylcholine (Milburn et al., 1989). (B) The apparatus. A $BC_3H1$ cell, of approximately 15-μm diameter, attached to an electrode for recording whole-cell currents, was equilibrated with caged carbamoylcholine. The beam from a Candela SLL500 dye laser with a 600-ns pulse length was introduced from an optical fiber. The cell-flow method was used before and after each laser pulse to determine the concentration of liberated carbamoylcholine and to detect cell damage. (C) A laser-pulse photolysis experiment with caged carbamoylcholine and a $BC_3H1$ cell, pH 7.4, 22–23 °C, and −60 mV. The whole-cell current was generated by photolysis of 400 μM caged carbamoylcholine. The laser excitation wavelength was 328 nm. The solid line through the points represents the rise of the current fitted to a single exponential ($k_{obs}$ = 2140 $s^{-1}$). The desensitization reaction, the falling phase of the current, is shown on a different time scale (Matsubara et al., 1992). (D) Evaluation of the kinetic parameters for channel opening. The relationship between the rate coefficient, $k_{obs}$, for the rising phase of the current and ligand concentration is plotted according to eq 9b (Table I). $k_{op}$, $k_{cl}$, and $K_1$ (listed in Table II) were evaluated using a nonlinear least-squares fitting procedure, and the values were used to construct the solid line. The different symbols represent data from different experiments using different laser dyes with outputs at 318 or 328 nm (Matsubara et al., 1992). [Figure 3 panels B–D were reprinted from Matsubara et al. (1992) with permission.]

and Jackson (1988)]. Despite elegant statistical methods (Katz & Miledi, 1972), including the powerful single-channel current-recording technique (Neher & Sakmann, 1976), and modern methods of data analysis (Colquhoun & Sigworth, 1983; Magleby & Weiss, 1990), the values reported for $k_{op}$ [reviewed in Madsen and Edeson (1988), Jackson (1988), Liu and Dilger (1991), and Matsubara and Hess (1992)] varied as much as 50-fold, even when measurements were made with the same cell line (for instance, $BC_3H1$ cells) (Sine & Steinbach, 1986, 1987; Steinbach et al., 1986; Papke et al., 1988; Liu & Dilger, 1991). The inconsistencies in the reported values of $k_{op}$, at least for the acetylcholine receptor in $BC_3H1$ cells, apparently arise mainly from the assignment of the measured rate constants to a particular step in the mechanism (Matsubara et al., 1992), a recognized and difficult problem in kinetic studies of complex reactions in *quasi*equilibrium (Eigen & Hammes, 1963; Eigen, 1967; Hammes, 1982; Fersht, 1985; Johnson, 1992).

An advantage of the photolysis technique is that one can observe individual, sequential steps of the reaction in different time regions (Figure 3C) (Matsubara et al., 1992), and the ability to know, and vary, the ligand concentration over a wide range allows one to use several criteria to relate an observed rate process to a particular step in a mechanism (Eigen & Hammes, 1963). At low concentrations, the *rising phase* of the current reflects the rate constants for the neurotransmitter-binding steps; at higher concentrations, when the ligand binding steps are much faster than the channel-opening step, the rise time reflects the rate constants for channel opening and closing. The *current maximum* is a measure of the concentration of open receptor–channels before desensitization, and the *falling phase* gives information about the rate of desensitization.

I shall first describe a general mechanism for the acetylcholine receptor and how it is obtained and then mention the implications of the mechanism for signal transmission.

## THE GENERAL MECHANISM

Several neurotransmitter receptors are structurally related (Stroud et al., 1990; Betz, 1990), but each recognizes a specific

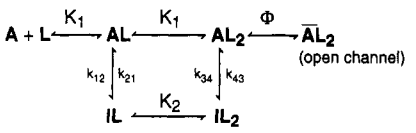


FIGURE 4: Chemical mechanism for the acetylcholine receptor from *E. electricus* electroplax. A and I represent the active and inactive (desensitized) receptor forms, respectively, and $K_1$ and $K_2$ the receptor–neurotransmitter (L) complex dissociation constants for the A and I forms, respectively. The subscript indicates the number of neurotransmitter molecules bound. $\Phi$ is the equilibrium constant. $AL_2/\overline{AL}_2 = k_{cl}/k_{op}$; $k_{op}$ and $k_{cl}$ are the rate constants for channel opening and closing, respectively. $k_{12}$, $k_{21}$, $k_{34}$, and $k_{43}$ are the rate constants for the interconversion between A and I receptor forms.

chemical signal (for instance, acetylcholine, glutamate, serotonin, glycine, GABA). We chose to concentrate initially on the chemical mechanism of the acetylcholine receptor from *E. electricus* for a number of persuasive reasons. The electroplax cells from which the vesicles were prepared are suitable, unlike those from *Torpedo* sp., for electrophysiological characterization of the receptor (Schoffeniels & Nachmansohn, 1957; Nachmansohn, 1959; Higman et al., 1963) and for measurement of single-channel currents (Hess et al., 1984; Pasquale et al., 1986). Thus, we could compare and augment the information obtained using two different approaches, one measures the average properties of many molecules (chemical kinetics) and one the properties of individual molecules (single channels). We also chose not to start with *Torpedo* sp. vesicles because their receptor concentration is much higher than that of the *E. electricus* vesicles we did use. The time resolution of the available mixing techniques was not adequate to measure directly the receptor-controlled flux in *Torpedo* vesicles; indirect methods had to be employed (Neubig & Cohen, 1980; Hess et al., 1982).

The chemical mechanism in Figure 4 is based on quench-flow measurements, with a 5-ms time resolution, made with *E. electricus* vesicles. The mechanism and its constants predict the concentrations of the open-channel and desensitized receptor forms, over a 5000-fold range of acetylcholine concentration (Cash et al., 1981), and account for independent electrophysiological (single-channel current recordings) measurements made with the cells from which the vesicles were prepared (Hess et al., 1984; Pasquale et al., 1986). The active form of the receptor, A, on binding the neurotransmitter, L, undergoes conformational changes to the open-channel, $\overline{AL}_2$, and inactive, desensitized, I, forms in the 1-ms and 50–150-ms time regions, respectively (Hess et al., 1979, 1983). $K_1$ and $K_2$ are the dissociation constants for the receptor:neurotransmitter complex for the A and I forms, respectively. $\Phi$ is the equilibrium constant for channel closing and is defined as $AL_2/\overline{AL}_2 = k_{cl}/k_{op}$, where $k_{op}$ and $k_{cl}$ are the rate constants for channel opening and closing. It was introduced (Cash & Hess, 1980; Hess et al., 1983) to account for (i) cooperativity observed in the effect of ligand concentration on receptor-controlled flux of inorganic ions and (ii) differences in the maximum flux rates measured in the presence of saturating concentrations of acetylcholine, carbamoylcholine, or suberyldicholine (Figure 5A) (Hess et al., 1983). Different maximum flux rates were observed, even though the number of ions that pass through the open receptor–channel is independent of the activating ligand used (Gardner et al., 1984). The requirement that two neurotransmitter molecules must be bound to the receptor for the channel to open was first suggested on the basis of electrophysiological measurements made with a low time resolution (Katz & Thesleff, 1957), presumably with mixtures of active and inactive forms. It is consistent with structural studies indicating the existence of two ligand-binding sites (Reynolds & Karlin, 1978) and with rapid chemical kinetic measurements made with non-desensitized receptors (Cash & Hess, 1980). The formation of open channels without a ligand molecule bound (Jackson, 1984, 1988) and the existence of desensitized receptors in absence of bound ligand [Boyd & Cohen, 1980; Prinz & Maelicke, 1983; reviewed by Ochoa et al. (1989)] have been suggested. The chemical kinetic measurements with *E. electricus* vesicles provide no evidence for or against these suggestions, and they have, therefore, been left out of the minimum mechanism in Figure 4. Similarly, we have no evidence for or against the formation of an open-channel form of the desensitized receptor, and this has also been left out of the mechanism. A slow desensitization process in the frog muscle acetylcholine receptor was first discovered by Katz and Thesleff (1957). In view of the rapid desensitization of the *E. electricus* receptor observed with fast reaction techniques (Hess et al., 1979) and a fast and a slow desensitization of the frog muscle receptor that was subsequently observed in single-channel current recordings (Sakmann et al., 1980), the slow process is considered to be an interconversion between two desensitized receptor forms. A very slow desensitization of the *E. electricus* receptor has also been observed (Aoshima, 1984). The reaction pathway leading to the slowly desensitizing state is unknown and is, therefore, not shown in Figure 4. The *E. electricus* receptor is inhibited by high concentrations of its specific neurotransmitter (acetylcholine) and analogues (Pasquale et al., 1983) in a voltage-dependent manner (Takeyasu et al., 1983; 1986; Shiono et al., 1984; Karpen et al., 1986a), as are other acetylcholine receptors [see, for instance, Neher and Steinbach (1978) and Ogden and Colquhoun (1985)]. The mechanism of this inhibitory process is not known, and it is, therefore, not included in Figure 4.

*Determinations of the Rate and Equilibrium Constants of the Mechanism Using the Quench-Flow Technique.* Four processes within the total mechanism (Figure 4A) can be measured in separate experiments, under conditions where they follow simple first-order kinetics and analytical expressions can be used to account for the dependence of each process on ligand concentration [reviewed by Hess et al. (1983)]. This allows one to evaluate all the rate and equilibrium constants associated with the individual steps (Table II). (The basic equations for the mechanism and definitions of constants are given in Table I.) The processes are (i) transmembrane flux of inorganic ions controlled by the active receptor form (Figure 1B), (ii) transmembrane flux of inorganic ions controlled by desensitized receptor forms (Figure 1C), (iii) receptor desensitization (Figure 1D), and (iv) recovery from desensitization.

*Determination of the Rate and Equilibrium Constants of the Mechanism Using the Cell-Flow Technique.* The reaction steps can be separated in cell-flow experiments in the same way as has been described for the quench-flow measurements [reviewed by Hess et al. (1987)]. (i) In cell-flow experiments (Figure 2A) (eq 8, Table I), the maximum amplitude of the current after correction for desensitization ($I_A$) is a measure of the concentration of open receptor–channels, as is the rate coefficient for inorganic ion flux prior to desensitization in vesicles, $J_A$ (eq 3a), that is determined in quench-flow experiments. In quench-flow experiments, $R_0$ is defined as moles of receptor per liter of internal vesicle volume, whereas in cell-flow experiments $R_M$ is defined as moles of receptors per cell. $I_M$ in eq 8 represents the current per moles of receptors (Udgaonkar & Hess, 1987a). (ii) In cell-flow experiments, the current remaining after the receptor has desensitized is

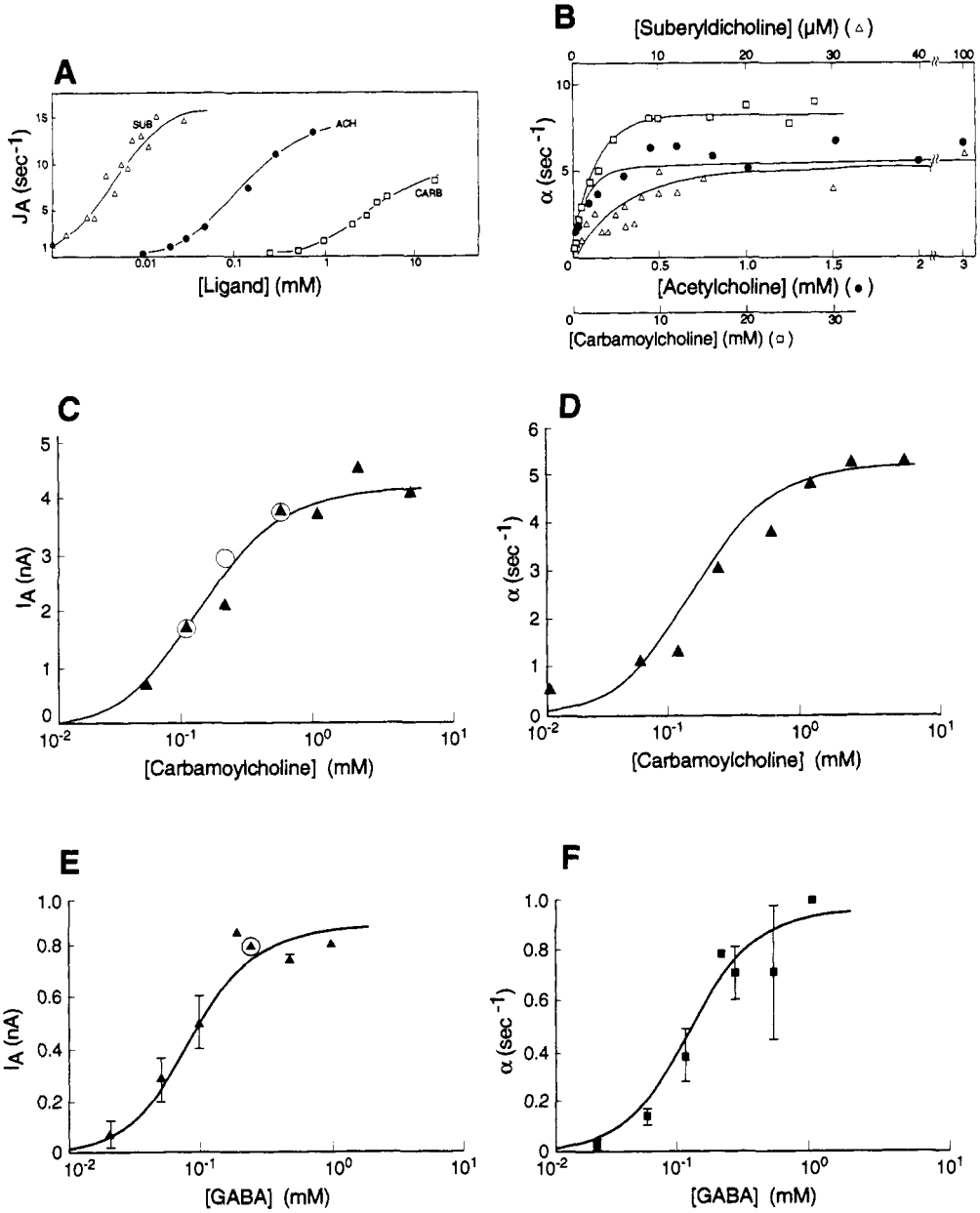


FIGURE 5: The chemical mechanism (Figure 4) accounts for results obtained with both excitatory and inhibitory neurotransmitter receptors. The solid lines were calculated using the chemical mechanism, and the constants from the experimental measurements are indicated by the various symbols. (A) Quench-flow measurements of the nicotinic acetylcholine receptor in vesicles prepared from *E. electricus* electroplax, at pH 7.0, 1 °C (Hess et al., 1983). The effect of suberyldicholine (△), acetylcholine (●), and carbamoylcholine (□) on the influx rate coefficient $J_A$ (eq 3a) or (B) the rate coefficient for desensitization $\alpha$ (eq 7) is shown. The maximum observed flux rates are 18.5 $s^{-1}$ (suberyldicholine), 14.8 $s^{-1}$ (acetylcholine), and 9.7 $s^{-1}$ (carbamoylcholine) (Hess et al., 1983). (C and D) Cell-flow measurements of the nicotinic acetylcholine receptor in $BC_3H1$ muscle cells, at pH 7.4, 23 °C, −60 mV (Udgaonkar & Hess, 1987a). (C) The effect of carbamoylcholine on the concentration of open channels as measured by $I_A$ (eq 8). (▲) Cell-flow measurements; (○) single-channel current recording measurements. (D) The rate coefficient for receptor desensitization $\alpha$. (E and F) Cell-flow measurements with inhibitory GABA receptors in mouse cerebral cortical cells, at pH 7.2, 21–23 °C, −70 mV (Geetha & Hess, 1992). The effect of GABA concentration on (E) the concentration of open receptor–channels as measured by $I_A$ (eq 8) [(▲) cell-flow measurements; (○) single-channel current recording measurements] and (F) the rate coefficient for receptor desensitization $\alpha$. [Figure 5 panels A and B, C and D, and E and F were reprinted from Hess et al. (1983), Udgaonkar and Hess (1987a), and Geetha and Hess (1992), respectively, with permission.]

a measure of the equilibrium concentration of active and inactive forms, as is the rate coefficient for inorganic ion flux in vesicles $J_I$ (eq 5a), which is proportional to the concentration of the active form of the receptor remaining after the receptor has desensitized. (iii) The rate coefficient for desensitization in cell-flow experiments is obtained from the falling phase of the current (Figure 2). (iv) Recovery from desensitization can be measured in cell-flow experiments using multiple mixing, just as with the quench-flow technique (Aoshima et al., 1981).

*Determination of the Rate Constants of the Channel-Opening Process Using Laser-Pulse Photolysis and Caged Neurotransmitter (Matsubara et al., 1992).* As can be seen from the experiment with a $BC_3H1$ muscle cell (Figure 3C), the current rise time in laser-pulse photolysis experiments is governed by a single exponential. When the ligand-binding steps are much faster than the channel-opening process, the relationship between the concentration of open receptor–channels and time is given by eq 9a, and the relationship between the observed rate coefficient $k_{obs}$ and ligand concentration is given by eq 9b. It can be seen from Figure 3D that eq 9b accounts for the effect of ligand concentration on $k_{obs}$. The constants and their values determined in quench-flow investigations of the excitatory nicotinic acetylcholine

Table I: Basic Equations for the Mechanism for the Nicotinic Acetylcholine Receptor[a]

(A) Quench-Flow Measurements of the Flux of Inorganic Ions into Membrane Vesicles (Hess et al., 1983)

general equation for receptor-controlled ion flux

$$[M^+]_t = [M^+]_{t\infty}\{1 - \exp-\bar{J}R_o[(\overline{AL_2})_o - (\overline{AL_2})_\infty]\frac{1 - e^{-\alpha t}}{\alpha} + (\overline{AL_2})_\infty t]\} \quad (1)$$

influx controlled only by the active receptor form A

$$[M^+]_t = [M^+]_{t\infty}(1 - e^{-J_A t}) \quad (2)$$

$$J_A = \bar{J}R_o(\overline{AL_2})_o \quad (3a)$$

$$(\overline{AL_2})_o = L^2[L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi]^{-1} \quad (3b)$$

influx controlled only by the desensitized receptor form I

$$(M^+)_t = (M^+)_{t\infty}(1 - e^{-J_I t}) \quad (4)$$

$$J_I = \bar{J}R_o(\overline{AL_2})_\infty \quad (5a)$$

$$(\overline{AL_2})_\infty = L^2K_{c2}\{K_{c2}[L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi] + \Phi(L^2 + 2LK_2)\}^{-1} \quad (5b)$$

quench-flow measurements of the rates of desensitization and reactivation

$$(\overline{AL_2})_t - (\overline{AL_2})_{t\infty} = [(\overline{AL_2})_{t=0} - (\overline{AL_2})_{t\infty}]e^{-\alpha t} \quad (6)$$

$$\alpha = \frac{k_{34}L + 2K_2k_{21}}{L + 2K_2} + \Phi\frac{k_{34}L^2 + k_{12}2LK_1}{L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi} \quad (7)$$

(B) Cell-Flow Measurements (Udgaonkar & Hess, 1987a)

$$I_A = I_M R_M(\overline{AL_2})_o \quad (8)$$

(C) Laser-Pulse Photolysis Measurements (Matsubara et al., 1992)

$$(\overline{AL_2})_t = (\overline{AL_2})_\infty(1 - e^{-k_{obs}t}) \quad (9a)$$

$$k_{obs} = k_{cl} + k_{op}(L/(L + K_1))^2 \quad (9b)$$

(D) Comparison between Chemical Kinetic Measurements and Single-Channel Current Recordings (Hess et al., 1984)

$$\bar{J} = \frac{RT}{F^2}\frac{\gamma}{[M^+]}N_A \quad (10a)$$

$$k_{cl} = \tau^{-1}_{(open)} \quad (10b)$$

$$(\overline{AL_2})_o = P_o \quad (10c)$$

$$\alpha \approx \tau^{-1}_{(burst)} \quad (10d)$$

[a] $[M^+]_t$ and $[M^+]_{t\infty}$ represent the molar concentration of inorganic ions in the vesicles at time $t$ and at equilibrium, respectively. $J_A$ is the rate coefficient for inorganic ion flux before the onset of desensitization. $\bar{J}$ is the specific reaction rate for receptor-controlled ion translocation (Hess et al., 1981). $R_o$ represents the moles of receptor per liter internal vesicle volume and $(\overline{AL_2})_0$ the fraction of receptors in the open-channel form. All other constants have been defined in the text or Figure 4 legend.

receptor from the *E. electricus* electric organ, and in cell-flow and laser-pulse photolysis investigations of the acetylcholine receptor in a mammalian muscle cell line ($BC_3H1$) and of the inhibitory GABA receptor in primary cells from the cerebral cortex of mice, are listed in Table II.

## COMBINING CHEMICAL KINETICS AND SINGLE-CHANNEL CURRENT-RECORDING EXPERIMENTS

How one chooses to define the constants evaluated in quench- and cell-flow experiments can be instructive, as in the case of the experimentally determined influx rate coefficient $J_A$ (eq 3a). In quench-flow experiments $\bar{J}$, the specific reaction rate of the receptor-controlled ion flux in eq 3a, is an intrinsic constant that is independent of other properties of the vesicles (for instance $R_0$, the moles of receptor per liter of internal vesicle volume) that also determine the observed rate of receptor-controlled ion translocation across the membrane (Hess et al., 1981). If we define the molar concentration of inorganic, monovalent cations that can diffuse through open receptor–channels as $[M^+]$, then $\bar{J}[M^+]$ gives the number of ions that diffuse through the open receptor–channel per unit time. $\bar{J}$, which we determined in chemical kinetic measurements, can also be obtained (Hess et al., 1984) by independent electrophysiological methods that allow one to determine the conductance ($\gamma$) of the receptor–channel (eq 10a) (Neher & Sakmann, 1976). Evaluation of $\bar{J}$ depends on the assumption that the rate coefficient for receptor-controlled ion flux was measured before desensitization occurred. Support for this assumption comes from the good agreement between the values of $\gamma$ and $\bar{J}$ (Table II) determined in single-channel currents recorded from electroplax cells (Hess et al., 1984) and in chemical kinetic measurements made with vesicles prepared from the electroplax cells (Hess et al., 1983).

The results obtained by the cell-flow method and the single-channel technique can also be compared. The fraction of receptors in the open-channel forms (determined in chemical kinetic measurements) (eqs 3b and 8) can also be obtained in single-channel measurements, from the conditional probability, $P_o$, that the receptor is in the open-channel form (eq 10c). In single-channel current recordings, under certain conditions, receptors display bursts of channel activity, separated by silent periods during which the channels do not open (Sakmann et al., 1980). Determining when a burst of channel activity begins and ends is often difficult (Sakmann et al., 1980) and is only possible under certain experimental conditions. $P_o$ has been determined at several concentrations of different ligands in single-channel current recordings from $BC_3H1$ cells (Udgaonkar & Hess, 1987a) and frog muscle (Marshall et al., 1991). In $BC_3H1$ cells we could compare the values obtained by two techniques. Good agreement was obtained between $P_o$ values obtained by single-channel current measurements and cell-flow measurements made with the same cell and experimental conditions (Udgaonkar & Hess, 1987a; Figure 5C).

The mean lifetime of a burst of channel activity, $\tau_{burst}$, is considered to reflect the rate constants for desensitization (Sakmann et al., 1980). When the rate constants for desensitization, $k_{12}$ and $k_{34}$ (Figure 4), are larger than those for recovery from desensitization, the rate coefficient for desensitization ($\alpha$; eq 7) may be compared to the mean lifetime of the burst of channel activity ($\tau_{burst}$; eq 10d) (Table II).

The results obtained by laser-pulse photolysis and by single-channel recordings can also be compared. The rate constant for channel closing ($k_{cl}$) (laser-pulse photolysis) is directly related to the mean lifetime of the open receptor–channel, $\tau_{open}$ (single-channel current recordings) (eq 10b) (Sakmann et al., 1980). When $k_{cl}$ was determined (Matsubara et al., 1992) with $BC_3H1$ muscle cells by both methods, the values were in agreement (Table II). Furthermore, the value of the dissociation constant of the receptor site controlling channel opening, $K_1$ (Figure 4), can be obtained by both laser-pulse

Table II: Some Constants of the Chemical Mechanisms for Excitatory (Acetylcholine) and Inhibitory (GABA) Receptors Determined by Rapid Chemical Kinetic Techniques, except for the Values in Parentheses, Which Were Determined by Single-Channel Current Recordings[a]

| | acetylcholine receptors | | | GABA receptor[d] | |
|---|---|---|---|---|---|
| | *E. electricus* electroplax | | $BC_3H1$ muscle cell | mouse cerebral cortical cells | |
| | acetylcholine (pH 7.0, 0 mV, 1 °C) | carbamoylcholine (pH 7, 0 mV, 12 °C) | carbamoylcholine (pH 7.4, −60 mV, 21–23 °C) | GABA[b] (pH 7.0, −70 mV, 21–23 °C) form 1 | GABA[b] (pH 7.0, −70 mV, 21–23 °C) form 2 |
| $K_1$ ($\mu$M) | 80 | 1000 (600)[a] | 240 210[c] | 40 | 320 |
| $K_2$ ($\mu$M) | 0.7 | | | | |
| $K_{c_1}$ | 0.07 | | | | |
| $K_{c_2}$ | $6 \times 10^{-4}$ | | | | |
| $\Phi$ | 1.5 | 3.4[a] 2.5[b] | 0.2 5.5 | 0.3 4.4 | 0.05 0.7 |
| $\alpha$ ($s^{-1}$) | 5.7 | 12 (20)[a] | | | |
| $k_{12}$ ($s^{-1}$) | 3.4 | | | | |
| $k_{21}$ ($s^{-1}$) | 0.2 | | | | |
| $k_{34}$ ($s^{-1}$) | 9.5 | | | | |
| $k_{43}$ ($s^{-1}$) | $6 \times 10^{-3}$ | | | | |
| $\bar{J}$ ($M^{-1}$ $s^{-1}$) | | $3 \times 10^{7}$[b] ($4 \times 10^{7}$) | | | |
| $k_{cl}$ ($s^{-1}$) | | (1000)[c] | 580[d] (350) | | |
| $k_{op}$ ($s^{-1}$) | | 440 | 9400[d] | | |

[a] Single-channel current measurements are extrapolated to 0 mV (Udgaonkar & Hess, 1987). [b] 1 °C (Hess et al., 1984). [c] Laser-pulse photolysis of caged carbamoylcholine. [d] Geetha and Hess (1992).

photolysis (eq 9b) and the cell-flow technique (eqs 3b and 8) (Table II).

## IMPLICATIONS OF THE CHEMICAL MECHANISM TO THE OPERATION OF THE NERVOUS SYSTEM. SUMMARY AND FUTURE PERSPECTIVES

Rapid chemical reaction techniques play an important part in the determination of the mechanism of enzyme-mediated reactions in solution (Eigen & Hammes, 1963; Eigen, 1967; Hammes, 1982; Fersht, 1985; Johnson, 1992) and provide a wealth of information about the regulation of *intra*cellular processes. Membrane-bound neurotransmitter receptors regulate *inter*cellular signal transmission crucial to the function of the nervous system. As indicated here, new knowledge, essential to an understanding of signal transmission between cells of the nervous system, is obtained when rapid-reaction techniques are used with membrane-bound receptors that must be studied in intact cells or vesicles. This new technology is relevant to many areas of research. Only a few examples can be given.

(1) Theories of nervous system function invoke use-dependent changes in signal transmission between cells (Hebb, 1949; Hopfield & Tank, 1986; Kandel et al., 1991). We know most about the mechanism of the acetylcholine receptor (Figure 4), and I shall start there. It has been observed that small amounts of acetylcholine can be released at cell junctions, sufficient to change the transmembrane voltage of the cell but not sufficient to initiate signal transmission (Thesleff & Molgo, 1984). This information combined with knowledge of the mechanism leads to the following suggestions, which have a bearing on short-term information storage. (i) The acetylcholine receptor is inhibited by high concentrations of acetylcholine, and this inhibition is strongly dependent on transmembrane voltage (Takeyasu et al., 1983, 1984; Sine & Steinbach, 1984; Shiono et al., 1984). Small transient changes in transmembrane voltage can, therefore, have a decisive effect on the transmission process. (ii) Receptor desensitization results from the binding of only one molecule of acetylcholine, while two are required for channel opening. It is, therefore, possible to desensitize the receptor without triggering signal transmission. This can have a decisive effect on the transmission process when subsequently larger amounts of acetylcholine, which normally induce signal transmission, are released at a synapse, but the concentration of nondesensitized receptors is no longer high enough for signal transmission to occur. (iii) The time needed for the receptors to recover from desensitization that reestablishes the signal transmission process is also determined by the rapid reaction technique described here.

Other suggestions for control mechanisms at the level of a single neuron and long-term information storage come from investigations of chemical mechanisms. For instance, cortical cells contain at least six receptors, both excitatory and inhibitory, activated by GABA, glycine, glutamate, and kainate. The two forms of the GABA receptor in these cells have dissociation constants for the site controlling channel opening that differ by a factor of 8, channel-opening equilibrium constants that also differ by a factor of $\sim$8, and desensitization rate coefficients that differ by a factor of $\sim$6 (Table II). The receptor form with the higher affinity for GABA is about twice as abundant in a cortical cell as the form with lower affinity. One form can, therefore, be activated without activating the other form. The other receptors probably also exist in these cells in different forms with different kinetic properties. Short-term changes in the firing threshold of the cell can, therefore, be obtained by variations in the combinations of neurotransmitters reaching the receptors and by the temporal sequence in which the neurotransmitters arrive at their targets. We may hypothesize that long-term variation can be obtained by changes in the biosynthesis of receptor forms, their concentration in the membrane, or both.

(2) Molecular biology has shown that five of the neurotransmitter receptors, both excitatory and inhibitory, are structurally related (Betz, 1990; Stroud, 1990; Maricq et al., 1992) and that isoforms of receptors responding to the same neurotransmitter occur in the same cell membrane [e.g., Boulter et al., (1990) and Keinanen et al. (1990); reviewed by Gasic and Hollmann (1992)]. Single-channel current recordings demonstrate that the open-channel forms of the isoforms may have different conductances and lifetimes. In all these cases, changes in chemical mechanism can be determined using the techniques described, which will tell us how signal transmission will be affected.

(3) From single-channel current recordings, one obtains information about changes in the lifetime and conductance of open channels that occur as a result of (i) development, (ii) genetically engineered receptors, and (iii) chemically modified receptor forms. In all these cases, changes in chemical mechanism can be detected and characterized by rapid reaction techniques. These experiments are expected to tell us how signal transmission will be affected.

(4) A range of nervous system diseases are attributed to receptor malfunction (Kandel et al., 1991). Elucidating the changes in receptor mechanism may help pharmacologists to develop drugs that modify or counteract the defects in mechanism.

(5) Pharmacological studies with compounds affecting receptor function are summarized in many textbooks [e.g., Gilman et al. (1990)]. In such studies, the effects of drugs are generally assayed by equilibrium binding studies with desensitized receptor forms or by measuring the lifetime of open receptor–channels. The mechanism by which the compounds inhibit or activate the active receptor form is crucially important in the development and testing of therapeutic agents. The importance of time resolution in these studies is demonstrated by the experiment in Figure 2C, which shows the unexpected result that one of the best characterized inhibitors of the GABA receptor, picrotoxin, can inhibit one form of the receptor without inhibiting the other form. This would not have been detected by the low-time resolution techniques used previously.

The results in Figure 5A–F demonstrate that the mechanism and constants obtained in chemical kinetic measurements account for the effects of neurotransmitter (or an analogue) concentration over a wide range of the concentration of the open-channel form. The chemical kinetic measurements also account for electrophysiological measurements: (i) the value of the specific reaction rate constant for transmembrane inorganic ion flux, $\bar{J}$, obtained by quench-flow agrees with the single-channel conductance, $\gamma$ (eq 10a), (Table II), (ii) the value of $(\overline{AL_2})_o$ (eq 3b) obtained by cell-flow agrees with the value of $P_o$ (eq 10c) determined in single-channel recordings (Figure 5c), (iii) the value of $\alpha$ (eq 7), obtained by either quench- or cell-flow agrees with $\tau_{burst}$ obtained in single-channel recordings (eq 10d), and (iv) the value of $k_{cl}$ (eq 9b) obtained by laser-pulse photolysis agrees with $\tau_{open}$ obtained in single-channel recordings (eq 10b; Table II). Agreement was also obtained in the values when $K_1$ was determined by laser-pulse photolysis and by the cell-flow technique.

Elucidation of receptor mechanism has a bearing on many current problems in neurobiology. The results obtained with the new techniques show that the approaches of chemical kinetics, well proven with soluble proteins, can also be used to investigate membrane-bound proteins that must be investigated in intact cells. Accounting for the initiation, inhibition, and alteration of signal transmission in basic units (cells) from different areas of the nervous system in terms of well-characterized chemical reactions is, therefore, becoming an attainable goal. The new knowledge is expected to have a major impact on our understanding of the coordinated response of cells responsible for perception of external stimuli, integration and storage of information, and reaction to the environment.

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