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How Fast Does an Acetylcholine Receptor Channel Open? Laser-Pulse Photolysis of an Inactive Precursor of Carbamoylcholine in the Microsecond Time Region with BC_3H1 Cells†

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ABSTRACT: The integrated function of the nervous system depends on specific and rapid transmission of signals between its constituent cells. The nicotinic acetylcholine receptor is the best known of a group of membrane-bound proteins responsible for such transmission; for this process to occur, a specific neurotransmitter, in this case acetylcholine, must bind to the receptor, which then forms transmembrane channels through which cations pass. The resulting change in transmembrane voltage determines whether or not a signal is transmitted. The question of how fast this process takes place in any neurotransmitter receptor has remained one of the interesting and most challenging in the field. To answer it, many attempts have been made to evaluate the rate constant for the opening of the acetylcholine receptor channel, but in almost all these studies the rate was measured after the receptor-mediated reaction, which involves the open channel and many intermediate states, had reached a quasi equilibrium. This resulted in a plethora of reported values for the rate constant that differ by a factor of up to 50-fold, even when the measurements were made with the same type of cell. The new approach described here involves the use of single cells of a mammalian cell line (BC_3H1), containing muscle-type acetylcholine receptors, and the rapid introduction of neurotransmitter to the cell surface. The rapid delivery was achieved by converting a previously synthesized photolabile precursor of carbamoylcholine to carbamoylcholine, a stable amino-group-containing analogue of acetylcholine, with a single laser pulse and an observed photolysis rate of 7300 s^{-1}. The resultant opening of the receptor channels creates a transmembrane current, which was measured in order to determine the rate constant for the formation of the open channel, k_{op}, the rate constant for channel closing, k_{cl}, and the dissociation constant of the receptor site controlling channel opening, K_1, the values of which were found to be 9400 s^{-1}, 580 s^{-1}, and 210 μM, respectively. Two of these constants, k_{cl} and K_1, were also measured by two independent methods, and good agreement was observed. The value of k_{op} is of interest because (i) it determines the rate at which signals can be transmitted between cells and (ii) signal transmission is determined by the concentration of receptors in the open-channel form. At any given concentration of neurotransmitter, the fraction of receptors in the open-channel form may be calculated from the values of K_1, k_{cl}, and k_{op}. These constants can now all be determined for the acetylcholine receptor, and for other receptors that are specific for neurotransmitters containing an amino group, using the laser-pulse photolysis technique described.

The nicotinic acetylcholine receptor is the best known member of a family of membrane-bound proteins responsible for transmission of signals at the junctions (synapses) between cells in the nervous system (Sakmann & Neher, 1984; Hess et al., 1987; Unwin et al., 1988; Claudio et al., 1989; Changeux, 1989; Stroud et al., 1990; Betz, 1990), ranging from about 10^{12} cells in humans (Kandel et al., 1985) to 302 cells in *Caenorhabditis elegans* (Chalfie et al., 1988). Here we describe a new approach to chemical kinetic investigations of receptor-mediated reactions, in which the reaction is initiated by a laser pulse, which converts an inactive precursor of carbamoylcholine (caged carbamoylcholine) (Walker et al., 1986; Milburn et al., 1989) to carbamoylcholine, a stable analogue of the neurotransmitter acetylcholine, with a $t_{1/2}$ value of 95 μs.

Rapid chemical reaction techniques, by virtue of their time resolution and the ability to vary the concentration of reactants, allow one to separate sequential steps of a complex reaction along the time axis. This has enabled kinetic studies to be made of individual steps of a reaction under conditions where simple rate laws are obeyed (Eigen, 1967; Hammes, 1982; Fersht, 1985). The approach has been used with considerable success in investigations of important biological reactions in solution (Eigen, 1967; Hammes, 1982; Fersht, 1985). A

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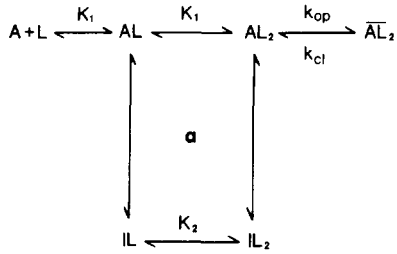


FIGURE 1: Mechanism based on quench-flow measurements with nicotinic acetylcholine receptor from the electric organ of *E. electricus* (Hess et al., 1983). A represents the receptor in its active form, L represents the concentration of acetylcholine (or another activating ligand, for instance, carbamoylcholine), and the subscript indicates the number of ligand molecules that are bound to the receptor molecule. The binding of at least two ligand molecules to the receptor prior to channel opening was first reported by Katz and Thesleff (1957). $\overline{AL}_2$ represents the open-channel form of the receptor which mediates the exchange of inorganic ions across the membrane, thus initiating an electrical signal and its transmission. K_1 is the intrinsic dissociation constant of the receptor site that controls channel opening; k_{op} and k_{cl} are the rate constants for channel opening and closing, respectively; $\Phi^{-1} = k_{op}/k_{cl}$ and is the channel-opening equilibrium constant (Cash & Hess, 1980). Two first-order transitions to inactive (desensitized) receptor forms have been observed: a rapid process (milliseconds) was first discovered for the receptor from the electroplax of *E. electricus* (Hess et al., 1979), and a slow process (seconds to minutes), which is not shown in the mechanism, was first discovered in investigations of the frog muscle receptor (Katz & Thesleff, 1957). α is the rate coefficient for the interconversion between active and inactive receptor forms. The inactive, desensitized receptor form, I, has ligand-binding properties characterized by the dissociation constant K_2. In addition to desensitization, the receptor is inhibited by its specific activating ligand (Pasquale et al., 1983; Shiono et al., 1984; Takeyasu et al., 1986; Udgaonkar & Hess, 1986), a process characterized by the dissociation constant K_R and not shown in the figure.

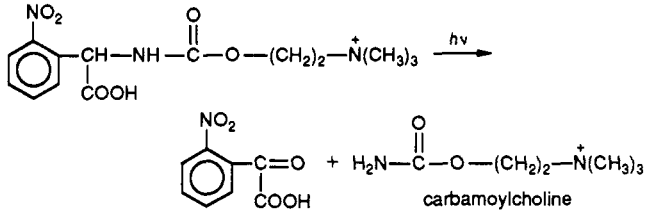
challenge has been to adapt this approach and the techniques to kinetic investigations of membrane-bound neurotransmitter receptors [Hess et al., 1979, 1987; Udgaonkar & Hess, 1987a,b; reviewed in Hess et al. (1983), Ochoa et al. (1989), and Changeux (1990)]. The chemical kinetic measurements described here are an extension of our previous studies in which a quench-flow technique with a 5-ms time resolution was used to investigate the acetylcholine receptor-controlled cation flux in membrane vesicles (Hess et al., 1979, 1983) prepared from the receptor-rich electric organ of *Electrophorus electricus* (Kasai & Changeux, 1971). In the quench-flow measurements it became possible to separate the reaction steps leading to channel opening from those leading to inactive (desensitized) receptor forms with altered biological activity and ligand-binding properties (Figure 1). Thus, the equilibrium constants determining channel opening, K_1, Φ, and K_R (Figure 1), and the rate constants for receptor desensitization could be measured in separate experiments. The constants pertaining to the minimum mechanism (Figure 1) were sufficient to predict the concentration of the open cation-conducting receptor channels at a known concentration of acetylcholine or carbamoylcholine over a large (2000-fold) range (Hess et al., 1983). The evaluated constants were also shown to account for the conductance and lifetime of the open receptor channel (Hess et al., 1984; Udgaonkar & Hess, 1987a,b) determined in independent measurements using the single-channel current-recording technique (Neher & Sakmann, 1976). As a continuation of these studies we developed a cell-flow technique suitable for making chemical kinetic measurements with single cells, with a 10-ms time resolution (Hess et al., 1987; Udgaonkar & Hess, 1987a,b). These investigations indicated that the minimum mechanism in Figure 1 accounts for chemical kinetic measurements made not only with cation-conducting muscle-type acetylcholine receptors in BC_3H1 cells (Udgaonkar & Hess, 1987a,b), and neuronal-type acetylcholine receptors in PC12 cells (Matsubara & Hess, 1992) but, more remarkably, also with a structurally related (Stroud et al., 1990; Betz, 1990) chloride ion-conducting inhibitory receptor, the γ-aminobutyric acid receptor in mouse cerebral cortical cells (Geetha & Hess, 1992).

We now report an improved (by a factor of $\sim$100) time resolution for chemical kinetic investigations of the acetylcholine receptor in BC_3H1 cells, and this is sufficient to enable us to measure the rate constants for the opening and closing of the transmembrane channel, k_{op} and k_{cl}, respectively (Figure 1). This evaluation is of interest, not only because of the importance of these constants in determining the rate with which signals can be transmitted between cells, and in delineating the time frame in which the nervous system can function, but also for the following reasons: (1) The equilibrium between the closed AL_2 and the open-channel form $\overline{AL}_2$ (Figure 1) is characterized by the equilibrium constant $\Phi = k_{cl}/k_{op}$. The values of both Φ and K_1, the dissociation constant of the site controlling channel opening, determine the number of receptor channels that are open at a given concentration of neurotransmitter (Hess et al., 1983) and, therefore, whether or not a signal is transmitted between cells at a given concentration of neurotransmitter. (2) The values of k_{op} and k_{cl} are also important to an understanding of the modulation of the receptor-mediated signal transmission process. (i) The lifetime of the open receptor channel, reflecting k_{cl} (Sakmann et al., 1980), is affected by the transmembrane voltage of the cell membrane (Sine & Steinbach, 1986). The effect of transmembrane voltage on k_{op} has yet to be determined. Changes in transmembrane voltage are expected to alter the ratio of k_{cl}/k_{op} and may, therefore, alter the number of receptor channels that will open at a given concentration of neurotransmitter. (ii) The equilibrium constant Φ is affected by the structure of the ligand bound to the receptor (Hess et al., 1983; Sine & Steinbach, 1986; Papke et al., 1988). This can account for a number of observations: In the presence of saturating concentrations of ligand, the receptor-controlled flux rate in *E. electricus* membrane vesicles is about twice as large in the presence of acetylcholine as it is in the presence of carbamoylcholine (Hess et al., 1983), while the number of ions that pass through the open receptor channel per unit time is independent of the activating ligand used (Gardner et al., 1984); compounds like *d*-tubocurarine that bind to the receptor can act as competitive inhibitors in the presence of the neurotransmitter but can also on their own induce open-channel formation (Takeda & Trautmann, 1984). (3) The values of k_{op} and k_{cl} reflect structural transitions associated with the formation and closing of the open transmembrane channel and, therefore, provide guidelines for interpretation of structural models.

Because of the importance of k_{op}, many attempts have been made to obtain its value. In spite of the availability of elegant statistical methods (Katz & Miledi, 1972), including the powerful single-channel current-recording technique (Neher et al., 1976), and modern methods of data analysis (Colquhoun & Sigworth, 1981, 1983; Magleby & Weiss, 1990), the values reported for k_{op} during the last decade in 21 publications [reviewed in Madsen and Edeson (1987), Jackson (1988), and Liu and Dilger (1991)] vary between 20- and 50-fold, even when the measurements were made with the same cell line. For instance, values of k_{op} obtained in experiments with the frog end plate vary by a factor of about 20 (Sakmann & Adams, 1979; Ogden & Colquhoun, 1983), and values obtained with a clonal mammalian BC_3H1 cell vary by a factor

of about 50 (Sine & Steinbach, 1986, 1987; Steinbach et al., 1986; Papke et al., 1988; Liu & Dilger, 1991).

Here we describe a different approach, using a rapid chemical reaction technique with a submillisecond time resolution. An important strategy for activating cell surface acetylcholine receptors in the submillisecond time region was introduced by Bartels and colleagues (Bartels et al., 1971), who synthesized an inactive receptor ligand, 3,3′-bis[(trimethylammonio)methyl]azobenzene (cis-Bis-Q), that can be photoisomerized to trans-Bis-Q, which activates the acetylcholine receptor channel (Bartels et al., 1971). This compound was used in kinetic investigations (Chaballa et al., 1986). We found, however, that "inactive" cis-Bis-Q desensitizes the receptor and "active" trans-Bis-Q becomes an inhibitor at concentrations greater than 50 μM (Delcour et al., 1986). Our approach is based on investigations in which photolabile precursors of biologically important phosphates were used (Kaplan et al., 1978; McCray et al., 1980; McCray & Trentham, 1989). The strategy was to develop a photolabile protecting group for amino groups in general, and we, therefore, synthesized precursors of several amino-group-containing compounds, the neurotransmitters glycine (Wilcox et al., 1990; Billington et al., 1992a), γ-aminobutyric acid, glutamic acid, and serotonin (D. Ramesh, B. K. Carpenter, and G. P. Hess, unpublished results), and an analogue of acetylcholine, carbamoylcholine (Walker et al., 1986; Milburn et al., 1989). The compound we used in this study is a nitrophenylacetic acid derivative of carbamoylcholine [*N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine], which is photolyzed to a 2-nitroso-α-keto carboxylic acid and carbamoylcholine (Milburn et al., 1989):



The carbamoylcholine derivative has no effect on BC_3H1 cells until it has been photolyzed (Milburn et al., 1989). The rate of photolysis in 100 mM Tris buffer, pH 7.0, or 100 mM HEPES buffer, pH 7.4, at room temperature is 17 000 s^{-1} (Milburn et al., 1989). The photolysis rate was found to be dependent on the concentration of the HEPES buffer used; in 25 mM HEPES buffer, pH 7.4, the photolysis rate decreased to a value of 7300 s^{-1}. HEPES inhibits the receptor at higher concentrations, and increasing the buffer concentration was, therefore, not practicable: the whole-cell current recorded in the presence of 100 μM carbamoylcholine and 25 mM HEPES buffer was reduced by 30% when the HEPES concentration was increased to 100 mM.

Materials and Methods

Clonal mammalian BC_3H1 cells containing nicotinic acetylcholine receptors (Schubert et al., 1974) were obtained from Professor E. Racker (Cornell University) and cultured as described by Sine and Taylor (1979). The cells were maintained in 25-cm^2 T-flasks (Corning) in Dulbecco's modified Eagle's medium (4500 mg/L glucose) (Gibco) (DMEM) with 10% fetal bovine serum (FBS) (Hazelton) at 37 °C in 10% CO_2. For the measurements reported here, cells were plated on 35-mm dishes (Corning or Falcon). After 1 day the cells were rinsed once with DMEM and then maintained in medium containing 1% FBS to induce differentiation (Olson et al., 1983). The cells were fed with DMEM containing 1% FBS about every 4 days and were used 4–12 days after plating.

A caged carbamoylcholine derivative, *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate, was synthesized as described (Milburn et al., 1989) and then obtained commercially from Molecular Probes. The compound was purified on a Dowex 50W-X8 column, 20–50 mesh, 8-mm internal diameter and 15 cm in height, which was preequilibrated with 10 mM HEPES buffer adjusted to pH 7.0 with sodium hydroxide. Caged carbamoylcholine dissolved (0.5 mL, 20 mM) in 10 mM HEPES and adjusted to pH 7 was loaded onto the column and eluted with 10 mM HEPES (pH 7.0). Free carbamoylcholine was retained by the column. The first 1–2-mL fraction contained the caged carbamoylcholine derivative; its concentration was determined at 266 nm (E_M = 5200 M^{-1} cm^{-1}).

Wavelengths of 318 and 328 nm were chosen for the experiments to avoid cell damage at lower wavelengths and lower product yields at longer wavelengths. A flash lamp pumped dye laser (Candela SLL500) using rhodamine 640 or sulforhodamine 640 laser dye (Exciton), together with frequency doubling of the laser output, produced the desired wavelength. The energy of the laser pulse emerging from the optical fiber was 200–600 μJ as measured by a Gentec ED200 joulemeter, resulting in a maximum fluence of 0.8 J cm^{-2}. The current amplitudes in the presence of carbamoylcholine measured in cell-flow experiments indicated that acetylcholine receptor function is not affected under these conditions [see also Marque (1989)] at the wavelengths used. Initial experiments with the system revealed that discharge of the laser was picked up by the channel current-recording system. To alleviate this problem, the laser was separated from the current-recording apparatus by using a long (>20-ft) optical fiber (Fiberguide Industries, SFS200N). Studies of the photolysis reaction were performed as described (Milburn et al., 1989) by using a dedicated laser-flash photolysis apparatus in which the excitation source was an excimer laser (Lumonics TE-861M).

For whole-cell current recording, a commercially available amplifier (List L/M-EPC7) (Sigworth, 1983) was used. The extracellular solution used in the measurements was 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 25 mM HEPES buffer (pH 7.4). Carbamoylcholine or caged carbamoylcholine solutions were made in this buffer unless noted otherwise. The solution inside the recording electrode was 145 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES buffer (pH 7.4). The electrodes were prepared on a homemade puller and fire-polished; the resistance of the electrode when filled with the above solution was typically 3–4 MΩ. The series resistance was typically 5–6 MΩ. The transmembrane voltage of −60 mV in the experiments was held constant (±6 mV) as described in detail by Sigworth (1983). The current obtained in a typical measurement of the channel-opening process is shown in Figure 3. Data were low-pass filtered (Krohn-Hite 3322) with a 1–10 kHz cutoff frequency (−3-dB point) and then digitized at a 2–20 kHz sampling frequency using a PDP 11/23 minicomputer; the data were transferred to a Convex C210 computer (Material Science Center, Cornell University), where the time constants for the rising and decaying phases of the whole-cell current were fitted, using the data analysis program PLOT (Material Science Center, Cornell University). All experiments were conducted at room temperature (22–23 °C), −60-mV transmembrane potential, and pH 7.4.

In order to measure the concentration of carbamoylcholine generated upon photolysis of caged carbamoylcholine, a cell-flow whole-cell recording technique was used (Udgaonkar

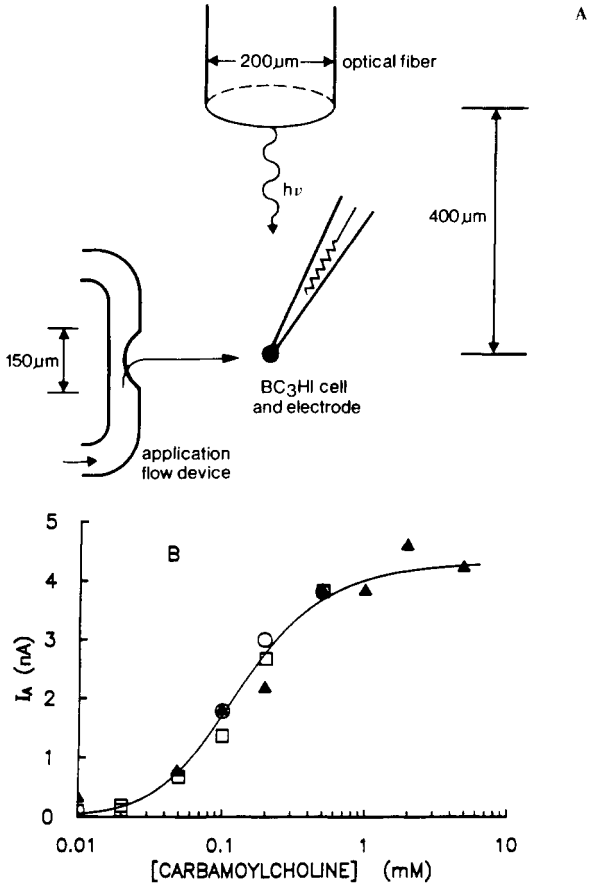


FIGURE 2: Experimental details of laser-pulse photolysis kinetic measurements with caged carbamoylcholine and BC_3H1 cells. (A) The BC_3H1 cell, of approximately 15-μm diameter, attached to an electrode for recording whole-cell currents was equilibrated with caged carbamoylcholine for 30 min. A Candela SLL500 dye laser with a 600-ns pulse length was used. The laser beam was introduced from an optical fiber of 200-μm core diameter. The fiber was adjusted to be about 400 μm away from the cell surface so that the area illuminated around the cell had a diameter of 300–400 μm. The porthole of the flow device (Krishtal & Pidoplichko, 1980), with a 150-μm diameter, is represented by the elipse. (B) Concentration dependence of the current amplitude corrected for receptor desensitization, I_A. The cell-flow technique utilizing whole-cell current recording was used at pH 7.4, 22–23 °C, and −60 mV transmembrane potential. The solid symbols and solid curve were obtained previously (Udgaonkar & Hess, 1987a,b); (△) cell-flow technique (Udgaonkar & Hess, 1987a,b); (○) I_A values were calculated from measurements of P_O, the conditional probability that the receptor is in the open-channel form providing the receptor is in its nondesensitized state (Sakmann et al., 1980). The open squares (□) were obtained in the present experiments. The new results and those previously obtained were normalized to the I_A value obtained at 500 μM carbamoylcholine. The experimental results were fitted to an equation (Udgaonkar & Hess, 1987a,b) relating I_A values to carbamoylcholine concentration based on the mechanism in Figure 1, and the coordinates of the solid line were calculated from the experimental measurements using this equation. Cell-flow experiments using known concentrations of carbamoylcholine were performed with each of the BC_3H1 cells used in the laser-pulse photolysis measurements. The I_A values observed were compared to the current amplitude produced by the laser-pulse photolysis experiments with caged carbamoylcholine and BC_3H1 cells. These two measurements, together with the solid curve shown above, were used to calculate the concentration of carbamoylcholine generated in the laser-pulse photolysis experiments.

& Hess, 1987a,b); a schematic drawing of the apparatus used in the present measurements with BC_3H1 cells is shown in Figure 2A (Billington et al., 1992b). Prior to photolysis a known concentration of carbamoylcholine was applied to a single cell using a cell-flow device (Krishtal et al., 1980), and the amplitude of the resulting current (Figure 2B) was recorded and used to calibrate the concentration of the carbamoylcholine subsequently liberated from the photolabile or caged precursor by a laser pulse. The carbamoylcholine was then removed from the dish containing the cell, and caged carbamoylcholine was added, all by means of the cell-flow device; the carbamoylcholine liberated by a laser pulse was calculated from the cell-flow calibration.

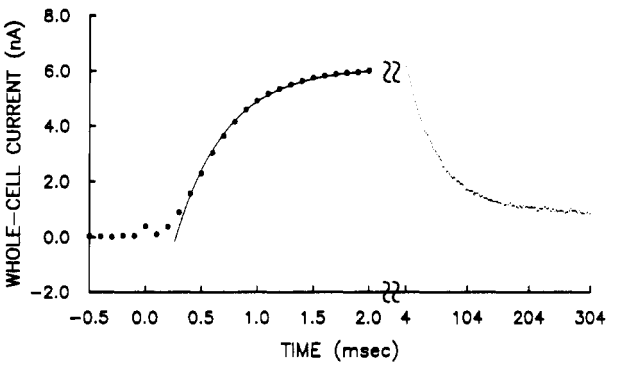


FIGURE 3: Laser-pulse photolysis experiment with caged carbamoylcholine and BC_3H1 cells at pH 7.4, 22–23 °C, and −60 mV. The whole-cell current was generated by laser-pulse photolysis of 400 μM caged carbamoylcholine. The excitation wavelength was 328 nm with a pulse length of 600 ns. This data set was obtained by low-pass filtering at 2.5 kHz (−3 dB) and was digitized at a sampling rate of 10 kHz. The solid line through the points represents the fit of the rise of the current to a single exponential (k_{obs} = 2140 s^{-1}) rate equation. The deviations of the current from the baseline subsequent to the laser pulse at time zero are discussed in the text. The desensitization reaction, the falling phase of the current, is shown on a different time scale.

Results and Discussion

Figure 2B shows the results produced in cell-flow experiments from which the dependence of I_A on carbamoylcholine concentration was established; I_A is a measure of the number of receptor molecules in the open-channel form before desensitization occurs. Data obtained previously by the same cell-flow method (Udgaonkar & Hess, 1987a,b) are represented by solid triangles and data acquired in cell-flow experiments in this study by open squares. The data depicted by open circles were obtained by recording single-channel currents (Neher & Sakmann, 1976) and represent the probability, P_O, that the channel is open while the receptor is in a nondesensitized, active state (Sakmann et al., 1980). P_O is directly related to I_A (Udgaonkar & Hess, 1987a,b) so both sets of values are shown on the same graph, and it is clear that there is excellent agreement between the results of the previous and present experiments.

In the experiment shown in Figure 3, 400 μM caged carbamoylcholine was allowed to equilibrate with the receptors on the surface of a BC_3H cell before photolysis was induced by a laser pulse at time zero. An initial deviation of the current from the baseline lasts for about 200 μs before the current rises smoothly; the time course for this deviation is consistent with the rate of photolysis of the caged compound, $t_{1/2}$ = 95 μs, in the presence of 25 mM HEPES. After this initial induction period, the current reaches its maximum value in about 1.5 ms. The desensitization reaction, indicated by the falling phase of the current, is shown on a different time scale; about 50% of the desensitization reaction occurs within 100 ms. These results (Figure 3) demonstrate that the time resolution of the laser-pulse photolysis technique is sufficient to resolve the individual, sequential steps of the reaction. The kinetics of individual reaction steps can, therefore, be measured (Udgaonkar & Hess, 1986), thus simplifying the evaluation of the pertinent constants of the mechanism. At low concentrations

of neurotransmitter the formation of receptor:neurotransmitter complexes is expected to be rate-limiting, and the current rise time then reflects the rate constants for the neurotransmitter-binding steps. At high concentrations of neurotransmitter the formation of the transmembrane channel is expected to be rate-limiting, and the current rise time then reflects the rate constants for the channel-opening process. The maximum amplitude of the current obtained in the absence of receptor desensitization, I_{max}, is a measure of the concentration of the receptor in the open-channel form, at a given concentration of neurotransmitter. From the dependence of the current amplitude on neurotransmitter concentration, it is possible to evaluate the equilibrium constants for neurotransmitter binding to the receptor and for the channel-opening process (Udgaonkar & Hess, 1986). From the falling phase we obtain the rate of receptor desensitization, which has previously been determined by use of the cell-flow technique (Udgaonkar & Hess, 1987a,b). Except for the initial deviation of the current from the baseline (Figure 3), the rising phase of the current (Figure 3) follows a single exponential rate equation:

$$I_t = I_{max}[1 - \exp(-k_{obs}t)] \quad (1)$$

I_t is the current observed at time t, and I_{max} is the maximal observed current in the absence of desensitization. For the mechanism in Figure 1, the relationship between the first-order rate coefficient for the channel-opening process and the constants of the mechanism has been derived (Udgaonkar & Hess, 1986). When the ligand-binding steps are fast compared to the channel-opening process and L represents molar concentrations:

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

A number of different criteria and independent measurements can be used to identify k_{obs} with the channel-opening process. (1) In the experiments (Figure 3) the photolysis of caged carbamoylcholine is followed by a single exponential rise of the whole-cell current due to the opening of transmembrane channels. In contrast, in single-channel current measurements as many as six different exponential processes have been observed in experiments with BC_3H1 cells (Steinbach et al., 1986; Sine & Steinbach, 1987). (2) In the case of the mechanism for the channel-opening process (Figure 1), the time resolution of the technique and the ability to conduct experiments at different known carbamoylcholine concentrations allow one to distinguish between four assumptions: (i) The ligand-binding and channel-opening processes occur in similar time regions. This assumption requires that as the carbamoylcholine concentrations are changed in the experiments, deviations from a single-exponential current rise time are observed. Excluding the initial deviations from the baseline (Figure 3) that occur at high concentrations of caged carbamoylcholine, the reaction obeys first-order kinetics for at least 90% of the observed maximum current amplitude. The solid line is calculated on the assumption that the current rise time follows a single-exponential rate process. The solid circles represent the measured current. (ii) The current rise time reflects the ligand-binding steps. This assumption requires a direct dependency of k_{obs} on carbamoylcholine concentration (Udgaonkar & Hess, 1986). This was not observed (see Figure 4A). (iii) The ligand-binding steps and the formation of AL_2 (see Figure 1) are fast compared to the channel-opening process. (iv) The binding of two ligand molecules to the receptor prior to channel opening is required. When conditions iii and iv are fulfilled, the relationship between k_{obs} for the current rise time and carbamoylcholine concentration is given by eq 2. This relationship is represented well by the data,

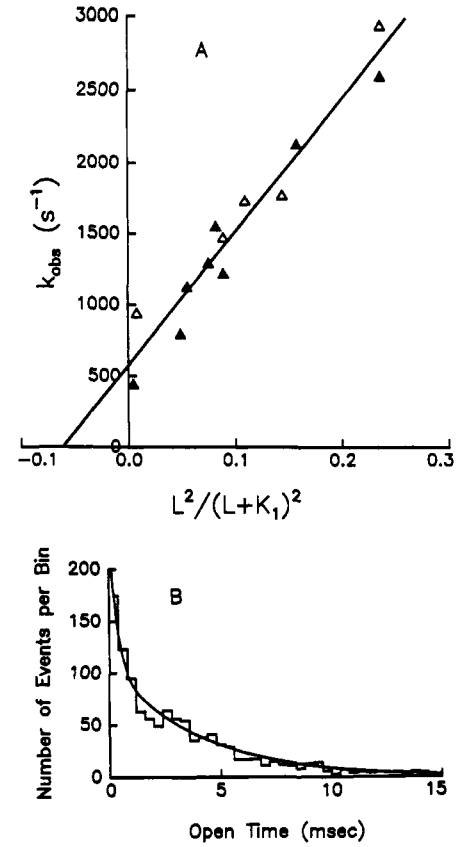


FIGURE 4: Evaluation of the kinetic parameters for the channel-opening process in BC_3H1 cells at pH 7.4, 22–23 °C, and −60 mV. (A) Relationship between the rate coefficient, k_{obs}, for the rising phase of the current and ligand concentration plotted according to eq 2 (see the text): $k_{obs} = k_{cl} + k_{op}[L/(L + K_1)]^2$. The parameters k_{op}, k_{cl}, and K_1 were evaluated by using a nonlinear least-squares computer program, and the values of these constants were used to construct the solid line. The symbols shown for the data represent different experiments using either sulforhodamine 640 (△) or rhodamine 640 (▲) as the laser dye, with output at 318 or 328 nm, respectively. A different cell was used for each measurement. The values of the constants are $k_{cl} = 580 \pm 140\ s^{-1}$, $k_{op} = 9400 \pm 3900\ s^{-1}$, and $K_1 = 210 \pm 90\ \mu M$. (B) Open-time histograms obtained with the single-channel current-recording technique. The recording electrode contained extracellular solution (see Materials and Methods) with 1.9 mM carbamoylcholine added. All values are given ± standard deviation. The histogram is represented by the sum of two exponentials: 1118 openings were measured; 10% of the openings had a mean lifetime of 0.33 ± 0.16 ms, and 90% had a mean lifetime of 3.61 ± 0.22 ms. In other experiments the mean open time (τ_{op}) of acetylcholine receptors in ten BC_3H1 cells measured at carbamoylcholine concentrations ranging from 1.9 to 500 μM was averaged (22 694 openings). $\tau_{op} = 2.85 \pm 1.05$ ms, observed at transmembrane potentials from −49 to −73 mV. The bin width was 0.4 ms. For comparison, measurements were made in the presence of 0.3 μM acetylcholine, pH 7.4, 22–23 °C, and −120 mV. Using one cell and measuring 831 openings, two types of open times were observed, with τ_{op} values of 0.24 ± 0.055 ms (25%) and 7.35 ± 0.52 ms (75%). Using similar conditions and acetylcholine concentrations of 0.02–10 μM, Papke and Oswald (1989) reported τ_{op} values of 0.20 and 10.6 ms.

which is shown in Figure 4A. Other assumptions in eq 2 are that, at the carbamoylcholine concentrations used, channel opening when only one ligand molecule is bound to the receptor can be neglected. At lower carbamoylcholine concentrations this assumption may not be valid (Steinbach et al., 1986; Sine & Steinbach, 1987; Jackson, 1988; Papke & Oswald, 1989). Single-channel current measurements (Sine & Steinbach, 1986) and cell-flow investigations (Udgaonkar & Hess, 1987a,b) indicate the presence of a minor component in BC_3H1 cells responsible for about 10%–20% of the observed current in the presence of carbamoylcholine. It is believed that

there is only a single population of receptors in BC_3H1 cells (Sine & Steinbach, 1986). No evidence for the existence of two forms associated with different values of k_{op} and k_{cl} was obtained from kinetic measurements of the current rise time in the experiments reported.

We noticed that, at the wavelength used in the laser-pulse experiments, increasing the laser energy to produce concentrations of carbamoylcholine greater than 200 μM often led to cell damage. Although this limited the concentration range of carbamoylcholine used, eq 2 contains, in addition to k_{op}, two constants that can be evaluated by independent measurements, k_{cl} and K_1. (i) The value of K_1 can be determined by the cell-flow technique (Udgaonkar & Hess, 1987a,b). There is good agreement between the values of K_1 determined by the two techniques using BC_3H1 cells and comparable experimental conditions; in the laser-pulse photolysis experiments the evaluation of K_1 is based on experiments covering a range of carbamoylcholine concentration from 15 to 200 μM (K_1 = 210 μM), but in the cell-flow technique a 500-fold range of carbamoylcholine concentration (K_1 = 240 μM) was used. (ii) The value of k_{cl} can be determined by an independent method, the single-channel current-recording technique (Neher & Sakmann, 1976), also using BC_3H1 cells and similar experimental conditions. Single-channel current measurements allow the mean lifetime, τ_{op}, of the open receptor channel to be evaluated, and $\tau_{op} = k_{cl}^{-1}$ (Sakmann et al., 1980). In the single-channel measurements the τ_{op} value obtained corresponds to a k_{cl} value of 350 ± 129 s^{-1}, and the k_{cl} value determined from the ordinate intercept of the graph in Figure 4A is 580 ± 140 s^{-1}. In order to compare our single-channel current-recording measurements to published values, we determined τ_{op} in the presence of the same ligand, acetylcholine, and with the same cell type, at the same temperature and transmembrane voltage, as were used by a different laboratory (Papke & Oswald, 1989), and the agreement is excellent (see the legend to Figure 4). (iii) The cell-flow measurements allow one to evaluate the ratio $k_{op}(k_{op} + k_{cl})^{-1}$ (Udgaonkar & Hess, 1987a,b), which can be compared to results obtained in the laser-pulse photolysis experiments. The ratio $k_{op}/(k_{op} + k_{cl})$ gives the fraction of the receptors in the open-channel form when the receptors are saturated with activating ligand (Hess et al., 1983). Using BC_3H1 cells and similar conditions, values of 0.94 and 0.84 were determined using the laser-pulse photolysis and the cell-flow techniques, respectively. A comparison of results using BC_3H1 cells and carbamoylcholine, but different techniques, is given in Table I.

Table I: Comparison of the Value of the Constants Obtained in Laser-Pulse Photolysis Experiments with BC_3H1 Cells and Those Obtained by Other Techniques[a]

constant	method	value of constants
k_{op}	LPP[b]	9400 ± 3900 s^{-1}
	SCC[c]	179 ± 89 s^{-1} [d] (11952 ± 2980)
k_{cl}	LPP	580 ± 140 s^{-1}
	SCC	350 ± 129 s^{-1}
$k_{op}(k_{op} + k_{cl})^{-1}$	LPP	0.94
	cell-flow technique	0.84
K_1	LPP	210 ± 90 μM
	cell-flow technique	240 μM (Udgaonkar & Hess, 1987a,b)

[a] Unless stated otherwise, experiments were performed at 22–23 °C, pH 7.4, and transmembrane voltage −60 mV. [b] LPP = laser-pulse photolysis (this paper). [c] SCC = single-channel current measurements. [d] The authors (Sine & Steinbach, 1986) were able to evaluate two rate constants from their measurements. The value of 11952 s^{-1} in parentheses was considered not to reflect k_{op}. Both measurements were obtained at 11 °C and −100 mV.

It is of interest to compare (Table I) the values of k_{op} obtained in chemical kinetic investigations of the acetylcholine receptor in BC_3H1 cells in the presence of carbamoylcholine (k_{op} = 9400 s^{-1} at 22–23 °C) with the rate constant determined from single-channel current measurements using the same cells. The authors (Sine & Steinbach, 1986) obtained two rate constants of 174 s^{-1} and 11952 s^{-1} at 11 °C. The first value was considered to reflect k_{op} (Sine & Steinbach, 1986) and is smaller by a factor of about 50 than the value obtained in these studies (Table I). The second value, in reasonable agreement with the value for k_{op} obtained in the experiments presented, was considered not to reflect the channel-opening rate constant (Sine & Steinbach, 1986). Values for k_{op} found in experiments with BC_3H1 cells in the presence of acetylcholine vary between 321 s^{-1} (Sine & Steinbach, 1986) and 12000 s^{-1} (Liu & Dilger, 1991) at 11 °C to 8280 s^{-1} (Papke et al., 1988) at room temperature. These results suggest that a major reason for the inconsistency in the reported values of k_{op} determined with BC_3H1 cells arises from the assignment of the rate constants to the channel-opening step. In the single-channel technique the activating ligand is applied relatively slowly (during 1 s or longer), and it is assumed that the reaction, involving active, inactive, and completely desensitized receptor states, is at quasi equilibrium (Colquhoun & Hawkes, 1981; Colquhoun & Sigworth, 1983; Magleby & Weiss, 1990). Among the possible receptor states of the proposed mechanism (Figure 1), only the open receptor channel, $\overline{AL_2}$, gives a measurable signal and is, therefore, easily identified. Thus, the lifetime of the open channel, a measure of the channel-closing rate constant k_{cl}, can conveniently be determined (Sakmann et al., 1980). Assigning any one of the many silent states (A, AL, AL_2, IL, IL_2; Figure 1) to a specific step of a complex reaction at equilibrium (for instance, Figure 1) is a major and well-known challenge in kinetics, and many attempts to do this in order to determine k_{op} have been described in great detail [reviewed in Madsen and Edeson (1987) and Jackson (1988)]. In another approach, solutions containing neurotransmitter at concentrations much larger than those required to saturate the receptor binding site were allowed to flow over small membrane patches (1–2-μm diameter) of BC_3H1 cells with the expectation that a small and unknown amount of the neurotransmitter in the flowing solutions will saturate the receptors in a time short compared to channel opening (Liu & Dilger, 1991). Since high concentrations of activating ligands are known to inhibit this receptor (Pasquale et al., 1983; Shiono et al., 1984; Sine & Steinbach, 1984; Ogden & Colquhoun, 1985; Takeyasu et al., 1986), and the concentration of the ligand binding to the receptor sites is not known in these experiments, interpretation of experimental results may be not straightforward (Liu & Dilger, 1991).

The chemical kinetic technique differs from the single-channel current technique in that it allows one to observe the individual sequential steps of the reaction in different time regions (Figure 3). The ability to know the ligand concentration, and to vary it, allows one to use a number of different criteria to relate an observed rate process to a particular step in a mechanism. It is also possible to use a number of independent measurements to assess the mechanism and the constants pertaining to the mechanism that are based on the results of laser-pulse photolysis experiments. Chemical kinetic measurements using fast reaction techniques for investigating biological processes in solution are well documented (Eigen, 1967; Hammes, 1982; Fersht, 1985). The adaptation of this approach to investigations of membrane-bound acetylcholine receptors (Hess et al., 1979; Hess et al., 1983) made it possible

to determine the rate and equilibrium constants of the active, nondesensitized receptor rather than of equilibrium mixtures of active and desensitized receptors [reviewed in Hess et al. (1987) and Ochoa et al. (1989)]. The use of fast reaction techniques also made it possible to detect and measure desensitization processes that occur in the millisecond time region and to observe the effects of various compounds or chemical modification on the nondesensitized receptor forms [reviewed in Udgaonkar and Hess (1986) and Ochoa et al. (1989)]. The increased time resolution of the laser-pulse photolysis technique described here now allows one to investigate the channel-opening process with an average error of less than ±50% with respect to the determination of the rate constants. This experimental error, calculated from the standard deviations from the mean given in Table I, is comparable to the error of rate constants evaluated in single-channel measurements.

The results presented here, and the availability of inert, photolabile precursors of other amino-group-containing neurotransmitters, suggest that investigations of the channel-opening process for all the receptors that respond to these amino-group-containing neurotransmitters may now be possible. Furthermore, the results of the laser-pulse photolysis experiments are expected to be helpful in the identification of some of the closed states in single-channel current measurements, for instance, the closed time which reflects the channel-opening rate constant (see Table I). This is expected to facilitate the identification of other closed times that are contained in these records and that reflect reaction steps that are not accessible to chemical kinetic investigations. The combined use of the techniques available now allows one to investigate the chemical mechanism of both inhibitory and excitatory receptors in single, specific cells of the nervous system. These mechanisms will allow one to predict the changes in transmembrane voltage brought about by the combined action of the receptors in a single cell. The predictions can be tested by photolyzing a mixture of specific caged neurotransmitters at varying concentrations and measuring the resulting changes in transmembrane voltage. Thus, it should become possible to account for the initiation and alteration of the signal transmission process in single cells, the basic units of the nervous system, in terms of well-characterized chemical reactions.

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Mechanics of Solute Translocation Catalyzed by Enzyme II^{mtl} of the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*†

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ABSTRACT: The kinetics of binding of mannitol to enzyme II^{mtl} embedded in the membrane of vesicles with an inside-out or a right-side-out orientation were analyzed at 4 °C in the absence of the phosphoryl group donor, P-HPr. The binding to the right-side-out oriented vesicles equilibrated too fast to be monitored by the flow dialysis technique. On the other hand, with the inside-out oriented membrane vesicles two conformational changes of the enzyme could be detected kinetically. One change involved a recruitment of binding sites from a state of the enzyme where the binding sites were inaccessible from the cytoplasmic volume. The second change involved a conformational change of the enzyme that followed upon the initial binding to the cytoplasmic-facing binding site leading to a state with a higher affinity for mannitol. Equilibrium binding to the inside-out and right-side-out oriented membrane vesicles at 4 °C indicated that the two transitions did not represent the translocation of the binding site, free and with mannitol bound to it, to the other side of the membrane. Instead, a model is proposed in which the conformational changes represent transitions from states with the binding pocket opened to the cytoplasmic side of the membrane to occluded states of the enzyme in which the binding sites, with or without mannitol bound, are not accessible to either side of the membrane.

Bacterial solute transporters may be classified according to the way energy is provided for the accumulation of the substrate. Accumulation may be achieved through the coupling of the transport to the downhill movement of an ion (H^+ or Na^+) in the secondary transport proteins, to the hydrolysis of ATP in the carriers that use periplasmic binding proteins or the ATPase type carriers, and finally, to the concomitant phosphorylation of the substrate in the case of the P-enolpyruvate-dependent phosphotransferase system. Though these different types of energy coupling to the transport may result in significantly different enzymes, they all have one particular

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