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Chemical kinetic measurements of a mammalian acetylcholine receptor by a fast-reaction technique

(BC$_3$H1 cells/procaine/cell flow)

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ABSTRACT **In the presence of acetylcholine, the nicotinic acetylcholine receptor undergoes two rapid conformational changes: one in the 1-ms time region, leading to the formation of a transmembrane channel and signal transmission between cells, and the other in the 100-ms time region, leading to an inactive "desensitized" form with altered ligand-binding properties. To determine the properties of the receptor that are relevant for channel opening and signal transmission, we have developed a cell-flow technique that allows measurements to be made with cells prior to receptor desensitization. Here we illustrate the usefulness of the technique. A wide concentration range of both a ligand that controls the opening of receptor channels (carbamoylcholine) and a receptor inhibitor (procaine) was used to measure the dissociation constant of the receptor site controlling channel opening (2.4×10^{-4} M), the channel-opening equilibrium constant (5.5), the inhibition constant for procaine (5.8×10^{-5} M), and the rate coefficients for two desensitization processes of 5 s^{-1} and 0.2 s^{-1}. The cell-flow technique illustrated here is of interest because, by rapid-reaction techniques, it extends the chemical kinetic approach from investigations of reactions in solutions to investigations of many different receptors that exist in membranes of central nervous system cells and whose properties are not well known.**

The regulation and control of signal transmission by the nicotinic acetylcholine receptor in muscle cells, and presumably by many other receptor proteins activated by chemical signals, depends ultimately on the concentration of transmembrane channels formed. The chemical reaction leading to the open-channel form is generally formulated as:

$$A + L \overset{K_1}{\rightleftharpoons} \overline{AL}_1 \overset{K_1}{\rightleftharpoons} \overline{AL}_2 \overset{\Phi}{\rightleftharpoons} \overline{AL}_2$$

Scheme I.

A represents the receptor in its active form. L represents acetylcholine (or another activating ligand), and the subscript indicates the number of ligand molecules bound to the receptor molecule. $\overline{AL}_2$ is the open-channel form of the receptor, which mediates the exchange of inorganic ions across the membrane, thus initiating an electrical signal and signal transmission. K_1 is the intrinsic dissociation constant of the ligand, and Φ^{-1} is the channel-opening equilibrium constant (1). Not shown in the scheme are two first-order transitions to inactive (desensitized) receptor forms: a slow process (seconds to minutes) first discovered for the frog muscle receptor (2) and a rapid process (milliseconds) first discovered for the *Electrophorus electricus* receptor (3, 4). The inactive desensitized receptor forms have ligand-binding properties that are different from those of the receptor form primarily involved in signal transmission (refs. 2–4; reviewed in ref. 5). We have shown that rapid mixing techniques commonly used in investigations of reactions in solution (6, 7) also can be used for kinetic investigations of the receptor in membrane vesicles prepared from the electric organ of certain fish (1, 4, 8). Thus, the constants relevant to channel opening and signal transmission could be determined prior to receptor desensitization (1). This approach is restricted, however, to only a few receptors that occur in sufficient abundance in nature to enable preparation of membrane vesicles. Recently a flow technique (9–11) in combination with the whole-cell current recording technique (12, 13) has been used to measure the falling phase of receptor-controlled cell currents, which reflects desensitization of the receptors. We have shown that the observed current rise time in these measurements can be corrected for receptor desensitization and, therefore, that it is possible to determine the concentration of the open-channel form of the receptor prior to desensitization, $[\overline{AL}_2]_O$ (14, 15). From the dependence of $[\overline{AL}_2]_O$ on ligand concentration, the constants pertinent to the channel opening process can be determined (reviewed in ref. 1).

To illustrate the new approach to cell-flow measurements, we have used BC$_3$H1 cells and have determined the effects of both carbamoylcholine (which induces channel opening) and an inhibitor, procaine, on $[\overline{AL}_2]_O$. From the dependence of $[\overline{AL}_2]_O$ on ligand concentration, we determined the values of the dissociation constant of the receptor–ligand complex controlling channel opening, K_1, the channel-opening equilibrium constant, Φ^{-1}, and the dissociation constant, K_P, of procaine. To compare the new chemical kinetic approach with a well-established electrophysiological technique, we show that the cell-flow measurements give results in good agreement with those obtained by the single-channel current recording technique (16).

The new approach to cell-flow measurements is of interest for investigations of the many different types of receptors (*i*) that are present at low concentrations in central nervous system cells (see, for instance, refs. 17–21), (*ii*) that cannot conveniently be incorporated into membrane vesicles, and (*iii*) about which comparatively little is known.

EXPERIMENTAL PROCEDURES

BC$_3$H1, a nicotinic acetylcholine receptor-containing clonal mammalian cell line (22), was cultured as described by Sine and Taylor (23). The cells were maintained in 35-mm dishes in a differentiating medium containing 0.5% fetal calf serum (GIBCO) as described by Sine and Steinbach (24) for 7 days before use.

The whole-cell current recording variant of the patch-clamp technique was used as described (12, 13). A commercially available amplifier (List L/M-EPC7) suitable for the

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current measurements (25) was used. The pipette solution contained 145 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM Hepes (pH 7.4), and the bath solution contained 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 25 mM Hepes (pH 7.4). Pipettes (electrodes) were prepared on a home-made puller and fire-polished; electrode resistance was typically 1–3 MΩ. Cell capacitances were typically 10–15 pF, and the series resistance was typically 1.5–2 times the electrode resistance. To ensure a good voltage-clamp (the voltage drop across the series resistance should be less than 10% of the holding voltage during maximum current flow), series-resistance compensation was used when necessary to cancel up to 90% of the series resistance as described in detail by Sigworth (25). The current amplitudes typically ranged between 1 and 5 nA at a holding voltage (V_m) of −60 mV. The whole-cell current signal from the patch-clamp amplifier was amplified and then digitized at a 200-Hz sampling frequency and then stored on a PDP 11/23 minicomputer for later analysis. The data was then transferred to a Prime 750 computer (Material Science Center, Cornell University) for actual analysis.

The fraction of time the receptor is in the open-channel state, P_O, was determined as described (26, 27). The data from single-channel current measurements were first passed through a low-pass filter (Krohn-Hite 3322) with the cutoff frequency [−3-decibel (dB) point] adjusted so that the average baseline deviation was 1/8th to 1/10th that of the unit amplitude of the single-channel current. After the filtering, the data were digitized, at a sampling frequency (10 or 20 kHz) at least 5 times the cutoff frequency of the low-pass filter, by using a PDP 11/23 minicomputer and were stored on a hard disc. The data then were transferred to a Prime 750 computer for actual analysis. An automated analysis program, based on and modified from the program developed by Sachs *et al.* (28), was used to detect events (the detection threshold was half the unit amplitude of events) and to prepare event amplitude and duration histograms. In the construction of burst-duration histograms, only bursts that met certain criteria were considered (27). Any period of channel activity was considered a valid burst and accepted for analysis only when (*i*) there were no overlapping open events, and (*ii*) it was preceded and followed by silent periods, the duration of which was at least 3 times the mean closed time measured within the burst. The flow method to achieve rapid application of ligand solutions to cells under whole-cell clamp and the technique for changing the composition of the solution emerging from the flow device have been described (9–11).

In our experiments the maximum current produced was corrected for the desensitization process that occurs during the current rise time (14, 15). The correction is based on theories of solution flow over submerged spherical objects (29, 30) and on the observation that many cells, including BC_3H1 cells, become spherical when detached from the dish. At the flow rates used in our experiments, 0.5 $cm \cdot s^{-1}$ to 4 $cm \cdot s^{-1}$, the rate-limiting step in equilibration of the cell surface with ligand is the velocity of a layer of the solution [the diffusion boundary layer, ≈2 μm above the cell surface (30)] that emerges from the flow device and from which the ligand diffuses to the cell surface. The buildup in ligand concentration on the cell surface below this solution layer is rapid (2–5 ms, depending on the flow rates used) (14, 15). Knowing the ligand concentration on the cell surface allows one to correct the observed current during the current rise time for receptor desensitization (14, 15), which is characterized by the rate coefficient α and can be measured independently in each experiment (10, 11). The corrected current, I_A, is defined as the amplitude of the current arising from receptors on the cell surface in absence of receptor desensitization and at a definite ligand concentration (14, 15).

To obtain the value of I_A from measurements of the observed current, I_{obs}, we divide the current time course into constant (5 ms) time intervals to take into account the equilibration time of small segments of the cell surface with ligand. The current is then corrected for the desensitization occurring during each time interval Δt. After n constant time intervals ($n\Delta t = t_n$), during each of which the current, $(I_{obs})_{\Delta t}$, is measured, the corrected current is given by (14, 15):

$$I_A = (e^{\alpha\Delta t}-1) \sum_{i=1}^{n} (I_{obs})\Delta t_i + (I_{obs})\Delta t_n. \quad [1]$$

$(I_{obs})\Delta t_i$ is the observed current during the i^{th} time interval, and t_n is equal to or greater than the current rise time (14, 15). The value of I_A was found to be independent of the solution velocities used in the cell flow method and could be determined with good precision (±10%) (14, 15).

RESULTS

The relationship between I_A and the concentration of receptors in the open-channel form in the absence of receptor desensitization is given by:

$$I_A = I_M R_M (\overline{AL_2})_O. \quad [2]$$

I_M represents the current produced by 1 mol of open receptor channels, R_M represents the moles of receptors on the cell surface, and $(\overline{AL_2})_O$ represents the fraction of receptors present that are in the open-channel form. In terms of Scheme I, $(\overline{AL_2})_O$ is given (31) by the following equation:

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

Fig. 1*a* shows the dependence of I_A ($\propto [\overline{AL_2}]_O$) on carbamoylcholine concentration over a 500-fold range. The circles in Fig. 1*a* show the dependence of $(\overline{AL_2})_O$ on the carbamoylcholine concentration when determined by an entirely different approach and methodology. The probability P_O that the channel is open while the receptor is in a nondesensitized active state (27, 32) was determined at three carbamoylcholine concentrations. The P_O values were obtained from single-channel current measurements (16) and represent the fraction of time the channel is open while the receptor is in a nondesensitized state (Fig. 2). P_O corresponds to $(\overline{AL_2})_O$, and its dependence on acetylcholine and carbamoylcholine concentration at the frog neuromuscular junction has been determined (33, 34).

In our experiments with 500 μM carbamoylcholine and a membrane potential of −60 mV, a P_O value of 0.75 was obtained. This value for P_O, together with with the value of I_A (3.8 nA) obtained from the cell-flow experiments, allowed us to calculate a value of 5.1 nA for $I_M R_M$ by using Eq. **1** and equating P_O with $(\overline{AL_2})_O$. The value for $I_M R_M$, together with the P_O values obtained at two other carbamoylcholine concentrations (100 μM and 200 μM), allows the determination of I_A at these concentrations (Eq. **2**). The I_A values calculated from P_O values are shown in Fig. 1*a* as open circles and there is good agreement between the values of I_A measured in the cell-flow experiments and those calculated from P_O values. The maximum value of P_O obtained in our experiments is in good agreement with the P_O value obtained at saturating carbamoylcholine concentrations with BC_3H1 cells by Steinbach *et al.* (35). The agreement between results obtained by the cell-flow method and the single-channel current recording technique shown in Fig. 1*a* confirms the validity of the analysis of the flow measurements. Fig. 1*b* shows the dependence of the inactivation rate coefficient α over a 500-fold range of carbamoylcholine concentration. In Fig. 1*c*, the data in Fig. 1*a* are replotted according to Eq. **4**. This

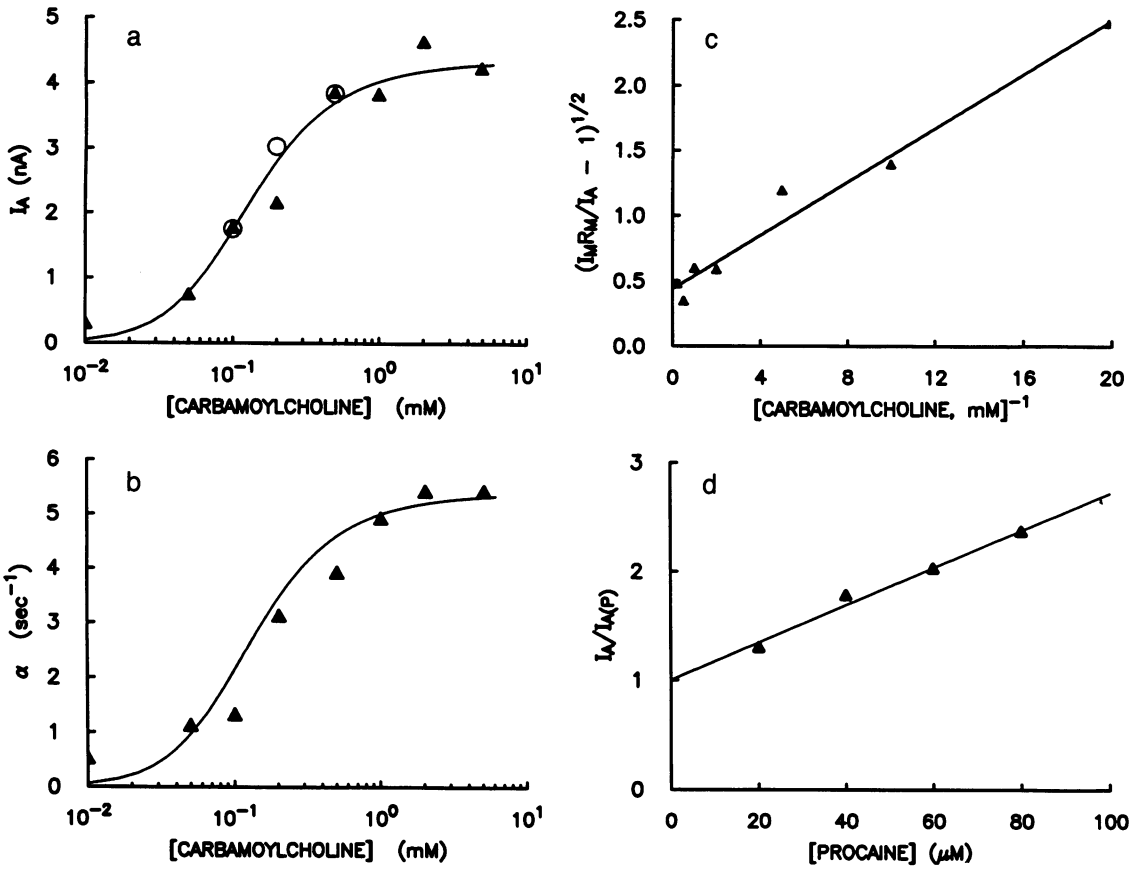

FIG. 1. Effect of carbamoylcholine concentration on I_A and α. Carbamoylcholine-induced whole-cell currents through the acetylcholine receptors of BC_3H1 cells were measured at 23°C and −60 mV. (*a*) The value for I_A, the current amplitude in the absence of desensitization, is plotted against carbamoylcholine concentration. The results represent measurements on 10 cells. Measurements on one cell were normalized to measurements on another cell as described in the text. The solid symbols represent the normalized I_A values. The open circles represent I_A values that have been calculated from P_O values at three carbamoylcholine concentrations. The solid line through the data points was drawn by using Eqs. 1, 2, and 3 and the values for K_1, Φ^{-1}, and I_MR_M of 2.4×10^{-4} M, 5.6, and 5.1 nA, respectively, obtained from the linear fit of the data in *c*. (*b*) The value for α, the rate coefficient for receptor desensitization, at each carbamoylcholine concentration was evaluated from the decay phase of the current as described in the text. The solid line through the data points was drawn by using Eq. 5; the values for Φ and K_1 were those used in fitting the I_A curve in *a*; $k_{34} = 35\ s^{-1}$ was also used. (*c*) Linear fits of I_A data. The data in *a* was replotted according to Eq. 4. A value of 5.1 nA for I_MR_M was used. From the slope and intercept of the line fitted through the data points, values of 2.4×10^{-4} M for K_1 and 5.5 for Φ^{-1} were obtained. (*d*) Inhibition of acetylcholine-induced currents by procaine. The whole-cell currents in BC_3H1 cells were measured at 23°C and −60 mV. The ratio of the current amplitudes in the absence (I_A) and presence [$I_{A(P)}$] of procaine is plotted against procaine concentration. The acetylcholine concentration (5 μM) was not varied. The solid line is a linear fit through the data points and satisfies Eq. 6. The slope of the line gives a value for K_P of 5.8×10^{-5} M.

equation is obtained by substitution of Eq. 3 into Eq. 2 and linearization to allow evaluation of Φ and K_1:

$$(I_MR_MI_A^{-1} - 1)^{1/2} = \Phi^{1/2} + \Phi^{1/2}K_1[L]^{-1}. \quad [4]$$

From the slope of the line in Fig. 1*c*, a value for K_1 of 2.4×10^{-4} M was calculated; from the ordinate intercept, a value for the channel-opening equilibrium constant, Φ^{-1}, of 5.5 was calculated. The solid line through the data points in Fig. 1*a* (concentration-dependence of I_A) was drawn by using the values for I_AR_M, K_1, and Φ. The solid line through the data points in Fig. 1*b* (concentration dependence of α) was drawn by using the following equation (36):

$$\alpha = k_{34}\Phi L^2[L^2(1 + \Phi) + 2K_1L\Phi + K_1^2\Phi]^{-1} \quad [5]$$

and the values of Φ and K_1 determined from the data in Fig. 1*c*. In Eq. 5, k_{34} represents the dominant rate constant in the rapid desensitization of the receptor (36). The data in Fig. 1 *a* and *b* indicate that the same K_1 and Φ values account for the concentration dependence of both I_A and α. This agreement also supports the validity of the method used to calculate I_A because the values of α are taken directly from the observed current measurements. The values of K_1 obtained in the cell-flow measurements are higher than the values obtained in ion-flux measurements with BC_3H1 cells (37, 38). In the cell-flow measurements, a rapid (Fig. 1*b*) and a slow desensitization process are observed; in ion-flux measurements in cells, only the slow process is seen (37, 38). The desensitization rates given in Fig. 1*b* indicate that, in ion-flux measurements on cells, a considerable amount of receptor desensitization occurs prior to the first ion-flux measurements, which are made several seconds after mixing cells with carbamoylcholine and tracer ions. The difference in the K_1 values observed by cell-flow and by ion-flux measurements in cells are characteristic of the difference between nondesensitized and desensitized electroplax receptors (reviewed in refs. 1 and 5).

To illustrate another use of the cell-flow technique, we show that measurements of I_A in the presence of inhibitors allow one to determine the dissociation constant of the inhibitor binding to the receptor prior to desensitization. In the presence of a noncompetitive inhibitor, procaine, (39):

$$[\overline{AL_2}]_O/[\overline{AL_2}]_{O(P)} = 1 + [P]/K_P, \quad [6]$$

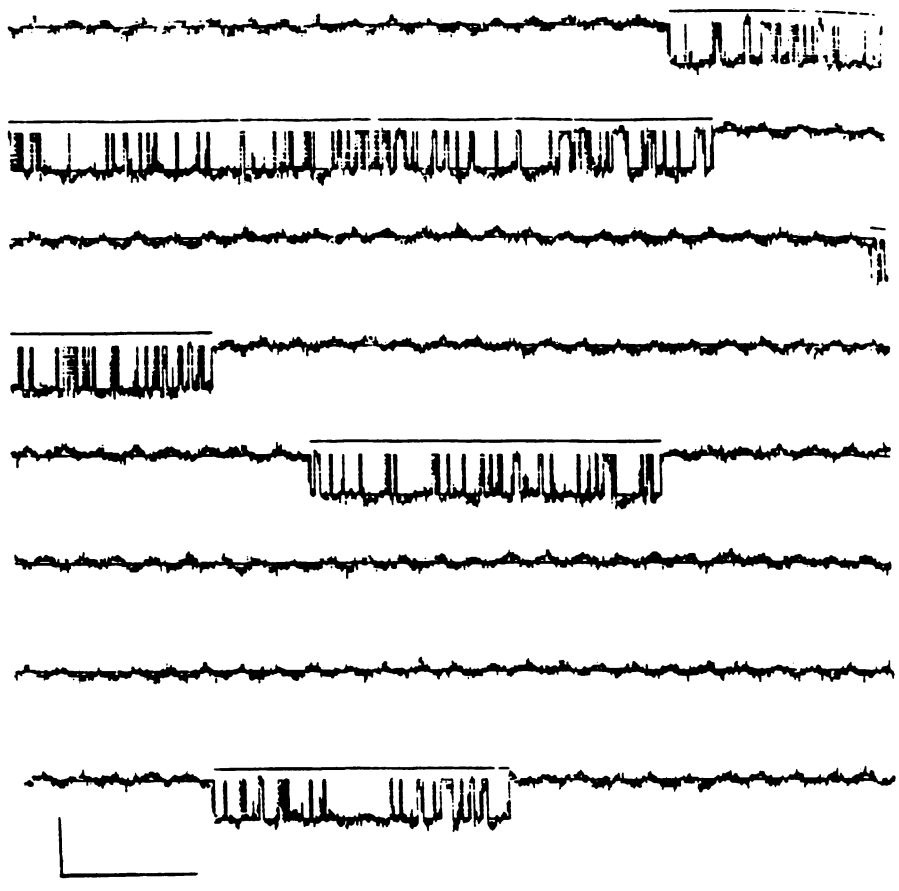

FIG. 2. Determination of P_O from single-channel current measurements. An example of a single-channel current recording of acetylcholine receptors on BC_3H1 cells that have been activated by 200 μM carbamoylcholine is shown. The recording was made at 23°C and at a membrane potential of −60 mV. Four bursts of channel activity are shown; a line has been drawn over each burst to denote the duration of the burst. Idealized data have been superimposed on the channel openings for identification purposes. A computer program was used to determine the duration of each burst and the sum of the durations of all channel openings in the burst. The procedure is repeated over many bursts to obtain the average fraction of time during a burst that the receptor channel is open. This is equal to P_O, the conditional probability that the channel is open when the receptor is in a nondesensitized state. The horizontal and vertical calibration bars indicate 50 msec and 5 pA, respectively.

where [P] represents the procaine concentration and the subscript P indicates that the measurements were carried out in the presence of procaine. Substituting I_A for $[\overline{AL_2}]_O$, we have

$$I_A/I_{A(P)} = 1 + [P]/K_P, \quad [7]$$

where K_P represents the receptor–procaine dissociation constant. In the experiment shown in Fig. 1*d*, I_A and $I_{A(P)}$ were determined at constant acetylcholine and various procaine concentrations and were plotted according to Eq. 7. From the slope of the linear fit of the data, a K_P value of 5.8×10^{-5}M at a transmembrane voltage of −60 mV and 23°C was obtained. Quench flow measurements at 1°C using membrane vesicles and in the absence of a transmembrane voltage gave K_P values of 1.0×10^{-4} M and 8.5×10^{-5} M for the *E. electricus* and *Torpedo californica* receptors, respectively (39–41).

DISCUSSION

Several methods have been used recently in which cells can be used to measure the effect of ligand concentration on $[\overline{AL_2}]_O$ in cells. The discovery of bursts of channel activity in single-channel current measurements (42, 43) permits evaluation of P_O and, therefore, also of $(\overline{AL_2})_O$ while the receptor is in a nondesensitized state (27, 32). Another approach (44) uses a flow technique in combination with the single-channel current recording technique (16). In both of these approaches, only a few receptors are sampled in the measurements and, therefore, require extensive data collection and analysis (26, 44). In contrast, the chemical kinetic approach takes into consideration the theory of solution flow over submerged objects (29, 30) and reflects the properties of a large number of receptors. The theory and the experimental results indicate that, at moderate rates of flow of solutions over a relatively large cell surface (typically over 10^3 μm^2), the equilibration of ligand with the cell surface is rapid compared to receptor desensitization (14, 15). Therefore, it is possible to determine the concentration of receptor sites in the open-channel form in the absence of desensitization with good precision (±10%). Because of the flow rates and precision of measurements, it is possible to make measurements with many different ligand concentrations using the same cell. Only a few cells were required to obtain the results shown in Fig. 1 in which we show the effect of ligand concentration on I_A. The applicability of the theory and the validity of the approach described have been tested by: (*i*) observing that, at the different solution-flow rates used in the experiments, the same calculated value of I_A is obtained; (*ii*) showing that similar results are obtained whether $(\overline{AL_2})_O$ is measured by determining I_A in the cell-flow method or by using the single-channel current recording technique; and (*iii*) showing that I_A [which is calculated from the observed current values (I_{obs})] and α [in which (I_{obs}) values are used directly] have the same dependence on carbamoylcholine concentration as has been found in intensive investigations of the electroplax receptors (1).

By using the approach described, investigations of the receptor of a mammalian muscle (BC_3H1) cell line show that the values of the constants measured are sufficiently similar to the results obtained with electroplax receptors (1, 5, 36) to confirm the common assumption that the electroplax receptor is a good model for muscle receptors in general.

The chemical kinetic approach using fast-reaction techniques described here has previously brought considerable insight into the mechanism by which proteins function in solution (45, 46). What we consider most important in the experiments presented is that they indicate that the same approach also can be used in investigations of receptor function in cell membranes. Thus, the scope of chemical kinetic investigations is extended to many different types of receptors that exist in central nervous system cells and whose properties are not well known.

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