On the Mechanism of Inhibition of the Nicotinic Acetylcholine Receptor by the Anticonvulsant MK-801 Investigated by Laser-Pulse Photolysis in the Microsecond-to-Millisecond Time Region†

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ABSTRACT: The mechanism of inhibition of the muscle nicotinic acetylcholine receptor is of interest because of the many drugs which are known to modify its function. The laser-pulse photolysis technique, using a photolabile, biologically inert ligand (caged carbamoylcholine) for the nicotinic acetylcholine receptor, and BC_3H1 cells have been used to investigate the mechanism of inhibition of the receptor by MK-801 [(+)-dizocilpine] in the microsecond-to-millisecond time region. MK-801 is an anticonvulsant and a known inhibitor of the *N*-methyl-D-aspartate and nicotinic acetylcholine receptors. Both the chemical kinetic and the single-channel current-recording measurements reported here indicate the existence of two inhibition processes, one occurring within 50 ms and the other within about 1 s of equilibration of the receptor with the inhibitor. Unless stated otherwise, here we characterize the receptor inhibition observed when MK-801 is equilibrated with the receptor for only 50 ms. We determined the effect of MK-801 on the concentration of the open receptor-channels and the apparent dissociation constant of the inhibitor from the closed-channel ($K_{I(obs)}$ = 180 μM) and open-channel ($\overline{K_{I(obs)}}$ = 950 μM) forms. Within a few milliseconds after inhibitor binding, $\overline{K_{I(obs)}}$ decreases to about 100 μM, due to an inhibitor-induced isomerization to an inactive receptor form. A mechanism that incorporates the new results is proposed. It includes the formation of an ion-conducting receptor:inhibitor complex with a channel-opening equilibrium constant that is unfavorable compared to the open-channel receptor form in the absence of inhibitor. In the MK-801 concentration range of 0−500 μM, this mechanism accounts for the observed MK-801-induced decrease in the concentration of open channels. At high concentrations of carbamoylcholine, when the receptor is mainly in the open-channel form, the conducting receptor:inhibitor complex isomerizes to a nonconducting state with a rate constant of about 2400 s^{-1} for the forward reaction and 230 s^{-1} for the back reaction. It is shown that the proposed new mechanism, based on transient kinetic measurements, also accounts for the results of previous investigations with other inhibitors (procaine, cocaine), which were carried out under both pre-steady-state and equilibrium conditions. A compound that binds to the same regulatory site on the receptor as MK-801 but does not affect the channel-opening equilibrium constant may have considerable use in protecting an organism from the effects of abused drugs.

Ligand-gated ion channels are inhibited by a variety of chemical substances, for example, natural toxins, local anesthetics, abused drugs (e.g., cocaine), clinically important compounds (e.g., tranquilizers), and metal ions (ref *1*; reviewed in refs *2*, *3*). The mechanism of inhibition of the nicotinic acetylcholine receptor (nACh receptor) has been intensively investigated, and evidence for more than one inhibitory site has been reported (reviewed in refs *3*−*5*). A proposed mechanism of the inhibition of the nACh receptor by positively charged inhibitors was based mainly on electrophysiological measurements (*6*−*8*) and on the effect of such inhibitors on the lifetime of the open receptor-channel (*1*, *9*, *10*) determined by the single-channel current-recording technique (*11*). A simple mechanism that arises from these studies involves the binding of the inhibitor in the open receptor-channel and sterically blocking it (*2*−*6*, *9*, *12*). Here we chose another inhibitor, MK-801 [(+)-dizocilpine], for studying the inhibition mechanism. At pH 7.4, used in our studies, MK-801, a secondary amine, is present mainly in the cationic form. MK-801 is of both pharmacological and clinical interest. MK-801 was first reported to be a neuroprotective agent, preventing NMDA-induced cell death (*13*). It also has anticonvulsant properties and alleviates the effects of cocaine intoxication and behavioral sensitization in rats (*14*, *15*), and is known to be a potent inhibitor of the NMDA (*N*-methyl-D-aspartate) receptor (*16*, *17*) that does not compete for the NMDA-binding site. The results obtained previously with the NMDA receptor, using electrophysiological and radiolabeling techniques, were considered to

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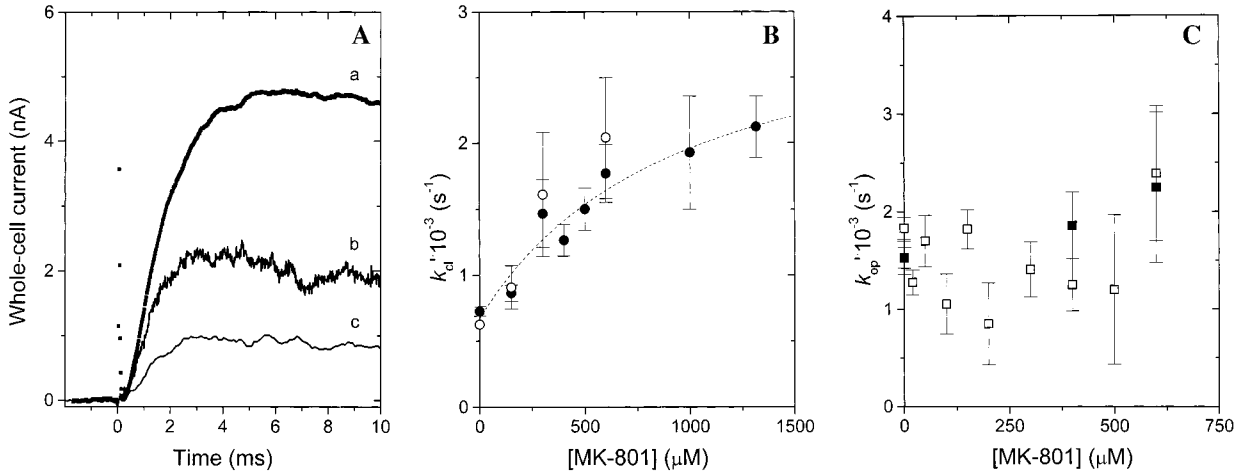

FIGURE 1: (A) Laser-pulse photolysis experiments with BC_3H1 cells in the absence (control, upper trace a, or closed squares) and presence of 300 μM (trace b) or 1 mM (trace c) MK-801 at 22 °C, −60 mV, and pH 7.4 at 25 μM released carbamoylcholine. The cell was preincubated with MK-801 for 200 ms. The rate constants for the whole-cell current rise and amplitude are as follows: Control (curve a) 660 ± 5 s^{-1}/4.8 ± 0.1 nA; 300 μM MK-801 (curve b) 1040 ± 30 s^{-1}/ 2.3 ± 0.1 nA; 1 mM MK-801 (curve c) 1590 ± 50 s^{-1}/ 1.1 ± 0.2 nA. Trace b was recorded with a different cell with the same k_{obs} as the control experiment and rescaled with respect to the maximum current amplitude of the control experiment. Traces a and c were recorded from the same cell. (B) Determination of k_{obs} for the current rise as a function of MK-801 concentration using the laser-pulse photolysis technique. At the concentration of carbamoylcholine used (25 μM), k_{obs} reflects mainly k_{cl} (k_{op}' was calculated to be 90 s^{-1} at 25 μM carbamoylcholine; eq I). The cells were equilibrated with 400 or 800 μM caged carbamoylcholine with and without MK-801 (200 ms preincubation with MK-801). The solid symbols (●) represent the laser-pulse photolysis experiments. Each data point represents the average of 3−10 experiments with at least 2 different cells. For comparison the inverse of the mean lifetimes of the open channel determined by single-channel recording are shown (○) (see Figure 3). The solid line represents the best fit according to eq 6 (appendix). The value of k_{cl} was taken as 580 s^{-1}; the apparent value k_{op}' is 90 s^{-1} at this carbamoylcholine concentration. A nonlinear least-squares fitting program (see Experimental Section) was used to obtain the values of $\overline{k_{cl}}$, 3150 s^{-1}, and $\overline{K_I}$, 950 μM, and to construct the dashed line. (C) Laser-pulse photolysis experiments. Plot of $k_{op}' = (k_{obs} - k_{cl})$ at 160 μM released carbamoylcholine as a function of MK-801 concentration determined from the values obtained with 200 ms (■) and 4 s (□) preincubation. k_{cl} was obtained from k_{obs} at 25 μM released carbamoylcholine (see text). Each data point represents the average of 3−10 experiments with at least 2 different cells.

be consistent with a mechanism in which the inhibitor enters the open channel and sterically blocks it (*9*, *18*). It is also known that MK-801 acts on the nACh receptor (*19*, *20*) in the low micromolar concentration range. To account for the observed voltage dependence of the inhibition and the shortening of the single-channel lifetime in the presence of MK-801, an extended channel-blocking mechanism was suggested for the inhibition of the nACh receptor (*19*). This model predicts association of MK-801 with the open-channel form which is blocked by the inhibitor. In contrast to the consecutive channel-blocking model (*9*), it was suggested (*19*) that the blocked channel can close before the inhibitor dissociates. This mechanism is based on the observation that the total channel-open time decreases in the presence of MK-801 (*19*), which is not consistent with the consecutive channel-blocking model (*9*).

Recently the existing techniques for investigations of the receptor mechanism have been supplemented by rapid chemical kinetic techniques, the cell-flow (*21*) and laser-pulse photolysis techniques (*22*), suitable for measurements of receptor-mediated reactions on cell surfaces in the microsecond-to-millisecond time region (*21*, *22*). The technique has a better time resolution than flowing solutions over outside-out membrane patches (*23*, *24*). An additional advantage is that in the laser-pulse photolysis technique the current from all the specific receptors on the surface of a cell can be recorded; this surface is on average several orders of magnitude larger than the surface of outside-out membrane patches. This allows one to observe and analyze the current arising from different receptor forms with different desensitization rates that are present in the cell membrane (*21*, *25*−*27*).

The rapid chemical kinetic method employed in this study, the laser-pulse photolysis technique (refs *22*, *28*, *29*; reviewed in ref *30*), entails equilibration of the muscle type nACh receptors on the surface of BC_3H1 cells (*31*) with a photolabile precursor of carbamoylcholine, caged carbamoylcholine (*22*, *28*−*30*). Carbamoylcholine is a stable and well-characterized analogue of acetylcholine. Caged carbamoylcholine and its photolysis side products have been shown to be biologically inert when tested with BC_3H1 cells (*22*, *28*−*30*). Upon photolysis the caged compound releases carbamoylcholine in the microsecond time region. The released carbamoylcholine binds to the nACh receptors, and the current resulting from the opening of cation-conducting transmembrane channels is measured. Three distinct phases of the reaction are resolved in the current versus time trace. (i) A rising phase of the current (Figure 1). From the effect of carbamoylcholine concentration on this phase it is possible to determine the rate constants for channel opening, k_{op}, and channel closing, k_{cl}, and the dissociation constant from the receptor site controlling channel opening, K_1 (*28*). (ii) A maximum current amplitude. From the effect of carbamoylcholine concentration on this amplitude one can also determine K_1 and the channel-opening equilibrium constant $\Phi^{-1} = k_{op}/k_{cl}$ (*28*). (iii) A falling phase of the current (not shown in Figure 1) is a measure of receptor desensitization (*28*). In the case of the nACh receptor in BC_3H1 cells, the values of the constants evaluated from the effect of carbamoylcholine concentration on the observed rate constant, k_{obs},

for the current rise (Figure 1A, eq I) and on the maximum current amplitude (reviewed in ref *30*) are self-consistent and in agreement with measurements made using two independent methods (*28*). (i) The single-channel-recording technique allows evaluation of k_{cl} (*11*), and (ii) the cell-flow technique allows evaluation of the maximum current amplitude (*21*). More recently, the laser-pulse photolysis technique has been used to determine the effect of inhibitor (procaine and cocaine) concentration on the rate constants for channel opening and closing, as well as on the maximum current amplitude (*32*, *33*). In agreement with the single-channel current measurements, it was found that procaine decreases the observed rate constant for channel closing (*32*). The effect of the inhibitor on the rate constant for channel opening, which could not be measured previously, indicated that this rate also decreased with increasing inhibitor concentration (*32*). This indicates that the inhibitors affect the receptor before the channel opens, presumably by binding to a receptor site on the closed-channel form of the receptor. The value of the apparent dissociation constant of the inhibitor from the closed- and open-channel forms of the receptor determined from the effect of the inhibitor on the maximum current amplitudes obtained in laser-pulse photolysis experiments, or in cell-flow experiments corrected for receptor desensitization (*21*), can also be obtained from the effect of inhibitors on the rate of channel closing or opening obtained from the laser-pulse photolysis experiments (reviewed in ref *30*).

The reaction steps that could be measured when MK-801 was used as the inhibitor are the following: (i) the effect of the inhibitor on the rate for the formation and closing of an open-channel form of the receptor:inhibitor complex; (ii) the effects of inhibitor and carbamoylcholine concentrations on the rate of formation of an inactive receptor:inhibitor complex (Figure 5); (iii) the effect of the inhibitor on the maximum current amplitude (Figures 1A and 2), a measure of the concentration of open receptor-channels; and (iv) the effect of time on the reaction of the inhibitor with the receptor.

Here we report that, when comparison is possible, the laser-pulse photolysis technique (*28*), single-channel current recordings (*11*), and the cell-flow technique (*21*) give results which agree with each other. We also show that the reaction scheme resulting from these studies accounts for results with a variety of inhibitors obtained earlier using the single-channel current-recording (*1*, *10*, *11*, *19*, *20*) and rapid chemical reaction (*32*−*35*) techniques.

MATERIALS AND METHODS

Carbamoylcholine was purchased from Sigma and (+)-dizocilpine (MK-801) from Research Biochemicals International. Caged carbamoylcholine (*N*-[2-α-carboxy]-2-nitrobenzyl-carbamoylcholine), synthesized according to Milburn et al. (*22*), was donated by Molecular Probes. The chemicals for the cell culture and the buffers were purchased from GIBCO and Sigma.

BC_3H1 cells were cultured according to published methods (*36*). Currents were recorded using the whole-cell configuration (*37*). The solution in the recording pipet contained 140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES, balanced to pH 7.4. The bath buffer solution contained 145 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 25 mM HEPES, pH 7.4. Single-channel currents were recorded in the cell-attached mode with the recording pipet containing bath buffer solution and the activating ligand alone or with inhibitor, and with the outside of the membrane facing the bath buffer solution in the outside-out patch configuration (*37*, *38*).

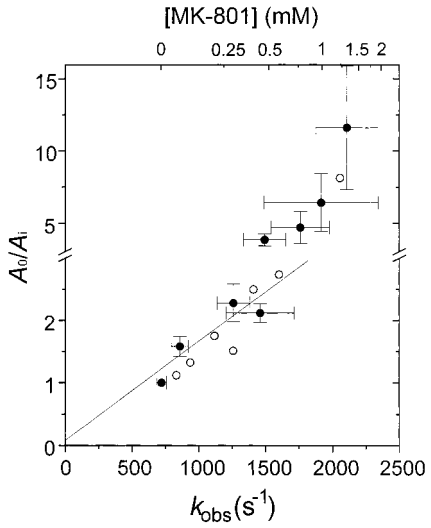

FIGURE 2: Reduction of the whole-cell current amplitude as a function of MK-801 concentration determined by the laser-pulse photolysis and cell-flow methods. The ratio of the maximum whole-cell current in the presence (A_i) and absence (A_0) of MK-801 is plotted versus the k_{obs} value obtained in the same laser-pulse photolysis experiment (see text; ●, 22 °C, −60 mV, pH 7.4, and 25 μM released carbamoylcholine). The corresponding MK-801 concentration is shown on the top axis (note the nonlinear scale). Each data point represents the average of 3−10 experiments with at least 2 different cells. The solid line was calculated according to eq 5 (appendix) with $k_{cl} = 580\ s^{-1}$ (*28*) and $(\overline{AL_2})_0 = 0.08$ (*28*). The open symbols (○) represent A_0/A_i values obtained in cell-flow experiments at 20 μM carbamoylcholine.

Rapid solution exchange was performed using the cell-flow method as described earlier (*21*). The cell attached to the recording electrode in the whole-cell mode was positioned in front of a solution exchange device (*39*). The time resolution of the method is typically 10 ms. The method for exposing the cell surface receptors to an inhibitor for various short periods of time has been described (*40*). The cell-flow/whole-cell-recording method was also used to calibrate the amount of carbamoylcholine released with the laser-pulse photolysis technique, to test for cell damage by the laser pulse, and to monitor the recovery of the receptor after the inhibitor has been removed by washing the cells using the cell-flow device (*32*). Typically, standard concentrations of carbamoylcholine (20, 100 μM) were applied before and after each laser-pulse experiment. The current amplitudes corrected for receptor desensitization (*21*) were compared to standard curves relating current amplitudes to carbamoylcholine concentration (*21*, *28*). The resistance of the recording electrode filled with buffer solution was typically 3−5 MΩ and the series resistance, 5−6 MΩ. The cells were held at a constant transmembrane voltage of −60 or +60 mV (*41*). Whole-cell currents were amplified with an Adams & List EPC-7 amplifier, and low-pass filtered (Krohn Hite 3322) at 0.5−2 kHz (cell-flow) or 10−30 kHz (laser-pulse photolysis). The filtered signal was digitized using a Labmaster

DMA 100 kHz digitizing board controlled by the Axon PClamp software. Typical digitizing frequencies were 0.5−100 kHz. Single-channel currents were amplified with a Adams & List EPC-7 amplifier and stored on magnetic tape. The data were low-pass filtered at 2 or 5 kHz using a Bessel filter (Dagan). The data were digitized at a rate of 20−40 kHz using the Axon PClamp software and the Labmaster DMA digitizing board.

The laser-pulse photolysis method was used as described (*22*). The photocleavage was initiated with a pulse of laser light generated by a Lumonics nitrogen laser ($\lambda = 337$ nm, pulse duration = 3 ns) or a flashlamp-pumped dye laser (Candela SLL650, $\lambda = 335-343$ nm after frequency doubling, 600 ns pulse duration) using oxazine 720 or sulforhodamine 640 as the dye (Lambda Physics). In the concentration range used, MK-801 does not contribute to the absorption of laser light at the wavelengths used. The output of the laser was coupled into an optical fiber (Fiberguide Industries 200 or 300 μm in diameter), which delivered the light to the cell. The concentrations of caged carbamoylcholine used were 200−800 μM, and the typical laser energies were 2−8 mJ/mm^2, depending on the required concentration of released carbamoylcholine. A major contribution to the error in the measurements arises because a cell can only be used for a limited number of measurements and different cells contain different numbers of receptors. The cell-flow technique (*21*) and a standard concentration of carbamoylcholine (20 or 100 μM) were used before and after each laser-pulse photolysis experiment to determine whether the receptors and/or cells were damaged. On the average the number of experiments performed with the same cell was 3 (200 ms equilibration with MK-801) or 1 (4 s equilibration with MK-801), respectively. When the response of the cell to carbamoylcholine decreased, as observed in cell-flow experiments, another cell was used. A standard concentration of carbamoylcholine (20 or 100 μM) was used with each cell to normalize the measurements to one another.

Linear regression and nonlinear least-squares fitting (Marquardt algorithm) were performed using the MicroCal Origin program.

RESULTS AND DISCUSSION

BC_3H1 cells were equilibrated with caged carbamoylcholine for 200 ms in the presence and absence of MK-801. Unless stated otherwise, MK-801 was equilibrated with the receptor for 50 ms (cell-flow) or 200 ms (laser-pulse photolysis). The whole-cell current obtained after photolytic release of 25 ± 10 μM carbamoylcholine is shown in Figure 1A. The $t_{1/2}$ for the liberation of free carbamoylcholine is 40 μs (*22*). The current rise is represented by a single exponential over 80−90% of the rising phase of the current according to the following equation (*28*):

$$(\overline{AL_2})(t) = (\overline{AL_2})_\infty[1 - \exp(-k_{obs}t)] \quad \text{(I)}$$

$$k_{obs} = k_{cl} + k_{op}\left(\frac{L}{L + K_1}\right)^2 \quad \text{(IA)}$$

$(\overline{AL_2})$ is the concentration of receptors in the open-channel form and is proportional to the measured whole-cell current (Figure 1). L is the concentration of activating ligand, and K_1 is the dissociation constant of carbamoylcholine from the receptor. k_{obs} is the observed first-order rate constant of the current rise. When the ligand-binding steps are fast compared to the channel-opening process and the ligand concentration is much larger than the concentration of receptors, k_{obs} reflects k_{op} and k_{cl} (eq. I). The rise of the whole-cell current is followed by a decay of the current due to transient inactivation of the receptors (desensitization) with a time constant which depends on the concentration of released carbamoylcholine (*28*). Because the liberated neurotransmitter diffuses out of the irradiated volume (300−400 μM in diameter), the desensitization process can be followed for only the first 30−50 ms after photolytic neurotransmitter release.

The whole-cell current amplitude and, therefore, the concentration of open receptor-channels decrease with increasing MK-801 concentration, as shown in Figure 1A. As compared to the control (curve a) the current amplitude decreases by about 52% in the presence of 300 μM MK-801 (curve b) but by only about another 29% when the MK-801 concentration is increased by a factor of 3.3 to 1 mM (curve c). At the latter concentration of MK-801 (curve c), the current amplitude is 23% of that recorded in the absence of MK-801 (curve a). The results in Figure 1A, therefore, suggest that at low carbamoylcholine concentrations the receptor saturated with the inhibitor can still form open receptor-channels.

The effect of MK-801 on the observed first-order rate constant for the whole-cell current rise, k_{obs}, indicates that the inhibition of the receptor by this compound can be accounted for by a decrease in the channel-opening equilibrium constant, $\Phi^{-1} = k_{op}/k_{cl}$, as a result of the formation of an open channel with the inhibitor bound, $\overline{IAL_2}$ (Figure 5). At the low concentration of 25 μM released carbamoylcholine and with equilibration of the receptors with MK-801 for 200 ms, k_{obs} increases with increasing inhibitor concentration, and at 1.3 mM MK-801 reaches a value that is about 3 times the control value (Figure 1B). In the presence of 25 μM carbamoylcholine and the absence of inhibitor the fraction of receptors in the closed-channel form is 0.95 (eq 3, appendix) and the k_{obs} value for the current rise reflects primarily the rate for channel closing (eq I; ref *28*). Similar results have been obtained when the receptors are equilibrated with MK-801 for 4 s (data not shown). It should be noticed (Figure 1B) that k_{cl}' (the apparent rate constant for channel closing in the presence of MK-801 is $k_{cl}\overline{K_I}/(\overline{K_I} + I_0)$) does not increase linearly with increasing MK-801 concentration but reaches a limiting value. At 500 μM MK-801 k_{cl}' reaches a value about 3 times that obtained in the absence of MK-801 and remains about the same at MK-801 concentrations up to 1.3 mM (Figure 1B).

In the presence of high concentrations (160 ± 30 μM) of released carbamoylcholine, the k_{obs} value for the current rise time represents a sum of k_{cl} and k_{op}, the rate constant for channel opening (eq I). The effect of MK-801 on the apparent rate constant for channel opening ($k_{op}' = k_{op}(L/(L + K_1))^2/K_I/(K_I + I_0)$) determined at high carbamoylcholine concentrations (Figure 1C) is obtained by subtracting the k_{obs} values obtained at low released carbamoylcholine concentrations, when $k_{op}' \rightarrow 0$ (eq I), from those obtained at high released carbamoylcholine concentrations. Within experimental error k_{op}' does not change in the range of MK-801 concentrations studied (Figure 1C).

Thus, the effect of MK-801 on k_{obs} at both low and high carbamoylcholine concentrations indicates the existence of a rapid process, which occurs within the time required for mixing of the receptors on the cell surface with MK-801. The rapid process leads to an increase in k_{cl}' without a concomitant change in k_{op}' and, therefore, a reduction in the channel-opening equilibrium constant. The reduction in the channel-opening equilibrium constant gives rise to a reduction in the current amplitude and, therefore, receptor inhibition. The increase in k_{cl}' can be accounted for by the rapid formation of an inhibitor:receptor complex that can form an open channel (Figure 5). This inhibitor:receptor complex has a rate constant for channel closing higher than that of the receptor without inhibitor bound, whereas k_{op} remains unchanged. The result is a shift of the channel-opening equilibrium to the closed-channel form in the presence of MK-801 and, therefore, a reduction in the fraction of receptors in the open form at a given neurotransmitter concentration (receptor inhibition). The k_{obs} value measured at low carbamoylcholine concentrations reflects k_{cl} at low MK-801 concentrations and $\overline{k_{cl}}$ (the rate constant of channel closing of the inhibitor:receptor complex) at high MK-801 concentrations (Figure 5). k_{obs} as a function of MK-801 concentration can be quantitatively described by eq 6 (appendix), which is consistent with the observed nonlinear dependence of k_{obs} on MK-801 concentration (Figure 1). From a nonlinear least-squares fit of eq 6 (dashed line in Figure 1B) and by using the experimentally obtained values for k_{cl}, the rate constant for channel closing of the receptor: inhibitor complex $\overline{k_{cl}}$, and $\overline{K_1}$, the dissociation constant of MK-801 from the open-channel receptor form (Figure 5), can be estimated: $\overline{k_{cl}} = 3150 \pm 600\ s^{-1}$ and $\overline{K_I} = 950 \pm 400\ \mu M$. From the principle of detailed balance (*42*) and the value for $\overline{K_I}$, the apparent dissociation constant of MK-801 from the closed-channel receptor forms can be estimated as 180 μM (see eq 2, appendix). Accordingly, MK-801 binds ~5 times more strongly to the closed- than to the open-channel form of the receptor.

Can the shift of the channel-opening equilibrium to the closed-channel form in the presence of inhibitor account for the reduction of the whole-cell current in the presence of MK-801 and, therefore, receptor inhibition? To answer this question, we have measured the ratio of the whole-cell current amplitude in the absence and presence of inhibitor, A_0/A_i, as a function of MK-801 concentration (Figure 2) using the laser-pulse photolysis technique. If the inhibition of the receptor is caused by the altered channel-opening equilibrium, A_0/A_i can be expressed as a linear function of the ratio of the channel-opening equilibrium constant in the absence, Φ^{-1}, and presence, $\Phi_{I_0}{}^{-1}$, of the inhibitor (eq 5). This ratio is given by k_{obs} obtained in the presence of the inhibitor divided by k_{cl} obtained in its absence (eq 5). k_{obs} is obtained from the current rise at low carbamoylcholine concentrations, at which the channel-opening rate constant does not contribute to the measurements (eq 6). The solid line shown in Figure 2 was calculated using eq 5, the experimentally determined values of A_0/A_i and of k_{obs} (eq 6), the known value of the fraction of receptor molecules in the open-channel form, $\overline{FAL_2}$ of 0.05 (calculated for 25 μM carbamoylcholine using eq 3, appendix), and a value for $k_{cl} = 580\ s^{-1}$ obtained from laser-pulse photolysis experiments (*28*). The results shown in Figure 2 are in agreement with the experimental data obtained in the MK-801 concentration range of 0−500 μM. The inhibition of the receptor at low carbamoylcholine concentration is, therefore, accounted for by the shift of the channel-opening equilibrium to the closed-channel form in the presence of up to 500 μM MK-801. At higher MK-801 concentrations, A_0/A_i curves upward (Figure 2). This upward curvature is due to the contribution of a slow inhibition process at high MK-801 concentrations. These measurements are discussed below.

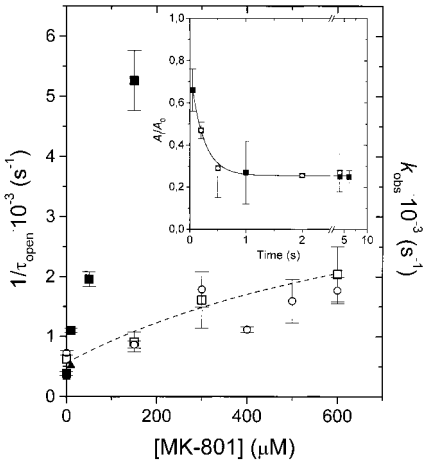

FIGURE 3: Single-channel current-recording experiments. Plot of $1/\tau_{op}$ versus MK-801 concentration. The open squares (□) represent the data obtained during the first 200 ms after rapid application of 20 μM carbamoylcholine and MK-801 to an outside-out patch. The dashed line represents the result obtained from a nonlinear least-squares fit according to eq 6a (appendix). $k_{cl} + k_f$ was estimated as 4500 s^{-1}. For comparison, the open circles (○) show the dependence of k_{obs} determined by the laser-pulse photolysis technique and obtained from the current rise with 200 ms preincubation with MK-801. The filled squares (■) represent data obtained under equilibrium conditions (the receptors were exposed to MK-801 for at least 30 s before data collection) with the cell-attached mode (the value reported in the literature (*21*) for 10 μM MK-801 with acetylcholine (0.5 μM) as activating ligand at −50 mV is shown for comparison, filled triangle). Inset: A_i/A_0 was measured as a function of time (A_i and A_0 are the maximum current amplitudes in the presence and absence of inhibitor, respectively) at a carbamoylcholine concentration of 100 μM and a MK-801 concentration of 200 μM at 22 °C, −60 mV, and pH = 7.4. The solid symbols (■) represent the data obtained with the cell-flow technique (50 ms equilibration time was used). The open symbols (□) represent a laser-pulse photolysis experiment with 200 ms exposure of the receptor to MK-801 before channel activation. Each data point represents the average of 1−3 experiments. The solid line represents the result obtained from nonlinear regression analysis according to eq 4 (appendix) (Figure 5). The rate constant obtained is $2.6 \pm 0.1\ s^{-1}$.

Evidence for the existence of a second inhibitory process for MK-801 (Figure 3) will be considered next. We shall consider first the effect of MK-801 on the lifetime of the open channel (τ_{op}), determined using the single-channel-recording method (*11*). We used outside-out membrane patches that can be rapidly equilibrated with carbamoylcholine and MK-801 to determine the mean channel-open time in the first 200 ms after application of MK-801. Experiments were performed at 150, 300, and 600 μM MK-801 and 20 μM carbamoylcholine. Only openings in the first 200 ms after simultaneous application of 20 μM carbamoylcholine and MK-801 to 2−3 outside-out membrane patches from different cells were used to calculate the mean open times. Multiple openings have been neglected. The open time

Table 1: Mean Open Times Obtained in the Presence of 20 μM Carbamoylcholine in the Absence and Presence of MK-801 of Different Concentrations, 22 °C, −60 MV, pH 7.4[a]

[MK-801] (μM)	τ_{op} (ms) equilibrium	N_{op}	τ_{op} (ms) first 200 ms	N_{op}
0	2.7 ± 0.2	1600	1.6 ± 0.1	75
10	0.9 ± 0.2	2800		
50	0.5 ± 0.1	330		
150	0.2 ± 0.1	451	1.1 ± 0.2	39
300			0.6 ± 0.1	50
600			0.5 ± 0.1	44

[a] τ_{op} values on the left, cell-attached mode; equilibrium conditions (>30 s exposure of the receptor to MK-801 before beginning data acquisition); τ_{op} values on the right, application of MK-801 to outside-out patches. Only openings in the first 200 ms after exposure to MK-801 were incorporated in the open-time histograms obtained from the outside-out patches. Typical openings consisted of single events in the presence of the inhibitor. N_{op} represents the number of openings in each measurement.

distributions can be described with a single-exponential decay function, that is a Poisson distribution, at all of the inhibitor concentrations used. The mean channel-open time was obtained from this exponential fit of the open time distribution. The results are summarized in Table 1.

The reciprocal of the lifetime of the open channel as a function of MK-801 concentration (Figure 3, open squares) is represented by eq 6a (appendix) and the dashed line in Figure 3. In the concentration range used only a slight deviation from linearity is observed (Figure 3, dashed line), as is expected from the conditions of the experiment ($\overline{K_I} > I$). Using a $\overline{K_I}$ value of 950 μM, obtained from the chemical kinetic experiments, $\overline{k_{cl}} + k_f$ can be estimated as 4500 s^{-1} (eq 6a), where k_f is the rate constant for the conversion of the open channel with inhibitor bound ($\overline{IAL_2}$) to the inactive form IAL_2* in Figure 5. Within experimental error, the effect of MK-801 on the lifetime of the open channel is in good agreement with the kinetic data obtained by laser-pulse photolysis (shown for comparison in Figures 1B and 3, open circles). The values differ from those that have been obtained under equilibrium conditions by Amador and Dani (*19*) and in the experiments in Figure 3 (solid squares).

We, therefore, determined the time dependence of the effect of MK-801 on the lifetime of the open channel (τ_{op}). The mean channel-open time at −60 mV transmembrane potential was measured under equilibrium conditions (cell-attached mode; MK-801 and carbamoylcholine were equilibrated with the receptors for t > 30 s before the single-channel current was recorded) at MK-801 concentrations of 10, 50, and 150 μM with 1 μM carbamoylcholine as the activating ligand. In the absence of the inhibitor the channel-open time distribution shows a fast (22%) and a slow (78%) component (*28*, *43*); only the mean open time determined from the slow component was used as the control value. $1/\tau_{op}$ is shown as a function of MK-801 concentration in Figure 3 (solid symbols). Under these conditions $1/\tau_{op}$ is dependent on MK-801 concentration, with a slope of (3.1 ± 0.2) × 10^7 M^{-1} s^{-1}. This value for the slope is in good agreement with the value reported for the nACh receptor for the same cell line at −50 mV under equilibrium conditions (*19*) using acetylcholine and MK-801. It is also in good agreement with the bimolecular rate constant for MK-801 binding to the open-channel form estimated for the NMDA receptor (*18*). Similar bimolecular rate constants have also been reported for the reaction of a variety of compounds with the open-channel form of the nACh receptor (*9*). It should be noted that when the channel-open time is determined within 200 ms of exposure of the receptor to MK-801 (Figure 3, open symbols), a different result is obtained. The reduction in the lifetime of the open channel in the presence of MK-801 is 1 order of magnitude lower than the reduction obtained from the equilibrium experiments (Figure 3, filled symbols). The reduction of the mean channel-open time within the mixing time of MK-801 with the cell surface receptors is in agreement with the values of $\overline{K_I}$, $\overline{k_{cl}}$, and k_f as measured with the laser-pulse photolysis technique (Figures 1B, 3, and 4). Thus, these single-channel current measurements provide evidence for the existence of two inhibitory processes, one occurring within the mixing time of MK-801 with the cell surface receptors and one associated with a slowly equilibrating receptor site.

In preliminary kinetic measurements on the effect of MK-801 concentration on the current amplitude (Figure 3, inset), the data are plotted as A_i/A_0 where A_i and A_0 are the maximum current amplitudes in the presence and absence of 200 μM MK-801. At this concentration about 60% of the receptors are inhibited. About 50% of the inhibition process has gone to completion within the mixing time of MK-801 and the cell surface receptors of 50 ms. A second, much slower process goes to completion within about 1 s. Preliminary experiments indicate that the equilibration time for this second process is slower at lower concentrations of MK-801 and faster at higher concentrations. Similar results were obtained at 100 μM carbamoylcholine but are not shown.

So far we have considered what happens at low carbamoylcholine concentrations, when the majority of the receptors are in the closed-channel form. We shall now consider the effect of carbamoylcholine concentration, and therefore the role of the open-channel form of the receptor, on the inhibition reaction. It can be seen in the laser-pulse photolysis experiments in Figure 4A that at a carbamoylcholine concentration of 160 μM and an MK-801 concentration of 400 μM, the falling phase of the current can no longer be described by a single-exponential decay function; instead the current decay can be represented by a sum of two exponentials (eq 7, appendix). An initial rapid decay phase of the current is followed by a slower phase with a time constant comparable to the time constant for receptor desensitization obtained in the absence of MK-801. When the receptor is pre-equilibrated with MK-801 for 4 s before exposure to both carbamoylcholine and MK-801, the current amplitude decreases, but the observed rates for the rapidly and slowly decaying phases of the current appear unchanged (curve b′ Figure 4A). The observed rate constant for the rapidly decaying phase of the current (Figure 4A) depends on both the carbamoylcholine and the MK-801 concentration. We shall examine first the effect of carbamoylcholine concentration on k_{obs} for the rapidly decaying phase.

The dependence of k_{obs}' for the rapidly decaying phase of the current on carbamoylcholine concentration at a constant concentration of 400 μM MK-801 has been investigated using the laser-pulse photolysis method. As shown in Figure 4B, k_{obs}' increases with increasing carbamoylcholine con-

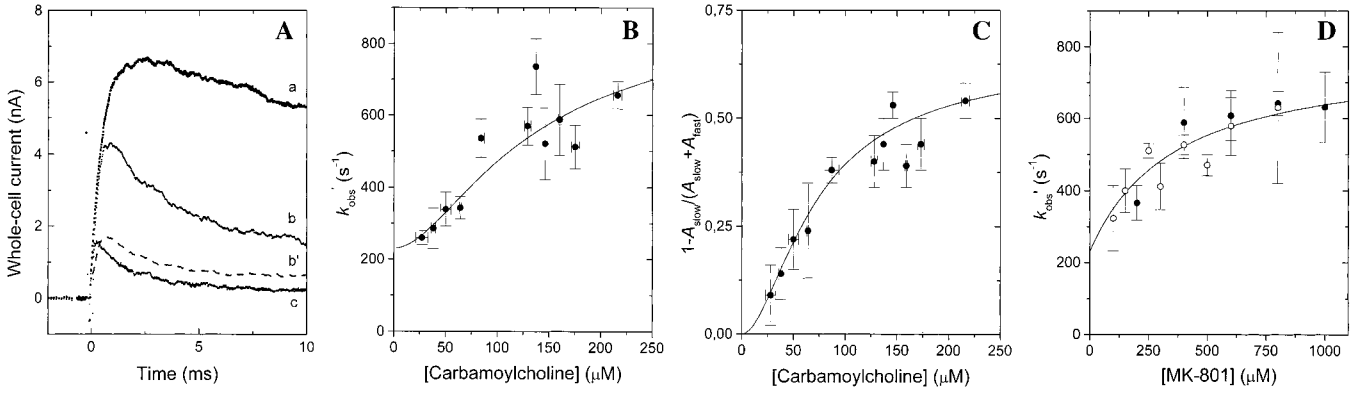

FIGURE 4: (A) Laser-pulse photolysis experiment (same conditions as in Figure 1) with 160 μM carbamoylcholine released from 800 μM caged precursor in the absence (upper trace, a) and presence of 400 μM MK-801 (traces b, b′) and 1 mM MK-801 (trace c). The cell was preincubated with MK-801 for 200 ms (traces a, b, and c) and 4 s (trace b′). The rate constants obtained for the rising phase of the whole-cell current and the amplitudes are the following: trace a, 1800 ± 20 s^{-1}/7.2 ± 0.1 nA (control); trace b, 3600 ± 100 s^{-1}/4.6 ± 0.1 nA (400 μM MK-801, 200 ms preincubation); trace b′, 3800 ± 100 s^{-1}/1.8 ± 0.1 nA (400 μM MK-801, 4 s preincubation); trace c, 10000 ± 300 s^{-1}/1.7 ± 0.1 nA (1 mM MK-801, 200 ms preincubation). The rate constants associated with the rapidly decaying phase and the amplitudes after decay of the fast phase are the following: trace b, 440 ± 10 s^{-1}/2.3 ± 0.1 nA (400 μM MK-801, 200 ms preincubation); trace b′, 660 ± 20 s^{-1}/0.9 ± 0.1 nA (400 μM MK-801, 4 s preincubation); trace c, 530 ± 5 s^{-1}/0.3 ± 0.1 nA (1 mM MK-801, 200 ms preincubation). Traces b and b′ were recorded from a different cell with the same k_{obs} as the control experiment and rescaled with respect to the maximum current amplitude of the control experiment. (B) Laser-pulse photolysis experiments. Dependence of the rate constant of the rapidly decaying phase on carbamoylcholine concentration. The concentration of caged carbamoylcholine was 800 μM. The conditions were identical to those in Figure 1. The concentration of released carbamoylcholine was varied by adjusting the laser energy. Each data point represents the average of 3−9 experiments with at least 3 different cells. The solid line was calculated according to eq 8 (appendix). The parameters were the following: $K_1 = 240$ μM, $\Phi = 0.18$ (*21*), $\overline{K_I} = 950$ μM, $K_I = 180$ μM (estimated from dependence of k_{cl}' on MK-801 concentration), and $k_b = 230$ s^{-1}. k_f was obtained by a nonlinear least-squares fitting procedure as 2900 s^{-1}. (C) Laser-pulse photolysis experiments. Dependence of the ratio of the amplitude of the rapidly decaying phase to the maximum current (eq 9, appendix) on the carbamoylcholine concentration. The conditions of the experiments were identical to those in Figure 4B. Each data point represents the average of 3−9 experiments with at least 3 different cells. The solid line has been calculated using eqs 8 and 9 (appendix) with the same parameters as stated in the legend of Figure 4B except that $k_f = 1800$ s^{-1} was calculated by nonlinear least-squares fitting. (D) Determination of k_{obs}' for the rapidly decaying current as a function of MK-801 concentration using the laser-pulse photolysis technique. Experimental conditions: 22 °C, −60 mV, pH 7.4, and 160 μM released carbamoylcholine (●, 200 ms preincubation; ○, 4s preincubation). Each data point represents 3−10 experiments with at least 3 different cells. The solid line was calculated according to eq 8 (appendix) with $k_f/\delta = 560$ s^{-1}, $k_b = 230$ s^{-1} and $\gamma = 360$ μM (see text for details). By using the known values of $K_1 = 240$ μM, $\Phi = 0.18$ (*21*), $\overline{K_I} = 950$ μM, and $K_I = 180$ μM, one can estimate a value for k_f of 2400 s^{-1}.

centration from 25 to 200 μM. Over this concentration range, the ratio of the amplitude of the rapidly decaying phase of the current to the maximum current in the presence of MK-801 (eqs 8 and 9, appendix) increases with increasing carbamoylcholine concentration and, therefore, increases with increasing concentration of the open-channel form of the receptor (Figure 4C). This is consistent with the MK-801-induced receptor inactivation taking place from the open-channel receptor form. The experimental data can well be described with eqs 8 and 9, which assume that inactivation of the open-channel form does not involve a bimolecular reaction of an inhibitor with the open receptor-channel but that instead an inhibitor-induced transition from the open-channel form with inhibitor bound $\overline{IAL_2}$ to an inactive receptor form IAL_2* (Figure 5) occurs. The effect of MK-801 concentration on the rapidly decaying phase of the current (Figure 4D) is consistent with this assumption and is discussed below. Using the estimate for $\overline{K_I}$ of 950 μM and for K_I of 180 μM, both obtained from the effect of MK-801 on the current rise time (Figure 1B) and the published values for the ligand dissociation constant and channel-opening rate constant given in the appendix, eq 3, a value for the forward rate constant of the transition to the inactive state can be estimated. The value obtained for k_f is 2900 s^{-1} from the rate constant (eq 8) and 1800 s^{-1} from the relative amplitude (eq 9). A value for k_f of 2400 s^{-1} is obtained from the dependence of k_{obs}' on MK-801 concentration (eq 8).

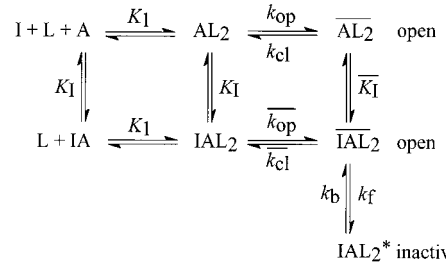

FIGURE 5: Proposed regulatory site mechanism for the inhibition by MK-801 of the nACh receptor in BC_3H1 cells. *A* represents the nondesensitized receptor, *L* the neurotransmitter, and *I* the inhibitor. $\overline{AL_2}$ and $\overline{IAL_2}$ are the open-channel forms and IAL_2* is the inhibited form. K_I and $\overline{K_I}$ are the dissociation constants from the closed- and open-channel receptor forms, respectively. k_{op}, $\overline{k_{op}}$, k_{cl}, and $\overline{k_{cl}}$ are the rate constants for channel opening and closing for the receptor in the absence of MK-801 and for the inhibitor-bound receptor form, respectively. k_f and k_b are the rate constants associated with the formation of the inhibited state.

The dependence of the observed rate constant k_{obs}' for the rapidly decaying phase on the MK-801 concentration at a concentration of 160 μM released carbamoylcholine is shown in Figure 4D. At low inhibitor concentrations, k_{obs}' increases with increasing MK-801 concentration. At high MK-801 concentrations, k_{obs}' does not increase linearly with increasing MK-801 concentrations as would be expected for a bimolecular process; instead it reaches a value of 630 ± 100 s^{-1} at 1 mM MK-801 (Figure 4D). The dependence of k_{obs}' on

MK-801 concentration is characteristic for a MK-801-induced transition to an inactive state that is preceded by a rapidly equilibrating inhibitor-binding step (Figure 5). k_{obs}' as a function of MK-801 concentration can be described by eq 8 (appendix). For the derivation of eq 8, it has been assumed that the MK-801-induced receptor inactivation takes place only from the open state, as is indicated by the results in Figure 4. By using the estimated values for the dissociation constants of MK-801 from the open- and closed-channel receptor forms (see above) and the known values for Φ and K_1 (eq 3, appendix), we calculated the γ term in eq 8 to be 360 μM. This is in good agreement with the experimental data (solid line, Figure 4D) obtained in laser-pulse photolysis measurements of the effect of MK-801 concentration on the observed rate constant for the rapidly decaying phase of the current (Figure 4A) at a carbamoylcholine concentration of 160 μM. It should be noted in Figure 4D that, within experimental error, the observed rate constants for the current decay are the same whether the receptor is equilibrated with MK-801 for 200 ms or 4 s. The relative contribution of the rapidly decaying phase to the maximum whole-cell current can be obtained from the ratio of the amplitudes of the rapidly (A_{fast}) and slowly (A_{slow}) decaying phases of the current (eq 9). The contribution of the pre-exponential factor of the rapidly decaying phase to the total current ($A_{fast}/(A_{fast} + A_{slow})$) depends on the MK-801 concentration. It reaches a mean value of 64% ± 8% at MK-801 concentrations greater than 600 μM when the receptors are equilibrated with the inhibitor for 200 ms. ($A_{fast}/(A_{fast} + A_{slow})$) can be described by eq 9 (appendix); by using eq 9 we can estimate a value for $k_b \approx 230\ s^{-1}$. This is in good agreement with the value of k_b estimated from the dependence of k_{obs}' on carbamoylcholine concentration (Figure 4B) at low carbamoylcholine concentrations, where the term $k_f I/(I + \gamma)\delta$ is small compared to k_b (eq 8). With the estimated values of k_b and k_f the equilibrium constant for the formation of the inhibited state, $1/K_b$, can be estimated as ≈10. The apparent binding affinity of the open state of the receptor for the inhibitor, which can be expressed as $1/(\overline{K_I} K_b)$, is, therefore, increased by a factor of 10 after the transition to the inactive state is completed.

The mechanism shown in Figure 5 represents a summary of the results obtained with the receptor site that equilibrates rapidly with MK-801. At low concentrations of carbamoylcholine, when the receptor is mainly in the closed-channel form, receptor inhibition is accounted for by an inhibitor-induced decrease in the channel-opening equilibrium; that is, the channel-closing rate increases in the presence of MK-801 while the channel-opening rate remains unchanged. At high concentrations of carbamoylcholine, when the receptor is mainly in the open-channel form, another step in the inhibition process is revealed. The effect of MK-801 concentration on this step is inconsistent with a bimolecular reaction of the inhibitor with the open-channel form. The results are consistent with the isomerization of the open-channel form of the receptor with inhibitor bound to a form in which the channel is closed.

The mechanism in Figure 5 can account for the single-channel measurements of the effect of other inhibitors on the lifetime of the open receptor-channel (*1*, *9*, *10*, *19*, *20*). When the receptor:inhibitor complex $\overline{IAL_2}$ (Figure 5) is nonconducting, measurements of the effect of inhibitors on the lifetime of the open receptor-channel will appear to be consistent with an open-channel-blocking mechanism (*9*). When $\overline{IAL_2}$ (Figure 5) represents an open channel, such measurements will indicate deviations from the channel-blocking mechanism (*9*, *10*, *19*). In view of the time-dependent effect of inhibitors on the open-channel lifetime (Figure 3), it is not yet clear which inhibition process has been observed in previous studies (*19*, *20*).

The use of rapid reaction techniques and the determination of the effect of the inhibitor on k_{op} and k_{cl} (*30*, *32*) and on the current amplitudes in kinetic measurements (*33*), when the receptor is mainly in the closed or mainly in the open-channel form (eqs 2, 6), have provided evidence that nACh receptor inhibitors such as procaine and cocaine bind to the closed- as well as the open-channel form of the receptor. A minimum mechanism that incorporates these results and the observation that, although MK-801 binds to the open-channel form, the rate constant for channel closing increases (Figure 1B) is shown in Figure 5. The principle of microscopic reversibility applied to the proposed mechanism in Figure 5, and the cyclic equilibrium involving the reaction intermediates AL_2, $\overline{AL_2}$, IAL_2, and $\overline{IAL_2}$, requires that when $K_I < \overline{K_I}$, the channel-opening equilibrium constant $\Phi_{I_0}^{-1} = \overline{k_{op}}/k_{cl}$ is smaller than Φ^{-1}. In the proposed mechanism in Figure 5, receptor inhibition is, therefore, accounted for by the formation of a receptor:inhibitor complex of the open-channel form $\overline{IAL_2}$ (Figure 5) with an unfavorable channel-opening equilibrium constant. A minor contribution to receptor inhibition involves the formation of a nonconducting receptor:inhibitor complex that proceeds from the open-channel form of the receptor (Figure 5).

We have shown here and previously (*32*, *33*) that the laser-pulse photolysis technique with a microsecond-to-millisecond time resolution (*22*, *28*−*30*) gives information about the effect of inhibitors on the rate constant of channel opening and closing, and in the same experiment about the effect of inhibitors on the extent to which the receptor is in the closed- or in the open-channel form. This allows one to compare the dissociation constants of the inhibitor determined in both kinetic and *quasi* equilibrium experiments and to arrive at more detailed information about receptor inhibition (Figure 5). The rapid chemical kinetic techniques may, therefore, be useful in answering many of the remaining questions regarding the chemical mechanism by which neurotransmitter receptors are inhibited by a large number of clinically interesting and abused compounds (*44*). A mechanism in which a compound inhibits by reducing the channel-opening equilibrium constant, as proposed here, suggests the development of therapeutic agents that compete with an inhibitor for its binding site, without affecting the channel-opening equilibrium constant. Such a compound can bind to this inhibitory site and protect the organism from the toxic effect of receptor inhibitors.

APPENDIX

Under equilibrium conditions, inhibition of the nACh receptor can be described by the following equation (*32*, *33*):

$$\frac{A_0}{A_i} = 1 + \frac{I}{K_{I,app}} \qquad (1)$$

A_i and A_0 represent the whole-cell current amplitudes in the presence and absence of inhibitor at the concentration I, respectively. $K_{I,app}$ is the apparent inhibition constant. According to Figure 5 the apparent inhibition constant can be expressed in terms of the dissociation constants of MK-801 from the individual states of the receptor:

$$\frac{1}{K_{I,app}} = \frac{FA}{(K_I)_I} + \frac{FAL}{(K_I)_{II}} + \frac{FAL_2}{(K_I)_{III}} + \frac{\overline{FAL_2}}{(K_I)_{IV}} \quad (2)$$

$(K_I)_N$ represents the apparent dissociation constant of MK-801 from the receptor with respect to the Nth state of the receptor. FA, FAL, FAL_2, and $\overline{FAL_2}$ are the fractions of receptor in each state.

For binding of the inhibitor only to the open-channel form

$$\frac{1}{K_{I,app}} = \frac{\overline{FAL_2}}{\overline{K_I}} \quad (3)$$

where

$$\overline{FAL_2} = \frac{L^2}{L^2 + \Phi(L + K_1)^2}$$

and represents the fraction of the receptors in the open-channel form (*21*). $\overline{K_I}$ represents the dissociation constant of the inhibitor from the open-channel form (*32*). L represents the concentration of activating ligand, K_1 the dissociation constant of the receptor site controlling channel opening (Figure 5), and Φ^{-1} the channel-opening equilibrium constant k_{op}/k_{cl} (Figure 5). Values for K_I of 240 μM and for Φ of 0.18 (*21*) have been used to calculate the fraction of the receptors in the open-channel form.

The inhibition of the receptor by MK-801 is time-dependent. The k_{obs}'' values for the time-dependent inhibition process (Figure 3, inset) have been obtained by a nonlinear least-squares fit according to the following equation:

$$\frac{A_i}{A_0}(t) = \frac{A_{i,t=\infty}}{A_0} + \frac{A_{i,t=0}}{A_0}\exp(-k_{obs}''t) \quad (4)$$

In this equation, $A_{i,t=\infty}/A_0$ was calculated from the whole-cell current amplitudes in the presence ($A_{i,\infty}$) and absence (A_0) of inhibitor at long preincubation times when A_i was constant. $A_{i,t=0}$ and k_{obs}'' were varied by the fitting routine.

The expression for the inhibition of the maximum current amplitude at 25 μM carbamoylcholine can be described by the following equation:

$$\frac{A_0}{A_i} = \overline{FAL_2} + \frac{(1 - \overline{FAL_2})}{k_{cl}}k_{obs} \quad \text{where} \quad \frac{k_{obs}}{k_{cl}} = \frac{\Phi^{-1}}{\Phi_{I_0}^{-1}} \quad (5)$$

$\overline{FAL_2}$ represents the fraction of the receptor-channel being open and k_{cl} the rate constant for channel closing; k_{obs} represents the observed rate constant for the current rise in the presence of MK-801.

The observed rate constant k_{obs} for the whole-cell current rise has been derived according to the scheme in Figure 5 (*33*):

$$k_{obs} = \left(\frac{L}{L + K_1}\right)^2 k_{op} + \left(k_{cl}\frac{\overline{K_I}}{I + \overline{K_I}} + \overline{k_{cl}}\frac{I}{I + \overline{K_I}}\right) \quad (6)$$

The lifetime of the open channel after formation of the inactive receptor:inhibitor complex can be expressed as follows:

$$\frac{1}{\tau_{op}} = k_{cl}\frac{\overline{K_I}}{I + \overline{K_I}} + (\overline{k_{cl}} + k_f)\frac{I}{I + \overline{K_I}} \quad (6a)$$

I represents the concentration of inhibitor and K_1 the dissociation constant of activating ligand from the receptor. K_I and $\overline{K_I}$ are the dissociation constants of MK-801 from the closed- and open-channel receptor forms, respectively. $\overline{k_{cl}}$ is the rate constant for channel closing of the inhibitor-bound open state (Figure 5), and k_f is the rate constant for formation of the inactive state.

The whole-cell current decay in the presence of MK-801 at high carbamoylcholine concentrations can be represented as a sum of two exponentials according to the following equation:

$$(\overline{AL_2})_t = (\overline{AL_2})_{t=0}[A_{fast}\exp(-k_{obsl}t) + A_{slow}\exp(-k_{obs2}t)] \quad (7)$$

$(\overline{AL_2})_t$ and $(\overline{AL_2})_{t=0}$ represent the concentration of open channels and are proportional to the current at times t and 0 after the current has reached its maximum value. A_{fast} and A_{slow} represent the fraction of the rapidly and slowly decaying exponentials of the total current, respectively, with the rate constants k_{obs1} and k_{obs2}.

The rate constant for the MK-801-induced transition (Figure 5) of $\overline{IAL_2}$, the receptor in the open-channel form with inhibitor bound, to IAL_2*, a closed-channel form of the receptor with inhibitor bound, can be derived according to Figure 5:

$$k_{obs}' = k_f\frac{I}{(I + \gamma)\delta} + k_b = k_f\frac{I}{I\left[1 + \Phi_{I_0}\left(\frac{L + K_I}{L}\right)^2\right] + \overline{K_I}\left[1 + \Phi\left(\frac{L + K_I}{L}\right)^2\right]} + k_b \quad (8)$$

where

$$\gamma = \frac{\overline{K_I}\left[1 + \frac{k_{cl}}{k_{op}}\left(\frac{L + K_I}{L}\right)^2\right]}{1 + \frac{\overline{k_{cl}}}{\overline{k_{op}}}\left(\frac{L + K_I}{L}\right)^2}$$

and

$$\delta = 1 + \frac{\overline{k_{cl}}}{\overline{k_{op}}}\left(\frac{L + K_I}{L}\right)^2$$

Here, k_f and k_b represent the rate constants for the formation and the back reaction of the inactive state (Figure 5). k_b (230 s^{-1}) can be estimated from the relative amplitudes of the

slowly (A_{slow}) and rapidly decaying (A_{fast}) phases as follows:

$$\frac{A_{fast}}{A_{fast} + A_{slow}} = \frac{k_f}{k_{obs}'} = 1 - \frac{k_b}{k_{obs}'} \quad (9)$$

where k_{obs}' is defined in eq 8.

The following assumptions have been made for the equations derived above: (i) The concentrations of activating ligand and inhibitor are large compared to the concentration of receptors. (ii) The ligand- and inhibitor-binding steps are fast compared to channel opening and closing and are in pre-equilibrium. (iii) The rate constant for channel opening is not affected by the inhibitor. (iv) k_{cl}, $\overline{k_{cl}}$, $k_{op} \gg k_f$, k_b (Figure 5, eq 8). (v) *A*, *IA*, *AL*, *IAL*, AL_2, IAL_2, $\overline{AL_2}$, and IAL_2 are in rapid pre-equilibrium. (vi) Receptor inhibition is mainly accounted for by the formation of a receptor:inhibitor complex with an unfavorable channel-opening equilibrium constant. A minor contribution to receptor inhibition involves the formation of a nonconducting receptor:inhibitor complex that proceeds only from the open-channel form of the receptor (Figure 5).

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