Mechanism-Based Discovery of Small Molecules that Prevent Noncompetitive Inhibition by Cocaine and MK-801 Mediated by Two Different Sites on the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The nicotinic acetylcholine receptor (nAChR) belongs to a group of five structurally related membrane proteins that play a major role in the communication between $\sim 10^{12}$ cells of the mammalian nervous system. The receptor is inhibited by both abused drugs and therapeutic agents. During the past two decades, many attempts have been made to find compounds that prevent cocaine inhibition of this protein. The use of newly developed transient kinetic techniques in investigations of the inhibition of the receptor by cocaine and MK-801 led to an inhibition mechanism not previously proposed. It was observed that the receptor contains two inhibitory sites: one that equilibrates with the tested noncompetitive inhibitors within ∼50 ms, and a second site that equilibrates with inhibitors within ∼1 s. The mechanism of inhibition of the rapidly equilibrating inhibitory site has been investigated, and based on that mechanism, the first evidence that small organic molecules exist that prevent inhibition of the rapidly equilibrating inhibitory site was obtained. These compounds did not prevent the inhibition due to the slowly equilibrating inhibitory site. Here, we present the first evidence that a compound (3-acetoxy ecgonine methyl ester) exists that prevents inhibition of the slowly equilibrating inhibitory site and that the mechanism of inhibition of this site differs from that of the rapidly equilibrating site. BC_3H1 cells containing a fetal mouse muscle-type nAChR were used, and the receptor was activated by carbamoylcholine. The resulting whole-cell current due to the nondesensitized nAChR was determined. Because the nAChR desensitizes rapidly, the measurements required the use of a transient kinetic technique with a time resolution of 10 ms; the cell-flow technique was used. Inhibitors and compounds that alleviate inhibition were tested by determining their effects on the whole-cell current due to activation of the nAChR by carbamoylcholine.

The nicotinic acetylcholine receptor (nAChR)[1] belongs to a family of structurally related proteins that form ligand-gated transmembrane ion channels (reviewed in ref *1*). They are involved in the rapid transmission of signals between $\sim 10^{12}$ cells of the nervous system and in the control of intercellular communication (*2*), a process considered basic to brain function (*3*). A variety of therapeutic agents, for instance, MK-801 [(+)-dizocilpine] and abused drugs, including cocaine, inhibit the nAChR and monoamine transporters and interfere with the nervous system function (*4*−*6*). Understanding the mechanism of inhibition of the nAChR is a longstanding (*7*) and intensively investigated problem (*8*) with major implications for medicine (*9*). Here, we consider the mechanism of action of MK-801 and cocaine on the fetal mouse muscle-type nAChR expressed in BC_3-H1 cells (*10*). MK-801 is of both pharmacological and clinical interest. It has anticonvulsant properties and alleviates the effects of cocaine inhibition in vivo (*11*, *12*). Cocaine, a commonly abused drug, can result in myocardial ischemia, seizures, hypothermia, and sudden death (*13*). The use of transient kinetic techniques suitable for investigating protein-mediated reactions on cell surfaces in the micro- to millisecond time region (*14*−*16*) led to the finding that the receptor contains two different inhibitory sites that equilibrate with noncompetitive inhibitors in two different time regions (*17*). One site equilibrates with an inhibitor within 50 ms, and the other one equilibrates within about 1 s. RNA polymers combinatorially selected on the basis of binding to the cocaine or MK-801 site of the receptor (*18*) displace inhibitors only from the rapidly equilibrating inhibitory site (*19*). These observations suggested that the slow inhibition process involves a different inhibitor-binding site than the fast process (*19*). In selecting the RNA polymers nAChR-rich *Torpedo californica* electroplax membranes (*20*) were used in which both the internal and external faces of the membrane were exposed (*18*). These RNA polymers do not interact with the slowly equilibrating inhibitory site, suggesting that it is on a part of the receptor not exposed on the membrane surfaces. Until now, we have investigated only the rapidly equilibrating inhibitory site (*17*−*19*, *21*−*24*). As predicted by the mechanism proposed for the rapidly

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; EME, ecgonine methyl ester; 3-acetoxy EME, 3-acetoxy ecgonine methyl ester.

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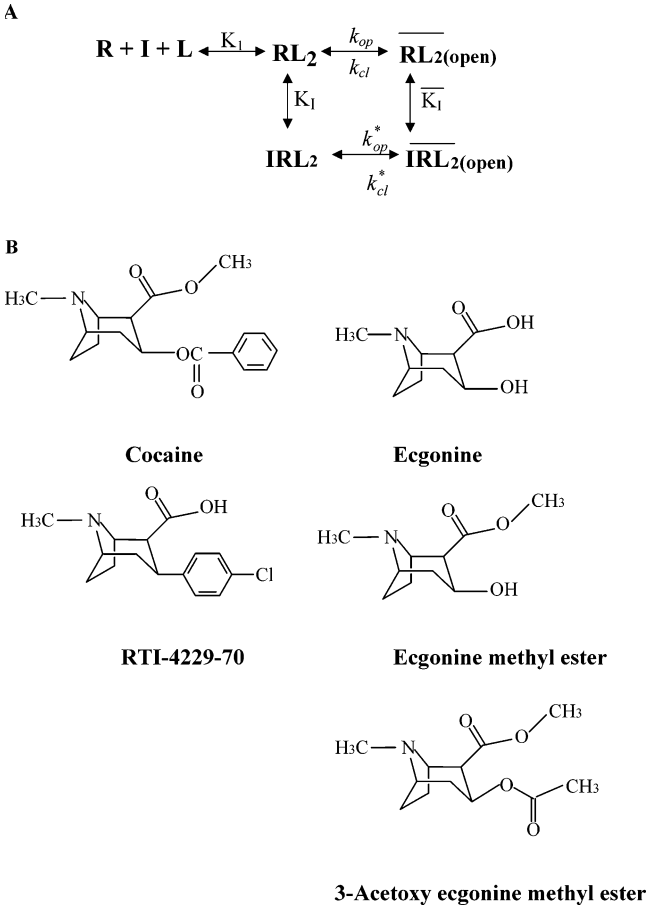

FIGURE 1: (A) Proposed cyclic mechanism of inhibition of the rapidly equilibrating site of the acetylcholine receptor. This mechanism accounts for the prevention of inhibition by ligands that can displace the inhibitor from the rapidly equilibrating site of the receptor (*17*, *19*). K_1 is the observed dissociation constant for the activating ligand L, K_I, the dissociation constant of the inhibitor from the closed-channel conformations RL or RL_2, and $\overline{K_I}$, the dissociation constant of the inhibitor from the open-channel conformation $\overline{RL_2}$. The subscript 2 designates the number of ligand molecules bound to the receptor. Φ is the equilibrium constant for the conversion of RL_2 to $\overline{RL_2}$ ($\Phi = \overline{RL_2}/RL_2$), and Φ_I is the equilibrium constant for the conversion of $\overline{IRL_2}$ to IRL_2 ($\Phi_I = \overline{IRL_2}/IRL_2$). The principle of microscopic reversibility requires that $\Phi_I/\Phi = K_I/\overline{K_I}$. This relationship indicates (i) ligands that bind to a regulatory site on the RL_2 form with higher affinity than to the site on the $\overline{IRL_2}$ form will shift the equilibrium from the open-channel conformation toward the closed-channel conformation and the receptor-mediated reaction will be inhibited; (ii) ligands that bind with equal or higher affinity to the open-channel conformation than to the closed-channel conformation will not change the channel-opening equilibrium in an unfavorable way (*19*). These ligands will not, therefore, inhibit the receptor-mediated reaction but can prevent the binding and action of inhibitors. K_a is the dissociation constant of these ligands. MK-801 and cocaine also bind to a slowly equilibrating inhibitory site, presumably on the interface between the receptor and the membrane (*19*). The mechanism of the inhibition involving this site is not yet known. (B) Structures of cocaine and derivatives used in this study.

equilibrating inhibitory site of the receptor (Figure 1A), compounds that inhibit the receptor were found to bind to a regulatory (allosteric) site on the closed-channel conformation with higher affinity than to the open-channel form (*17*, *21*, *23*). Using rapid chemical kinetic techniques (*15*, *16*), it was possible to determine the rate constants for channel opening (k_{op}) and closing (k_{cl}), in the absence and presence of inhibitors (*17*, *21*). These measurements indicated that the channel-opening equilibrium (k_{op}/k_{cl}) is changed unfavorably by noncompetitive inhibitors and accounts for receptor inhibition. Accordingly, compounds that bind to this allosteric site with equal affinity for the closed- and open-channel forms are not expected to change the channel-opening equilibrium constant. These compounds are, therefore, expected to displace noncompetitive inhibitors without themselves inhibiting the receptor (*19*). The existence of such compounds was verified first using combinatorially synthesized RNA ligands (*18*, *19*).

Can small organic molecules be found that have the same properties as the RNA aptamers? During the last 20 years, many attempts have been made to find compounds that prevent cocaine toxicity, including the synthesis of hundreds of cocaine analogues and derivatives (*9*, *25*), so far without success (*9*). Searching through the literature, we found that one of the compounds previously synthesized, RTI-4229-70 (*26*), bound to receptor-containing membranes with an affinity two orders of magnitude weaker than did the other cocaine analogues studied, and it was, therefore, not tested further (*27*). The mechanism of inhibition of the nAChR suggested that we should test this compound on both the closed- and open-channel forms of the receptor. Using the transient kinetic techniques that we developed, we determined that this compound binds with equal affinity to the open- and closed-channel forms. Therefore, it does not affect the channel-opening equilibrium and can prevent inhibition of the muscle nAChR in BC_3H1 cells by both MK-801 and cocaine (*19*). The compound (RTI-4229-70) and four synthesized analogues of it, all tropane analogues, prevented inhibition of the rapidly equilibrating inhibitory site of the receptor by MK-801 and cocaine (*24*). So far, all of the compounds investigated, the RNA polymers (*18*, *19*) and the tropane analogues (*24*), protect only the rapidly equilibrating inhibitory site of the muscle-type nAChR in BC_3H1 cells. A neuronal-type nAChR, whose $\alpha 3\beta 4$ subunits were expressed in HEK 293 cells, exhibited a mechanism of inhibition by cocaine and MK-801 (*28*) essentially the same as that described for the muscle-type nAChR. It also exhibits two inhibitory processes, one fast and one slow. Alleviation of inhibition of only the fast inhibitory process of this receptor was investigated (*28*). Both RNA aptamers and a tropane derivative [ecgonine methyl ester (EME)] were found to alleviate the rapid inhibitory process (*28*). Here, we present the first evidence that a compound also exists that alleviates the inhibition of the slowly equilibrating site of the muscle-type nAChR.

MATERIALS AND METHODS

Materials. Carbamoylcholine, EME, ecgonine, and cocaine were purchased from Sigma (St. Louis, MO), and (+)-dizocilpine (MK-801) was purchased from Research Biochemicals International (Natick, MA). The chemicals for the cell culture were purchased from Invitrogen/Gibco (Grand Island, NY), Fisher Scientific (Chicago, IL), and Sigma, unless otherwise indicated.

The mammalian clonal BC_3H1 cell line that expresses muscle-type nAChRs was cultured as described (*10*).

Synthesis of 3-Acetoxy EME. EME (0.016 g, 0.33 mM) was added to 3 mL of acetic anhydride, and the solution

was stirred in a boiling water bath at 95 °C for 5.5 h. The remaining acetic anhydride was removed under low pressure (vacuum pump) at about 60 °C and then placed under high vacuum for 24 h at room temperature to remove all of the acetic acid or the anhydride. The resulting oil was dissolved in small amounts of water (1−2 mL) and filtered using Whatman #1 filter paper to remove any insoluble residues. The aqueous solution was frozen and lyophilized to give 3-acetoxy EME, as a yellow oil. Yield: 0.019 g (100%). ^{1}H NMR (D_2O, 300 MHz): δ 2.06 (s, 3H), 2.11−2.46 (m, 6H), 2.83 (s, 3H), 3.51 (dd, $J_1 = 1.9$ Hz, $J_2 = 7.3$ Hz, 1H), 3.80 (s, 3H), 4.01 (br s, 1H), 4.17 (br d, $J = 5.9$ Hz, 1H), 5.32 (dt, $J_1 = 7.5$ Hz, $J_2 = 10.2$ Hz, 1H). ^{13}C NMR (D_2O, 75.5 MHz): δ 17.4, 19.7, 20.8, 29.6, 35.9, 43.2, 50.5, 60.2, 61.0, 61.1, 170.1, 170.4. EIMS *m*/*z* (relative intensity): 242.2 (M + 1, 4), 241.2 (M^+, 26), 210.1 (11), 198.1 (10), 182.2 (100), 168.1 (13), 122.1 (10), 96.1 (35), 94.1 (32), 83.1 (40), 82.1 (90), 67.1 (20), 55.1 (17). HRMS (high-resolution mass spectroscopy, GALBRAITH Laboratories, Inc., Knoxville, TN) calculated for $C_{12}H_{19}NO_4$, 241.1314; found, 241.1315.

Whole-Cell Current Recording. Currents were recorded using the whole-cell configuration (*29*) at room temperature, −60 mV, and pH 7.4. The solution in the recording pipet contained 140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM Hepes, adjusted to pH 7.4. The bath solution contained 145 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 25 mM Hepes at pH 7.4. The resistance of the recording electrode filled with the buffer solution was typically 3−5 MΩ, and the series resistance was 5−6 MΩ. The cells were held at a constant transmembrane voltage of −60 mV and room temperature (22 °C). Whole-cell currents were amplified by using an Axopatch 200B (Axon Instruments) amplifier and filtered at 0.5−2 kHz by using a 40-pole, low-pass, Bessel filter incorporated in the amplifier. The filtered signal was digitized by using a Labmaster DMA 100 kHz digitizing board (Scientific Solutions) controlled by Axon pCLAMP software.

Cell-Flow Method. The use of the flow device for rapid solution exchange at the surface of a cell has been described in detail (*14*, *30*, *31*). The device, consisting of a U-tube and a preincubation tube, allows one to preincubate a solution of a compound with a cell before a solution containing this and/or other compounds, such as the receptor-activating ligand, are allowed to flow over the cell (*31*). Unless stated otherwise, MK-801 or cocaine was equilibrated with the receptor for 50 ms before the carbamoylcholine solution flow over the cell was initiated when investigating the rapidly equilibrating inhibitory site or for 4 s (for MK-801) or 2 s (for cocaine) when investigating the slowly equilibrating site.

Current Correction for Desensitization in Cell-Flow Measurements. The maximum amplitude of the current is a measure of the concentration of open-receptor channels. In cell-flow experiments, we correct the observed maximum current amplitude for the desensitization that occurs while the receptors equilibrate with the channel-activating ligand in the solution flowing over the cell surface (*14*, *32*). This provides the maximum current amplitude corrected for the desensitization.

Data Analysis. The assumptions made in deriving the equations are based on (a) the simplest inhibition mechanism of the nAChR obtained from chemical kinetic measurements (*17*, *21*−*23*) and (b) the mechanism-based alleviation of receptor inhibition (*19*, *24*). Analysis of the data and nonlinear least-squares fitting were done using Origin software (MicroCal, Northampton, MA) (*14*).

$$\frac{A_0}{A_I} = 1 + \frac{I_0}{K_I(\text{obs})} \tag{I-A}$$

$$\frac{A_I}{A_{II}} = 1 + \frac{I_0}{K_{II}(\text{obs})} \tag{I-B}$$

A_0 and A_I or A_{II} are the corrected current measured in the absence and presence of the inhibitor, respectively. A_I represents the current associated with the rapidly equilibrating site and A_{II}, the current associated with the slowly equilibrating inhibitory site. I_0 represents the inhibitor concentration. K_I(obs) and K_{II}(obs) represent the dissociation constants of the inhibitor from the rapidly and slowly equilibrating sites, respectively. K_a and K_{a2} represent the dissociation constants of compounds that alleviate inhibition of the rapidly and slowly equilibrating inhibitory sites of the receptor, respectively.

$$\frac{A_0}{A_I} = 1 + \frac{I_0}{K_I(\text{obs})}\frac{K_a}{K_a + [\text{EME}]} \tag{II-A}$$

When we study the rapidly equilibrating site, we use eq II-A (*19*) to fit the curve and calculate K_I(obs) and K_a; equation II-A assumes a competitive mechanism for the inhibitor and the compounds that prevent inhibition. [EME] represents the concentration of EME. In some experiments, indicated in the text, 3-acetoxy EME was used.

$$\frac{A_I}{A_{II}} = 1 + \frac{I_0}{K_{II}(\text{obs})}\frac{K_{a2}}{K_{a2} + [\text{EME}]} \tag{II-B}$$

When we study only the slowly equilibrating site and after we evaluate K_I(obs) and K_a for the rapidly equilibrating site, we use eq II-B to evaluate K_{II}(obs) and K_{a2} for the slowly equilibrating site.

The smooth lines in the figures were obtained by use of the equation mentioned in the figure captions, and the value of the constants given in the figure captions was evaluated from the experimental data.

RESULTS AND DISCUSSION

The results in Figure 2 indicate that ecgonine has a weak effect in alleviating inhibition of the rapidly equilibrating site by MK-801. The A_0/A_I value obtained when 500 μM MK-801 was preincubated with the receptor for only 50 ms indicates that about 77% of the receptors were inhibited. In the presence of 40 μM ecgonine, ∼60% of the receptors were still inhibited. When the receptors were preincubated with only 150 μM MK-801 but for 4 s, 50 μM ecgonine did not alleviate inhibition of the slowly equilibrating inhibitory site (data not shown).

The effects of EME on the inhibition of the nAChR in BC_3H1 cells by cocaine are shown in Figure 3. When the receptor in BC_3H1 cells is preincubated with 200 μM cocaine for 50 ms, it is inhibited by a factor of about 2 (Figure 3A). The inhibition by 200 μM cocaine is prevented by ∼10 μM EME. The coordinates of the solid line were obtained by

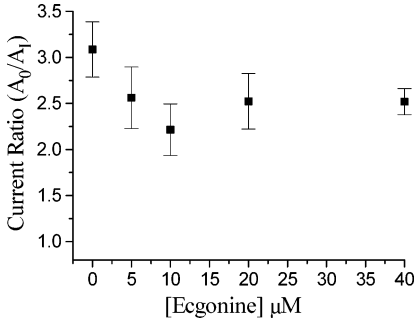

FIGURE 2: Alleviation by ecgonine of the MK-801-induced inhibition of the nAChR (at pH 7.4, −60 mV, and 22 °C). At a constant concentration (100 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the absence, A_0, and presence, A_I, of a constant concentration of 500 μM MK-801 was determined as a function of the concentration of ecgonine. Equation II-A was used to evaluate K_I(obs) and K_a. The cells were preincubated with 500 μM MK-801 for 50 ms before the carbamoylcholine solution flow over the cell was initiated. K_I(obs) = 259 ± 36 μM, and K_a = 35 ± 26 μM. Each data point is the average of 3−10 measurements with at least 3 different cells.

evaluating K_I(obs) and K_a from the measurements using eq II-A. It may be noticed that, at the highest concentration of EME, somewhat less alleviation of inhibition is obtained than expected. Alleviation of receptor inhibition depends on a shift in the channel-opening equilibrium induced by the alleviating compound (*19*). EME may not induce a sufficient shift in this equilibrium when cocaine is the inhibitor. It should be noticed that 3-acetoxy EME completely alleviates inhibition of the rapidly equilibrating inhibitory site when 500 μM MK-801 is the inhibitor in the presence of 100 μM carbamoylcholine (solid line of Figure 5A) but not in the presence of 500 μM carbamoylcholine (dotted line of Figure 5A).

When the receptor is preincubated with 200 μM cocaine for 2 s (Figure 3B), inhibition by the same concentration of cocaine increased by a factor of about 4. In this case, EME in the concentration range of 5−20 μM abolishes only ∼50% of the inhibition (Figure 3B). The results in Figure 3C indicate that EME affects mainly the rapidly equilibrating inhibitory site. In the absence of EME, the ratio of the maximum current amplitude when the receptor is preincubated with 200 μM cocaine for 50 ms, A_I, divided by the maximum current amplitude when the receptor is preincubated with 200 μM cocaine for 2 s, A_{II}, is ∼2.25. This means that the interaction of 200 μM cocaine with the slowly equilibrating inhibitory site decreases the receptor activity by another factor of ∼2. Upon addition of EME, the ratio A_I/A_{II} decreases only slightly. This result indicates, therefore, that the additional inhibition because of the binding of cocaine to the slowly equilibrating inhibitory site is essentially not alleviated by EME.

Similar results to those with cocaine are obtained when MK-801 is the inhibitor (Figure 4). Figure 4A presents the effects of EME on the inhibition of the nAChR by 500 μM MK-801 at low and high concentrations of carbamoylcholine. In these experiments, the receptors were exposed to MK-801 for only 50 ms, before the addition of carbamoylcholine. In the presence of 500 μM carbamoylcholine (dotted line of Figure 4A), a concentration at which the open-channel conformation predominates, 20 μM EME relieves most of the inhibition by 500 μM MK-801, which is a powerful inhibitor of the receptor. Furthermore, as predicted by the

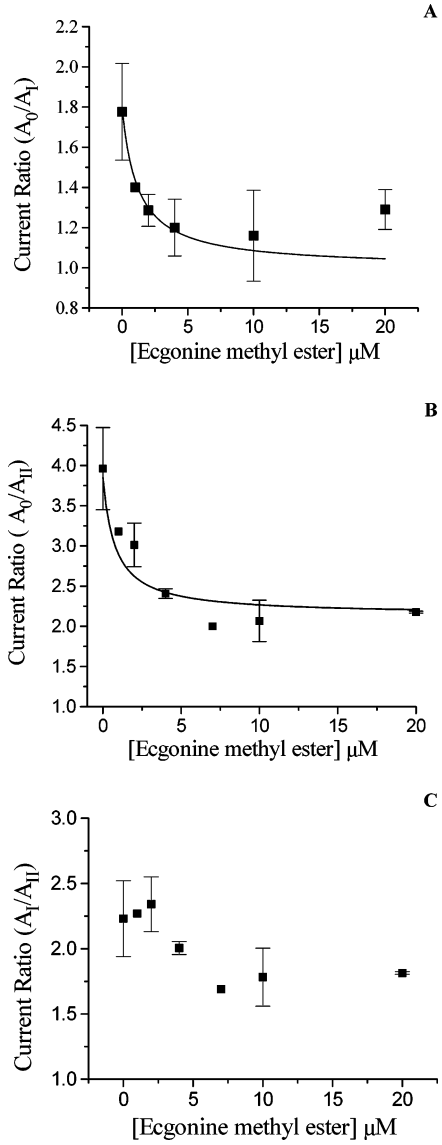

FIGURE 3: Alleviation by EME of cocaine inhibition of the nAChR (at pH 7.4, −60 mV, and 22 °C). (A) At a constant concentration (100 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the absence, A_0, and presence, A_I, of a constant concentration (200 μM) of cocaine was determined as a function of the concentration of EME. Equation II-A was used to evaluate K_I(obs) and K_a. The cells were preincubated with 200 μM cocaine for 50 ms before the carbamoylcholine solution flowed over the cell. K_I(obs) = 252 ± 52 μM, and K_a = 1.2 ± 0.8 μM. (B) Slowly equilibrating inhibitory site. The cells were preincubated with 200 μM cocaine for 2 s. Equations I-B and II-A were used to evaluate the data. K_I(obs) = 251 ± 30 μM, K_{II}(obs) = 175 ± 4 μM, and K_a = 0.8 ± 0.2 μM. A higher concentration of EME (40 μM) did not alleviate the cocaine inhibition more (data not shown). (C) Ratio of the maximum current amplitudes obtained in the presence of 200 μM cocaine without (A_I) and with (A_{II}) preincubation with 200 μM cocaine for 2 s was determined as a function of the concentration of EME. Each data point represents the average of 3−10 measurements with at least 2 different cells.

mechanism of MK-801 inhibition of the rapidly equilibrating site of the nAChR (*19*), EME is as effective in relieving the receptor inhibition in the presence of 100 μM carbamoylcholine when the concentration of receptors in the open-channel form is low (solid line of Figure 4A), as it is at high concentrations (500 μM carbamoylcholine), when most receptors are in the open-channel conformation. The mechanism of MK-801 inhibition of the rapidly equilibrating site

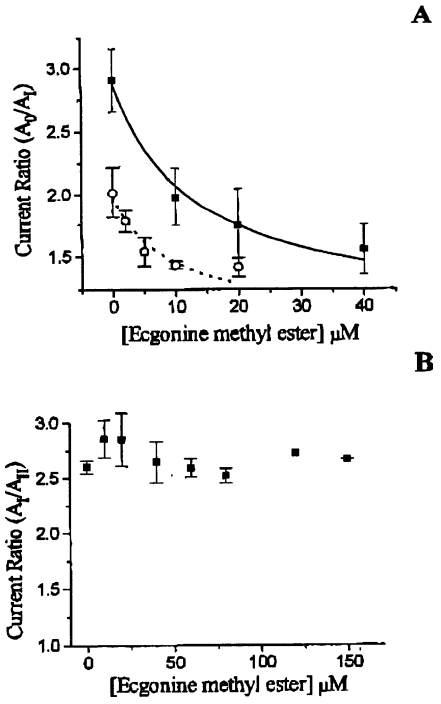

FIGURE 4: Alleviation by EME of MK-801 inhibition of nAChR (at pH 7.4, −60 mV, and 22 °C). (A) Cells were preincubated with 500 μM MK-801 for 50 ms before the carbamoylcholine solution flow over the cells was initiated. Equation II-A was used to evaluate K_I(obs) and K_a. (—) 100 μM carbamoylcholine, with K_I(obs) = 264 ± 13 μM and K_a = 12.7 ± 2 μM; (••••) 500 μM carbamoylcholine, with K_I(obs) = 511 ± 41 μM and K_a = 8.7 ± 2.5 μM. (B) Ratio of the maximum current amplitudes obtained in the presence of 150 μM MK-801 without (A_I) and with (A_{II}) preincubation with MK-801 for 4 s was determined as a function of the concentration of EME. Each data point represents the average of 3−10 measurements with at least 3 different cells.

indicated that inhibition occurs by the compound binding to its site with higher affinity when the receptor is mainly in the closed-channel form (at low carbamoylcholine concentrations) than when the receptor is mainly in the open-channel form (*17*). As observed earlier, this expectation is realized (*19*). In the experiments reported here, the K_I value for MK-801 is 264 ± 13 μM at low concentrations of carbamoylcholine and 511 ± 41 μM at higher concentrations of carbamoylcholine (Figure 4A).

The inhibition mechanism of the rapidly equilibrating inhibitory site of the nAChR also predicts (*19*) that compounds that bind to the inhibitory site with equal affinity for the closed- and open-channel receptor forms will not inhibit the receptor and at the same time can displace inhibitors from their site. The alleviation of MK-801 inhibition of the rapidly equilibrating site of the receptor by EME is shown in Figure 4A. The apparent dissociation constant of EME, K_a, is 12.7 ± 2 μM at low concentrations of carbamoylcholine and about 8.7 ± 2.5 μM at high carbamoylcholine concentrations, indicating that EME binds with about the same affinity whether the receptor is mainly in the closed- or open-channel form conformation.

In the experiments shown in Figure 4B, the receptor was preincubated with 150 μM MK-801 for 4 s before the measurements were made. Considerably more inhibition results in these experiments than when the receptors were equilibrated with 500 μM MK-801 for only 50 ms (Figure 4A). The results in Figure 4B show that EME interacts with only the rapidly equilibrating inhibitory site. They indicate that the ratios of the current amplitude A_I/A_{II}, obtained in the presence of 150 μM MK-801 after preincubation for 50 ms (A_I) and 4 s (A_{II}) are not affected by increasing the EME concentration to 150 μM, meaning that EME does not alleviate inhibition of the slowly equilibrating inhibitory site.

Previous experiments (*17*−*19*) suggested that the second inhibitory site for MK-801 on the nAChR is within the cell membrane rather than on the cytoplasmic surface of the receptor and, therefore, may not be accessible to the negatively charged molecules. To test this suggestion and to make an analogue that is more soluble in the membrane, we modified the structure of EME by acetylating its 3-hydroxy group to obtain 3-acetoxy EME (Figure 1B).

The results in parts A−C of Figure 5 show that, at 100 μM carbamoylcholine, 3-acetoxy EME interacts not only with the rapidly equilibrating inhibitory site (Figure 5A), but also prevents inhibition when MK-801 is allowed to interact with the slowly equilibrating inhibitory site (parts B and C of Figure 5). This is the first small molecule reported that prevents an inhibitor, MK-801, from binding to the slowly equilibrating inhibitory site. Furthermore, 3-acetoxy EME binds to the rapidly equilibrating site with higher affinity than does EME. Under the same conditions of carbamoylcholine and MK-801 concentrations, the value of K_a for EME is 12.7 ± 2 μM (Figure 4A) and for 3-acetoxy EME (Figure 5A) it is 0.76 ± 0.3 μM.

The observation that 3-acetoxy EME prevents MK-801 inhibition of both the rapidly and slowly equilibrating sites of the nAChR indicates the importance of an acetoxy group at the C-3 position of the tropane ring in the interaction of the compound with the nAChR. The experimental results suggest that a hydrogen bond to the C═O group of the acetyl group and/or hydrophilicity may play an important role in the interaction between the drug and the receptor. Interestingly, 3-acetoxy EME alleviates cocaine inhibition of only the slowly equilibrating inhibitory site (Figure 6A) but not of the rapidly equilibrating inhibitory site (Figure 6B). The observation that 3-acetoxy EME alleviates MK-801 inhibition of the rapidly equilibrating site (solid line of Figure 5A) but not cocaine inhibition (Figure 6B) suggests that the rapidly equilibrating inhibitory sites for cocaine and for MK-801 are not identical.

At a high concentration of carbamoylcholine, when the open-channel form is dominant, 3-acetoxy EME did not alleviate the inhibition of the nAChR by MK-801 at the rapidly equilibrating inhibitory site (open circle of Figure 5A). At high carbamoylcholine concentrations, it has only a minor effect in alleviating the inhibition of the slowly equilibrating inhibitory site by MK-801 (dotted line of Figure 5B). These are in contrast to the experimental results obtained with MK-801 in the presence of low, i.e., 100 μM, carbamoylcholine (solid lines of parts A and B of Figure 5). The mechanism of the rapidly equilibrating site indicates, however, that noncompetitive inhibitors bind with higher affinity to the closed-channel form of the receptor (low carbamoylcholine concentrations) than to the open-channel form (high carbamoylcholine concentrations) and, thereby, shift the channel-opening equilibrium toward the closed-channel form (*19*). Consequently, compounds that alleviate receptor inhibition and bind equally to the closed- and open-

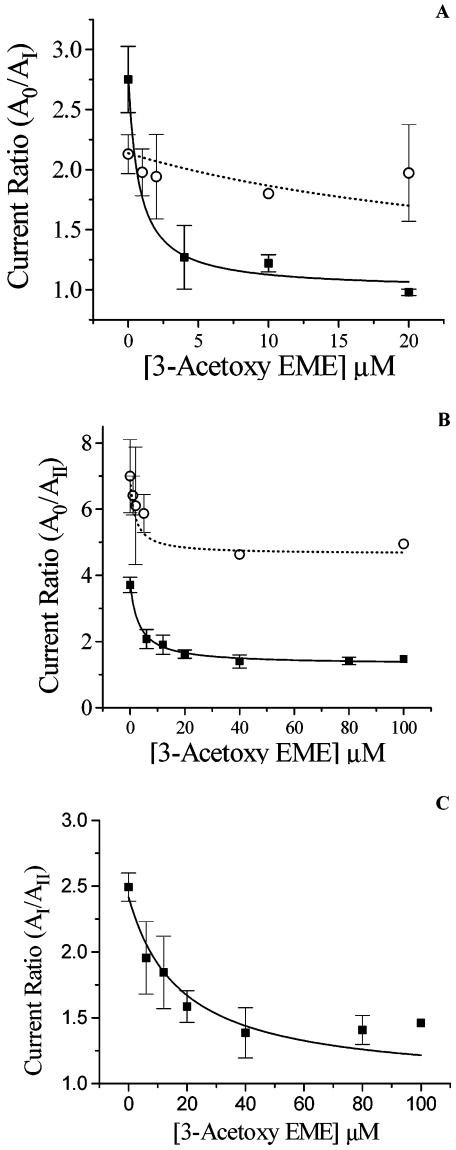

FIGURE 5: Alleviation by 3-acetoxy EME of MK-801 inhibition of the nAChR (at pH 7.4, −60 mV, and 22 °C). (A) Cells were preincubated with 500 μM MK-801 for 50 ms. At a constant concentration (—, 100 μM and ····, 500 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the absence, A_0, and presence, A_I, of a constant concentration (500 μM) of MK-801 was determined as a function of the concentration of 3-acetoxy EME. Equation II-A was used to evaluate K_I(obs) and K_a for the solid line. K_I(obs) = 281 ± 16 μM, and K_a = 0.76 ± 0.3 μM. (B) Cells were preincubated with 150 μM MK-801 (—) or 500 μM MK-801 (····) for 4 s before the flow of a solution of 100 μM (—) or 500 μM (····) carbamoylcholine and the inhibitor over the cell was initiated. At a constant concentration (100 or 500 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the absence, A_0, and presence, A_{II}, of a constant concentration (150 or 500 μM) of MK-801 was determined as a function of the concentration of 3-acetoxy EME. (C) Ratio of the maximum current amplitudes obtained in the presence of 150 μM MK-801 without (A_I) and with (A_{II}) preincubation with MK-801 for 4 s was determined as a function of the concentration of 3-acetoxy EME. Equation II-B was used to evaluate the apparent dissociation constant for the inhibitor and the compounds K_{II}(obs) and K_{a2}(app). K_{II}(obs) = 106 ± 10 μM, and K_{a2} = 18 ± 5 μM. Each data point represents the average of 3−10 measurements with at least 3 different cells.

channel forms are not expected to affect the channel-opening equilibrium but are expected to be effective in displacing the inhibitor (*19*). Indeed, both RNA polymers (*19*) and small

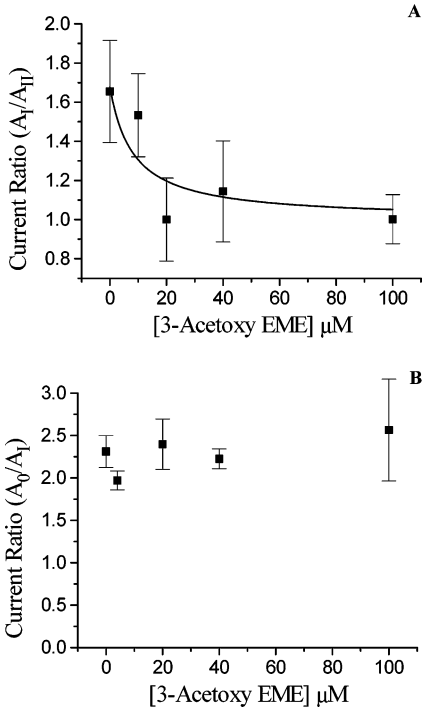

FIGURE 6: Alleviation by 3-acetoxy EME of cocaine inhibition of the nAChR (at pH 7.4, −60 mV, and 22 °C). (A) At a constant concentration (100 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the presence of 200 μM cocaine without (A_I) and with (A_{II}) preincubation with cocaine for 2 s was determined as a function of the concentration of 3-acetoxy EME. Equation II-B was used to evaluate K_{II}(obs) and K_a. K_{II}(obs) = 295 ± 91 μM, and K_a = 8.2 ± 8 μM. (B) At a constant concentration (100 μM) of carbamoylcholine, the ratio of the maximum current amplitudes obtained in the absence, A_0, and presence, A_I, of 200 μM cocaine preincubated with the receptor for 50 ms was determined as a function of the concentration of 3-acetoxy EME. Each data point represents the average of 3−10 measurements with at least 3 different cells.

organic molecules (*24*) were found that fulfill this expectation. As can be seen, EME exhibits this behavior in alleviating MK-801 inhibition at both low and high concentrations of carbamoylcholine at which the open channel exists in low and high concentrations, respectively (Figure 4A), as predicted by the mechanism of inhibition associated with the rapidly equilibrating site (*19*, *24*).

The results in Figure 5 indicate that the mechanism of inhibition of the slowly equilibrating inhibitory site of the muscle-type nAChR is different from that of the rapidly equilibrating inhibitory site. In contrast to the rapidly equilibrating site, considerably less alleviation of receptor inhibition is obtained at high concentrations of carbamoylcholine than at low concentrations. It can be seen that, at even low concentrations of carbamoylcholine, the data for the alleviation of the slowly equilibrating inhibitory site by 3-acetoxy EME (Figure 5C) do not quite fit the calculated solid curve. The solid curve, however, is based on eq II-B derived on basis of the mechanism of the rapidly equilibrating inhibitory site.

SUMMARY

The mechanism of inhibition of the rapidly equilibrating inhibitory site of the nAChR can be investigated by transient kinetic techniques (reviewed in ref *16*). The mechanism

predicted the properties of compounds that would alleviate this inhibition (*19*, *24*). EME shares these properties (parts A and B of Figure 3 and Figure 4A) with the compounds investigated previously (*19*, *24*) and does not alleviate inhibition of the slowly equilibrating inhibitory site (Figure 3C and Figure 4B). The alleviation experiments (*19*, *24*) and those presented here are good indications that one is dealing with two different inhibitory sites. If the slow and rapid inhibitory processes represent two consecutive reactions, one fast and one slow, compounds that alleviate the fast step of the inhibition reaction are also expected to prevent the subsequent slower reaction step. The same argument can be made for parallel inhibition reactions that originate from the same inhibitory binding site. These considerations and the relative slowness of the second inhibition reaction (*17*) suggest that the second inhibitory site is within the cell membrane and not accessible to RNA polymers (*18*, *19*). We, therefore, designed a compound, 3-acetoxy EME, that we expected to be more soluble in the cell membrane than the parent compound EME (Figure 1B), which does not alleviate inhibition of the slowly equilibrating site (Figure 4B). 3-Acetoxy EME is the first known compound that alleviates inhibition of the slowly equilibrating inhibitory site by cocaine and MK-801 (parts B and C of Figure 5 and Figure 6A). In contrast to the other compounds mentioned (*19*, *24*) that alleviate only the rapidly equilibrating inhibitory site, 3-acetoxy EME shows an effect only at low carbamoylcholine concentrations when the receptor is mainly in the closed-channel conformation.

Investigation of neurotransmitter receptor inhibition is a long-standing (*4*, *7*) and intensively investigated problem with major implications for medicine (*4*−*6*). There are more than 5 million cocaine users in the United States alone at an estimated cost to society of ∼60 billion dollars annually (*33*). It is expected that in the case of cocaine users both the rapidly and slowly equilibrating sites of the nAChR will be affected. Here, we presented the first evidence that compounds exist that can prevent cocaine inhibition not only of the rapidly equilibrating inhibitory site, but also of the slowly equilibrating inhibitory site of the nAChR. What the mechanism of inhibition of the slowly equilibrating receptor site is, as well as the development of compounds that alleviate cocaine poisoning in whole animals, present interesting problems for future investigations.

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