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An Acetylcholine Receptor Regulatory Site in BC_3H1 Cells: Characterized by Laser-Pulse Photolysis in the Microsecond-to-Millisecond Time Region†

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ABSTRACT: When a neurotransmitter binds to its specific receptor, the protein forms transmembrane channels through which ions flow, leading to changes in transmembrane voltage that trigger signal transmission between neurons. How do inhibitors affect this process? Interesting and extensive information comes from investigations of the acetylcholine receptor, the best known of these proteins. This receptor is inhibited by cationic inhibitors, including local anesthetics, and acetylcholine at high concentrations. The accepted mechanism, elegant in its simplicity, is that these compounds enter the receptor–channel after it opens and block inorganic ion flux. This mechanism requires that the inhibitors affect only the apparent rate constant for channel closing (k'_{cl}). An alternative mechanism invokes a specific regulatory (inhibitory) site to which inhibitors bind before the channel opens and the signal is transmitted. This mechanism requires that the inhibitors affect the apparent rate constants for both channel opening (k'_{op}) and closing. The effect of inhibitors on k'_{op} has not been determined previously. This report describes the use of a newly developed laser-pulse photolysis technique with a dead time of approximately 120 μs to determine the effect of a local anesthetic, procaine, one of the best studied cationic inhibitors of the acetylcholine receptor, on both k'_{op} and k'_{cl}. Both k'_{op} and k'_{cl} were found to decrease with increasing procaine concentration. This effect of the inhibitor of k'_{op} cannot be explained by the open-channel-blocking mechanism but is consistent with the existence of a regulatory (inhibitory) receptor site.

The integrated functioning of the nervous system depends on specific and rapid transmission of signals between constituent cells. The nicotinic acetylcholine receptor is the best known member of a group of membrane-bound proteins responsible for such transmission (Galzi et al., 1991; Karlin, 1991). For transmission to occur, a specific neurotransmitter, in this case acetylcholine, must bind to the receptor, which then forms a transmembrane channel. The resulting flux of inorganic cations through the channels leads to changes in transmembrane voltage that trigger signal transmission. How is this process regulated? The results of many electrophysiological and chemical labeling experiments led to the suggestion that organic cations, including clinically important compounds such as local anesthetics, abused drugs such as cocaine, and acetylcholine at high concentrations, enter the receptor–channel after it has opened and then block it (Adams, 1976; Neher & Steinbach, 1978; Ogden & Colquhoun, 1985; Galzi et al., 1991; Karlin, 1991). Deviations from this simple model were suggested on the basis of electrophysiological experiments (Adams, 1977; Tiedt et al., 1979; Neher, 1983; Gage & Wachtel, 1984; Papke & Oswald, 1989) and chemical kinetic measurements with a 5-ms time resolution (Shiono et al., 1984; Karpen & Hess, 1986). The latter measurements

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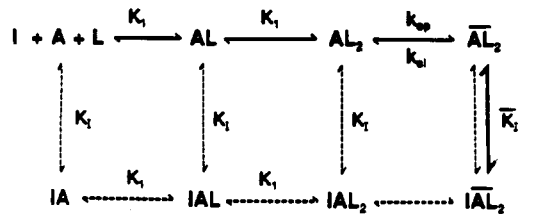

FIGURE 1: Alternative mechanisms for inhibition of the acetylcholine receptor. "L" represents the channel-activating ligand. "A" represents the active, nondesensitized receptor forms, K_1 the dissociation constant for the receptor:ligand complex, and $\overline{AL}_2$ the open-channel form of the receptor. I represents the inhibitor, and K_I and $\bar{K}_I$ the dissociation constants of the receptor–inhibitor complexes of the closed- and open-channel forms of the receptor. For other explanations see the text.

suggested that the inhibitors bind to a regulatory site on the receptor before the channel opens. Knowledge of the inhibition mechanism is needed for (i) understanding the regulation of signal transmission in the nervous system and (ii) ongoing structural studies, using chemical labels or cDNA technology, designed to delineate the receptor sites important in inhibition (Galzi et al., 1991).

The upper line in Figure 1 shows the commonly accepted mechanism for activation of the acetylcholine receptor–channel. An activating ligand (L) binds to the active, nondesensitized receptor A. The binding of two ligand molecules is followed by the formation of the open receptor–channel $\overline{AL}_2$, characterized by the rate constants k_{op} and k_{cl}. The solid lines indicate the generally accepted mechanism for inhibition: an inhibitor I binds only to the open-channel form to give $\overline{IAL}_2$ (mechanism I, channel blocking). The dashed lines indicate a noncompetitive mechanism in which an inhibitor binds to a regulatory site before the channel opens (mechanism II, regulatory site). The two mechanisms make the same predictions regarding the effect of inhibitors on k'_{cl} but entirely different predictions regarding the effect of inhibitors on k'_{op}. (For definitions of k'_{cl} and k'_{op}, see eq 1e in the legend to Figure 2). In the channel-blocking mechanism, only k'_{cl} will be affected (eq 1c in Figure 2 legend). In mechanism II, in which the inhibitor binds to the receptor before and after the channel has opened, both k'_{op} and k'_{cl} will be affected (eq 1d, Figure 2 legend).

Chemical kinetic techniques for determining the rate constant for channel opening (k_{op}), a process which occurs in the submillisecond time region, were not available; many different values for k_{op} have been reported using electrophysiological methods (Madsen & Edeson, 1988). The effect of the inhibitors on k'_{op} is unknown. Recently, we developed a laser-pulse photolysis technique, for use with single cells (Milburn et al., 1989; Matsubara et al., 1992). This technique allows measurements of the channel-opening process to be made in the microsecond-to-millisecond time region. For this we synthesized (Milburn et al., 1989) an inactive precursor of carbamoylcholine (caged carbamoylcholine). Photolysis of this compound to carbamoylcholine with a $t_{1/2}$ value of 40 μs combined with the whole-cell current-recording technique (Hamill et al., 1981) allowed us to measure the rate constants for the opening (k_{op}) and closing (k_{cl}) of the acetylcholine receptor–channel in BC_3H1 muscle cells. The value of k_{op} (9400 s^{-1}) was found to be about 16 times larger than that of k_{cl} (580 s^{-1}) (Matsubara et al., 1992). It may, therefore, be possible to measure separately the effects of inhibitor concentration on k'_{op} and k'_{cl}. At low carbamoylcholine concentration k'_{cl} is expected to dominate the observed rate of channel opening, whereas at high carbamoylcholine concentration k'_{op} is expected to dominate (eq 1d, Figure 2 legend). This report deals with the question of whether procaine binds to the receptor before the channel opens, as determined by its effect on the channel-opening rate constant, measured by the laser-pulse photolysis technique (Matsubara et al., 1992).

MATERIALS AND METHODS

A mammalian clonal cell line, BC_3H1 (Shubert et al., 1974), was cultured as described (Sine & Taylor, 1979).

The cell-flow whole-cell recording technique has been described (Udgaonkar & Hess, 1987; Hess et al., 1987). The pipette solution contained 145 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES (pH 7.4). The extracellular 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).

Caged carbamoylcholine (*N*-[(α-2-carboxy)-2-nitrobenzyl]carbamoylcholine) was synthesized as described (Milburn et al., 1989). A Candela SLL 500 flash-lamp-pumped dye laser, with oxazine 720 perchlorate (Exciton) as the laser dye, was used as described (Matsubara et al., 1992). The pulse length was 600 ns. The laser was tuned at 343 nm by the secondary harmonic generator. The laser-pulse output from the optical fiber was adjusted to be 200–400 μJ. The whole-cell current generated by the photorelease of carbamoylcholine was detected by a commercially available amplifier (List L/M-EP7).

The cell-flow technique (Udgaonkar & Hess, 1987) was used with a known concentration of carbamoylcholine before each laser pulse to calibrate the concentration of carbamoylcholine liberated in the photolysis experiment. Damage to the cell and/or receptor was monitored by comparing the current amplitudes in successive experiments with the same cell, using either laser-pulse photolysis and known concentrations of caged carbamoylcholine or the cell-flow method and known concentrations of carbamoylcholine. The resistance of the electrode when filled with 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 ($\pm$6 mV) (Sigworth, 1983). Data were low-pass-filtered (Krohn-Hite 3322) with a 1–20-kHz cutoff frequency (–3-dB point) and then digitized at a 2–63-kHz sampling frequency. The rate constants for the rising phase of the whole-cell current were evaluated using the LOTUS program.

The cells were equilibrated with caged carbamoylcholine alone, or with caged carbamoylcholine and procaine, before measurement of the channel-opening rate. The observed rate coefficient and the current amplitudes at a constant procaine concentrations but different preincubation times, between 1 s and 1 min, were found to be the same within experimental error. Therefore, for all experiments the cells were equilibrated for 1 s with caged carbamoylcholine with or without procaine.

RESULTS

Figure 2A shows the current produced by the opening of acetylcholine receptor–channels in BC_3H1 cells when 115 μM carbamoylcholine was released from caged carbamoylcholine by laser-pulse photolysis. The measurements were made in the absence and presence of procaine. In the experiments, the increase in current follows a single-exponential rate equation for over 85% of the reaction (Matsubara et al., 1992)

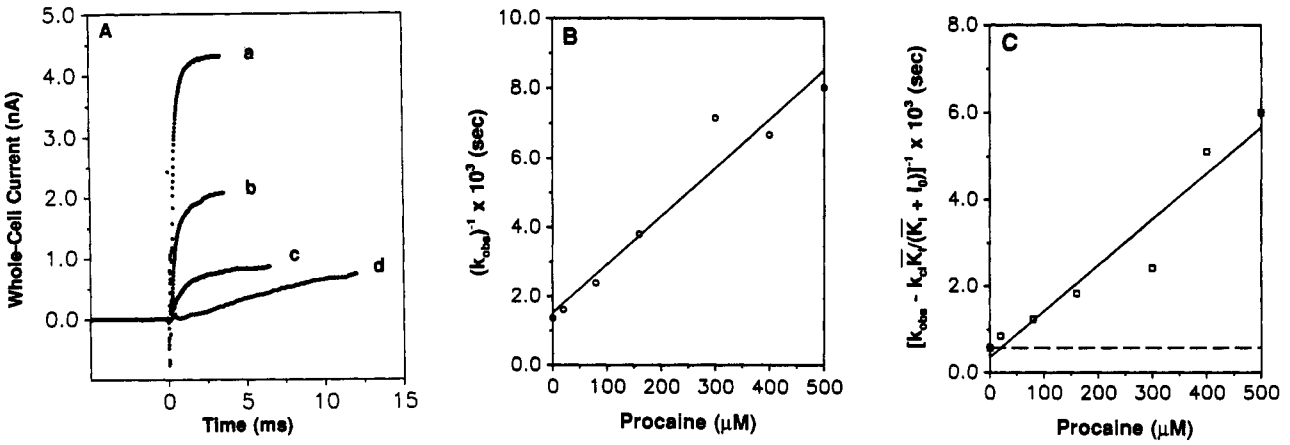

FIGURE 2: Laser-pulse photolysis of caged carbamoylcholine with BC_3H1 cells at pH 7.4, 22 °C, and −60 mV. In panels B and C each data point is the mean value of three measurements made with each of three cells. (A) Whole-cell currents generated by 115 μM released carbamoylcholine in the absence (a) and presence of (b) 160, (c) 300, and (d) 500 μM procaine. The observed first-order rate constants and maximum amplitudes of the whole-cell current for a, b, c, and d, are 2500, 1100, 610, and 200 s^{-1} and 4.40, 2.80, 0.865, and 0.730 nA, respectively. In all experiments, the cell was exposed to procaine for 1 s before the measurements were made. The current spike below the base line is an instrumentation artifact. The observed rate constant for the rise time of the current was evaluated under conditions where L, I, $\gg R_0$, the molar concentration of receptors, and the ligand binding steps are fast compared to the channel opening steps (Matsubara et al., 1992).

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

$(\overline{AL_2})$ represents the concentration of open channels at time t and at equilibrium t_∞.

$$k_{obs} = k_{cl} + k_{op}\left(\frac{L}{L + K_I}\right)^2 \tag{1b}$$

For the channel-blocking mechanism (mechanism I):

$$k_{obs} = k_{cl}\left(\frac{\bar{K}_I}{\bar{K}_I + I_o}\right) + k_{op}\left(\frac{L}{L + K_I}\right)^2 \tag{1c}$$

I_o represents the molar concentration of procaine. For the mechanism in which inhibitor binds before the channel opens, as well as to the open-channel form (regulatory site, mechanism II):

$$k_{obs} = k_{cl}\left(\frac{\bar{K}_I}{\bar{K}_I + I_o}\right) + k_{op}\left(\frac{L}{L + K_I}\right)^2\left(\frac{K_I}{K_I + I_o}\right) \tag{1d}$$

(i) For mechanisms I and II:

$$k'_{cl} = k_{cl}\frac{\bar{K}_I}{\bar{K}_I + I_o}$$

(ii) For mechanism I:

$$k'_{op} = k_{op}\left(\frac{L}{L + K_I}\right)^2$$

(iii) For mechanism II:

$$k'_{op} = k_{op}\left(\frac{L}{L + K_I}\right)^2\frac{K_I}{K_I + I_o} \tag{1e}$$

(B) $(k_{obs})^{-1}$ as a function of procaine concentration in the presence of 20 μM released carbamoylcholine. When $k_{obs} \approx k_{cl}$, rearrangement of eq 1c gives

$$\frac{1}{k_{obs}} = \frac{1}{k_{cl}} + \frac{1}{k_{cl}}\frac{I_o}{\bar{K}_I} \tag{2a}$$

A linear least-square fitting program was used to evaluate k_{cl} (670 ± 300 s^{-1}) and $\bar{K}_I$ (110 ± 50 μM). (C) $(k_{(obs)} - k'_{cl})^{-1}$ as a function of procaine concentration in the presence of 115 μM released carbamoylcholine. k'_{cl} is defined by eq 1d(i). (i) Rearrangement of eq 1c (mechanism I) gives

$$(k_{obs} - k'_{cl})^{-1} = \{k_{op}[L/(L + K_I)]^2\}^{-1} \tag{2b}$$

(ii) Rearrangement of eq 1d (mechanism II) gives

$$(k_{obs} - k'_{cl})^{-1} = [k_{op}L/(L + K_I)^2]^{-1}(1 + I_o/K_I)$$

The value of k'_{cl} is obtained from the data in panel B. A linear least-squares fitting program was used to evaluate K_I (40 ± 60 μM) and $k_{op}(L/(L + K_I)^2 = 2800 \pm 4800$ s^{-1}. The dashed line indicates the results expected for the channel-blocking mechanism, in which k'_{op} (eq 1c) is not affected by procaine concentration. The measurement of k_{op} and k_{cl} by the laser-pulse photolysis technique has been described (Matsubara et al., 1992).

(eq 1a, Figure 2 legend). The upper curve is a control; the lower curves were obtained when 160, 300, or 500 μM procaine was also present. The kinetic constants evaluated in such experiments (Figure 2A) are plotted as a function of procaine

concentration in Figure 2, panels B and C. In Figure 2B, the k_{obs} value for the current rise in the presence of 20 μM released carbamoylcholine is plotted according to eq 2a (Figure 2 legend). At this concentration k_{obs} reflects k_{cl}. As can be seen, k_{obs} decreases as the concentration of procaine is increased. An apparent dissociation constant for procaine of 110 μM is obtained from the slope of the line. Figure 2C represents a similar experiment, except that the concentration of released carbamoylcholine was 115 μM. Under these conditions, $k_{op}[L/(L + K_I)]^2$ is three times larger than k_{cl} (eq 1b, Figure 2 legend) and dominates the k_{obs} value for the current rise time. A plot of $k_{obs} - k'_{cl}$ as a function of procaine concentration is expected to be independent of procaine concentration for the channel-blocking mechanism in which the rate for channel opening is expected to be unaffected by the presence of procaine (eq 2b–i, Figure 2 legend). The dashed line in Figure 2C shows the results expected for the channel-blocking mechanism. However, it can be seen in Figure 2C that $k_{obs} - k'_{cl}$ decreases as the procaine concentration is increased. This result is expected (eq 2b–ii, Figure 2 legend) if the inhibitor binds to the receptor both before and after the channel has opened. A value for K_I of 40 μM is calculated from the slope of the line.

A control experiment is illustrated in Figure 3. The ratios of the maximum current amplitude obtained in the absence (A) and presence [$A(I_o)$] of procaine are plotted versus procaine concentration according to eq 3a (Figure 3 legend). The experiments were performed at a constant carbamoylcholine concentration of first 20 μM and then 115 μM; both the laser-pulse photolysis and the cell-flow technique (with a 5-ms time resolution) were used to determine the current amplitudes. The apparent K_I value for procaine, calculated from the slope of the line, is 88 μM. The dashed line shows the results expected if the inhibitor binds only in the open channel and then blocks it. The slope of the dashed line is determined by using the K_I value that would be observed at 115 μM carbamoylcholine if the inhibitor binds only in the open channel. This K_I value is calculated from the K_I value observed at 20 μM carbamoylcholine and eq 3b (Figure 3 legend). The value of $(\overline{AL_2})_o$, the fraction of receptors in the open-channel form (eq 3b) is calculated using eq 3c (Figure 3 legend) and the constants K_I and Φ (Figure 1) determined for the acetylcholine receptor in BC_3H1 cells (Udgaonkar & Hess, 1987; Matsubara et al., 1992).

The results obtained by the laser-pulse photolysis and cell-flow techniques are identical within experimental error (Figure 3). This indicates that neither the laser pulse nor the photolysis products other than carbamoylcholine affects the kinetic measurements. Further control experiments have been done to establish this point. Caged carbamoylcholine by itself does not induce the opening of receptor–channels, desensitize the receptor, or act as an inhibitor of the acetylcholine receptor in BC_3H1 cells (Milburn et al., 1989). We used reversed-phase HPLC chromatography to show that (i) the same amount of carbamoylcholine is released from 750 μM caged carbamoylcholine in the presence and absence of 500 μM procaine and (ii) procaine is not converted to detectable side products when subjected to laser pulses in presence and absence of caged carbamoylcholine. After 1 mM caged carbamoylcholine was photolyzed ($\sim$13%), we separated free carbamoylcholine from the reaction mixture, using HPLC, and incubated the remaining compounds with a cell for 1 min. The receptor activity was then determined in cell-flow experiments using 100 μM carbamoylcholine; the products had no detectable effects.

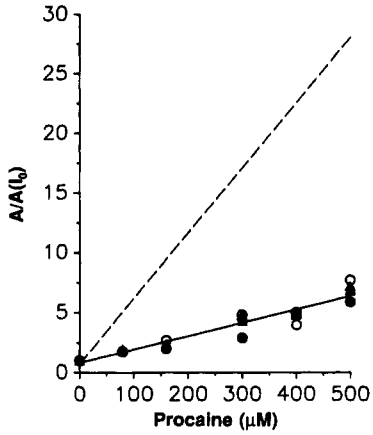

FIGURE 3: Ratio of current amplitudes in the absence, A, and presence, $A(I_o)$, of procaine. The circles and triangles represent measurements made by the laser-pulse photolysis and cell-flow techniques, respectively (Matsubara et al., 1992; Udgaonkar & Hess, 1987; Hess et al., 1987) at –60 mV, 22 °C, pH 7.4. The open and filled symbols represent experiments in the presence of 20 μM and 115 μM released carbamoylcholine, respectively. Each data point is the mean value of three measurements made with each of three cells. The data are plotted according to the equations below (Karpen & Hess, 1986). For mechanism II, the inhibitor binding to a regulatory site,

$$\frac{A}{A(I_o)} = 1 + \frac{I_o}{K_I} \tag{3a}$$

The solid line is a best fit of the data and yields a K_I value of 88 ± 10 μM. The dashed line is a simulation of the channel-blocking mechanism according to eq 3b at a carbamoylcholine concentration of 115 μM and with a K_I value for carbamoylcholine of 240 μM (Udgaonkar & Hess, 1987). For the channel-blocking mechanism

$$\frac{A}{A(I_o)} = 1 + I_o\frac{(\overline{AL_2})_o}{K_I} \tag{3b}$$

where $(\overline{AL_2})_o$ represents the fraction of receptors in the open channel form (Hess et al., 1983):

$$(\overline{AL_2})_o = \frac{\overline{AL_2}}{A + AL + AL_2 + AL_2} = \frac{L^2}{L^2(1 + \Phi) + 2K_IL\Phi + K_I^2\Phi} \tag{3c}$$

DISCUSSION

The effect of procaine on k'_{cl} was determined by laser-pulse photolysis (Figure 2B) and is in agreement with measurements of the effect of cationic inhibitors on the lifetime of the open acetylcholine receptor–channel, a measure of k'_{cl} (Neher & Steinbach, 1978; Neher, 1983; Papke & Oswald, 1989). The apparent K_I values we measured for procaine are within the range of K_I values reported previously (Adams, 1977; Gage et al., 1983, 1984; Karpen & Hess, 1986; Udgaonkar & Hess, 1987; Forman & Miller, 1989). The inhibition constant determined by measuring the effect of inhibitor on the lifetime of the open channel or on k'_{cl} may reflect the binding of the inhibitor to the regulatory site, to a site within the open channel, or a combination of both. The inhibitor may also bind with different affinities to the closed- and open-channel forms. Additional information is obtained from measurements of the effect of inhibitor concentration on k'_{op} (Figure 2C) and the current amplitude (Figure 3). The laser-pulse photolysis experiments show that (i) the apparent rate constant for channel opening, k'_{op}, is decreased in the presence of procaine (Figure 2C) and (ii) the observed K_I value for procaine does not decrease with increasing carbamoylcholine concentration, as determined from the maximum current amplitudes obtained

in either cell-flow or laser-pulse photolysis experiments (Figure 3). These results are inconsistent with the inhibitor binding only in the receptor channel after it has opened (mechanism I, channel blocking). The results are, however, consistent with the inhibitor binding prior to channel opening and the existence of a regulatory (inhibitory) site on the acetylcholine receptor. Thus, inhibitors like procaine can bind to this site before the channel opens and regulate the ion permeability of the receptor. Anesthetics and other cationic inhibitors of the acetylcholine receptor (including acetylcholine at high concentrations) vary widely in structure; whether they all bind to a regulatory (inhibitory) site of the receptor is not yet known.

This and previous experiments suggest [reviewed in Hess (1993)] that the rapid chemical kinetic techniques that are essential in elucidating mechanisms of reactions mediated by soluble proteins (Hammes, 1982; Fersht, 1985; Johnson, 1992) may be equally important in elucidating mechanisms mediated by proteins that must be studied in a membrane-bound form in a cell. A number of methods that can be used in such studies are now available (Udgaonkar & Hess, 1987; Matsubara et al., 1992; Hess, 1993).

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