Reversing the Action of Noncompetitive Inhibitors (MK-801 and Cocaine) on a Protein (Nicotinic Acetylcholine Receptor)-Mediated Reaction†

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ABSTRACT: The nicotinic acetylcholine receptor (nAChR) is one of five structurally related membrane proteins required for communication between ~10^{12} cells of the mammalian nervous system. The receptor is inhibited by both therapeutic agents and abused drugs. Understanding the mechanism of noncompetitive allosteric inhibitors of the nicotinic acetylcholine receptor is a long-standing and intensely investigated problem. During the past two decades, many attempts have been made to find drugs that prevent cocaine inhibition, including the synthesis of hundreds of cocaine analogues and derivatives, so far without success. The use of newly developed transient kinetic techniques in investigations of the inhibition of the receptor by the anticonvulsant MK-801 [(+)-dizocilpine] and the abused drug cocaine led to an inhibition mechanism not previously proposed. This mechanism indicates the properties of compounds that would prevent allosteric inhibition of the receptor and how to test for such compounds. Here we present the first evidence that small organic compounds (cocaine derivatives) exist that prevent cocaine and MK-801 inhibition of this receptor. These compounds are RTI-4229-70, a previously synthesized cocaine derivative, and based on its structure four newly synthesized cocaine derivatives, RCS-III-143, RCS-III-140A, RCS-III-218, and RCS-III-202A. Because the nAChR desensitizes rapidly, to make the required measurements a cell-flow technique with a time resolution of 10 ms was used to equilibrate BCH_3 cells containing the fetal mouse muscle-type nAChR with carbamoylcholine. The resulting whole-cell current pertaining to the nondesensitized nAChR was determined. Inhibitors and compounds that alleviate inhibition were tested by their effect on the whole-cell current.

The nicotinic acetylcholine receptor (nAChR)[1] is a member of a family of five structurally related membrane proteins that regulate intercellular communication between approximately 10^{12} cells of the nervous system (*1*), a process that is crucial for brain function (*2*). Many therapeutic agents and abused drugs affect its function (*3*). Understanding the functional mechanisms of neurotransmitter receptor function and their inhibition is a long-standing (*4*) and intensively studied problem with major implications for medicine (*3*, *5*, *6*).

Until recently, receptor inhibition has been investigated mainly using techniques in which equilibration of the protein with ligands is slow compared to ensuing reaction steps. Among the techniques that are employed is the widely used single-channel current-recording technique (*7*). Two decades ago, measurements made using this technique led to the proposal of a simple and widely accepted mechanism in which inhibitors enter the open channel and block it (*4*, *8*−*10*) (Figure 1A). Several variations of this open-channel blocking mechanism have been proposed, including the conversion of an inhibitor-bound, closed-channel conformation to a blocked open-channel form (*11*−*14*).

Although a range of well-established methods has been available for decades for investigating rapid chemical reactions of proteins *in solution* (*15*−*18*), techniques in which *membrane-bound* proteins on the surface of cells can be equilibrated with their respective ligands rapidly (microseconds) compared to the ensuing reaction steps have been developed only recently (*19*, *20*). The mechanism of receptor inhibition that is proposed (*25*; Figure 1B) is based on the

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; LaPP, laser-pulse photolysis; SELEX, systematic evolution of ligands by exponential enrichment.

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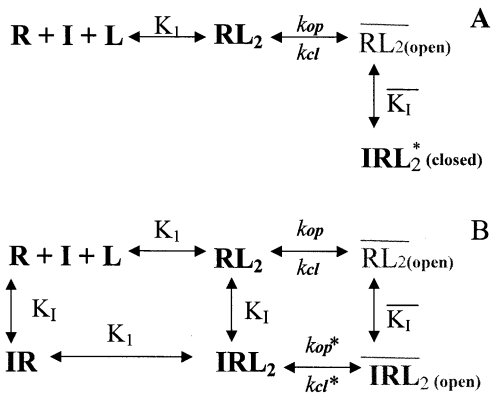

FIGURE 1: Proposed mechanisms for the inhibition of the nAChR by MK-801 and cocaine. In each case, the top line represents the minimum mechanism for the opening of the receptor channel (*47*). Receptor R binds the neurotransmitter L (or another activating ligand, for instance, carbamoylcholine). RL and RL_2 represent the closed-channel conformations. $\overline{RL_2}$ represents the open-channel conformation of the receptor that allows inorganic cations to cross the cell membrane. I represents the inhibitor, and $\overline{IRL_2}$ and IRL_2* represent the open-channel form of the receptor with inhibitor bound and the inhibited open-channel form of the receptor with inhibitor bound, respectively. K_1 is the observed dissociation constant for the activating ligand. K_I and $\overline{K_I}$ are the observed dissociation constants of the inhibitor from the closed- and open-channel receptor forms, respectively. k_{op} and k_{cl} are the rate constants for channel-opening and closing, respectively. k_{op}* and k_{cl}* are the rate constants for the opening and closing, respectively, of the receptor channel when the inhibitor is bound to the open-channel form. The channel-closing equilibrium constants in the absence (Φ) and presence of the inhibitor (Φ*) are given by k_{cl}/k_{op} and k_{cl*}/k_{op*}, respectively. For clarity, the desensitization reaction, which in the case of the nAChR occurs in the 100−500 ms time region (*20, 41*), is not shown. (A) Channel-blocking mechanism in which the inhibitor binds in the open channel and blocks it (*4, 8*−*10*). (B) Proposed cyclic inhibition mechanism involving a complex of the inhibitor with the open-channel conformation in which the open channel is not blocked by the inhibitor (i.e., it conducts ions) (*25*).

use of fast reaction techniques. In the laser-pulse photolysis technique (LaPP), the receptors on the cell surface are equilibrated with a biologically inactive, photolabile precursor of the neurotransmitter, and the precursor is then photolyzed to the neurotransmitter by a laser pulse in the microsecond time region (*19, 20*). The ensuing current due to the opening of receptor channels is measured by the whole-cell current-recording technique (*21*). The LaPP technique allows one to determine not only the rates at which a receptor, such as the nAChR, opens and closes its transmembrane channel but also the channel-opening equilibrium constant, the observed dissociation constants of the ligands that determine the concentration of open receptor channels, and the rate constant of receptor desensitization (*20*). It also allows one to determine the effect of inhibitors {such as the anticonvulsant MK-801 [(+)-dizocilpine] (*22*) and the abused drug cocaine (*23*)} on these rates and consequently on the dissociation constants of inhibitors from both the closed- and open-channel forms of the receptor (reviewed in ref *20*). The use of such transient kinetic studies with the fetal mouse muscle nAChR led to the discovery of two types of inhibition processes induced by MK-801 (*22*). One occurs within 50 ms of equilibration of MK-801 with the receptor, and the other within ~1 s, regardless of whether an inhibitor is applied from inside or outside the cell membrane (*22, 24*). Two types of processes, also within different time regimes, in the inhibition of the same receptor by cocaine have also been identified (*23*). The existence of two inhibitory sites on the nAChR was not described in previous studies (e.g., refs *4* and *8*−*14*). The inhibition mechanism of only the rapidly equilibrating site has been characterized so far (Figure 1B) (*22, 24, 25*) and is the process considered here.

According to this mechanism, a noncompetitive inhibitor of the nAChR binds with higher affinity to the closed-channel form than to the open-channel receptor form, and thereby shifts the channel-opening equilibrium to the closed-channel form and inhibits the receptor (*25*). The mechanism and equations pertaining to it (eq 2) indicated that if one could identify ligands that bind with equal or higher affinity to the inhibitor site on the open- than the closed-channel forms, such a ligand would displace the inhibitor, without changing the channel-opening equilibrium constant unfavorably and, therefore, without inhibiting the receptor (*25*) (eq 2).

To test this concept, we first adapted the elegant systematic evolution of ligands by the exponential enrichment (SELEX) technique of Tuerk and Gold (*26*) and of Ellington and Szostak (*27*) to select combinatorially synthesized RNA ligands that bind to specific sites of the *membrane-bound* protein (*28*). Two classes of RNA ligands, each containing 90 bases, that displace cocaine from the receptor were obtained (*28*). Each class is characterized by a different consensus sequence of six (class II) or seven (class I) nucleotides (*25, 28*). Members of one class bind with higher affinity to the closed-channel form of the nAChR and inhibit the receptor; members of the other class bind with equal affinity to the closed- and open-channel forms and prevent inhibition of the nAChR by cocaine (*25*). This was the first demonstration that molecules that can prevent the action of noncompetitive inhibitors on a protein-mediated reaction can be identified. Although this demonstrated that by combinatorial synthesis at least high-molecular weight RNA ligands can be identified that prevent the action of noncompetitive inhibitors, it does not directly confirm the proposed mechanism (Figure 1B). Because the combinatorially synthesized RNA ligands contain 90 bases and are much larger than the inhibitors they displace from the nAChR, they may exert their action by an as yet unknown mechanism.

Therefore, we asked the following question. Can small molecules, i.e., inhibitor derivatives, be found that act in a manner predicted by the mechanism (Figure 1B)? Are there compounds that bind to the same site as do inhibitors, but with equal or higher affinity for the open-channel receptor conformation than for the closed-channel conformation, and, thereby, prevent receptor inhibition? Here we demonstrate that such compounds exist. Many hundreds of cocaine analogues and derivatives have been synthesized during the past two decades and tested for their effects on the nervous system. Compounds that alleviate cocaine inhibition of a protein have not previously been identified (*5, 29*−*33*).

MATERIALS AND METHODS

Materials. The reagents used were of the highest available quality. Carbamoylcholine was purchased from Sigma, MK-801 [(+)-dizocilpine] from Research Biochemicals International, and cocaine hydrochloride from Sigma. RTI-4229-70 was synthesized as previously reported (*34*). The chemicals for cell culture, DMEM (Dulbecco's modified Eagle's

medium) and FBS (fetal bovine serum), were purchased from Invitrogen/Gibco, and a 10 000 units/mL penicillin and 10 mg/mL streptomycin solution was from Sigma. The reagents for the preparation of the buffers that were used were purchased from Sigma.

BC_3H1 Cell Culture and Electrophysiology. The BC_3H1 cell line expressing the fetal mouse muscle-type nAChR was cultured as described by Sine and Taylor (*35*). Carbamoylcholine-induced currents were recorded using the whole-cell current configuration (*21*) together with the cell-flow technique, which has a time resolution of 10 ms (*36*). In the cell-flow technique, a U-tube, originally developed by Krishtal and Pidoplichko (*37*), is modified and used to apply ligand solutions in a laminar flow, and the maximum observed current is corrected for receptor desensitization that occurs while the ligands in the solution flowing over the cells equilibrate with the cell surface receptors (*36*). Further experimental details have been published (*38*). The currents were amplified using an Axopatch 200B amplifier (Axon Instruments) and filtered at 1−2 kHz (using a four-pole low-pass Bessel filter incorporated in the Axopatch 200B amplifier). The filtered signal was digitized using a Labmaster DMA 100 kHz digitizing board (Scientific Solutions). Typical digitizing frequencies were 2−5 kHz. The recording pipet contained BC_3H1 intracellular buffer (140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2 \cdot 6H_2O$, 1 mM EGTA, and 25 mM HEPES), adjusted to pH 7.4; the bath buffer solution contained 145 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.7 mM $MgCl_2 \cdot H_2O$, and 25 mM HEPES, adjusted to pH 7.4. Typical pipet resistances were 2−3 MΩ. A series resistance compensation of 60−80% was used, via an Axopatch 200B amplifier (Axon Instruments). Typical maximum currents at a carbamoylcholine concentration of 100 μM (control concentration) were 1−4 nA.

All electrophysiological experiments were carried out at 22−23 °C, pH 7.4, and a transmembrane voltage of −60 mV. The cells were pre-equilibrated with the compounds to be tested, for 2 s in the case of RTI-4229-70, RCS-III-143, and RCS-III-140A, for 50 ms in the case of RCS-III-218 and RCS-III-202A, and for 50 ms with the inhibitors. The whole-cell currents presented in the figures have been corrected for desensitization (transient inactivation) of the receptor that occurs during the measurements (*36*).

At a constant concentration of carbamoylcholine, the ratio of the maximum current amplitude obtained in the absence (Amp) and presence (Amp_I) of a constant concentration of MK-801 or cocaine was determined as a function of the concentration of the different compounds to be tested (see Figures 4−6). Each data point represents the average of at least two measurements with at least two cells per point, except in the cases where error bars are not shown; those represent single data points.

Data Analysis. Analysis of the data and nonlinear least-squares fitting were carried out using Origin software (MicroCal, Northampton, MA) according to the method described by Udgaonkar and Hess (*36*). The curves were fitted using eq 1 (*20*) where Amp and Amp_I represent the maximum amplitude of the current in cell-flow experiments (*22*, *36*), in absence and presence of inhibitor, respectively, corrected for desensitization that occurs when the receptors on the cell surface equilibrate with ligand (*36*).

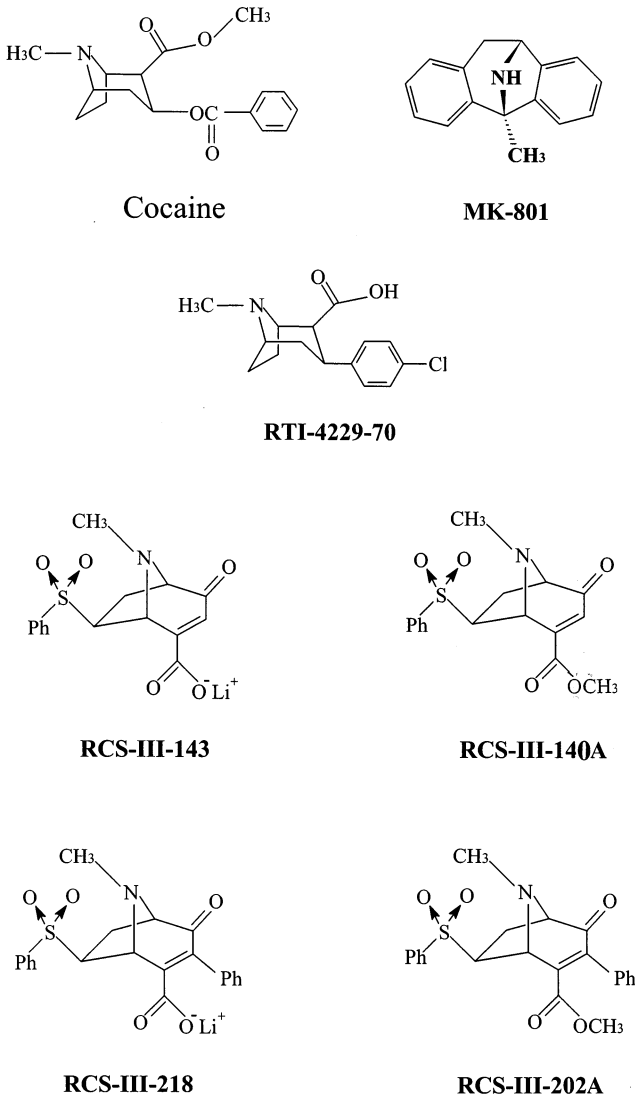

FIGURE 2: Structures of the inhibitors cocaine and MK-801 and the compounds that alleviate inhibition used in this study. RTI-4229-70 was synthesized previously (*34*). RCS-III-143, RCS-III-140A, RCS-III-218, and RCS-III-202A were synthesized in this study and are based on the structure of RTI-4229-70.

$$\mathrm{Amp}/\mathrm{Amp}_I = 1 + ([I]/K_I)\{K_{D(Alv)}/[K_{D(Alv)} + [Alv]]\} \quad (1)$$

The equation assumes that the inhibitor and the compound being tested compete for the same binding site. It also assumes that the maximum current amplitude obtained in transient kinetic experiments is a measure of the concentration of open receptor channels (*20*, *36*). [I] is the molar concentration and K_I the observed dissociation constant of the inhibitor. [Alv] is the molar concentration and $K_{D(Alv)}$ the observed dissociation constant of the compound being tested (RTI and RCS compounds).

Synthesis. The structure of the compounds synthesized is given in Figure 2. RCS-III-140A was synthesized as an inseparable 3:2 mixture of *exo*-7-benzenesulfonyl-2-carbomethoxy-2-tropen-4-one (major) and *exo*-6-benzenesulfonyl-2-carbomethoxy-2-tropen-4-one (minor) cycloadducts by the heteroaromatic [3 + 2] dipolar cycloaddition (*39*) of *N*-methyl-3-carbomethoxy-5-hydroxypyridinium iodide (*40*) with phenyl vinyl sulfone (triethylamine, THF, reflux, 5 days). Hydrolysis of the mixture of cycloadducts (LiOH, 2:1

CH_3OH/H_2O, room temperature, 2 h) afforded RCS-III-143 (Figure 2).

For RCS-III-140A. Major product: ^{1}H NMR ($CDCl_3$) δ 7.97−7.88 (m, 2 H), 7.71−7.54 (m, 3 H), 6.63 (d, 1 H, J = 1.6 Hz), 4.44 (s, 1 H), 3.68 (s, 3 H), 3.63 (d, 1 H, J = 6.4 Hz), 3.54 (dd, 1 H, J = 4.3, 9.1 Hz), 2.94 (ddd, 1 H, J = 4.3, 7.5, 14.5 Hz), 2.39 (s, 3 H), 1.97 (dd, 1 H, J = 9.1, 14.5 Hz). Minor product: ^{1}H NMR ($CDCl_3$) δ 7.97−7.88 (m, 2 H), 7.71−7.54 (m, 3 H), 6.54 (d, 1 H, J = 1.6 Hz), 4.24 (d, 1 H, J = 6.4 Hz), 3.97 (s, 1 H), 3.80 (s, 3 H), 3.38 (dd, 1 H, J = 7.5, 8.6 Hz), 2.77 (m, 1 H), 2.46 (s, 3 H), 2.08 (dd, 1 H, J = 8.6, 12.9 Hz). Mixture MS: FABMS m/z 336 (M + 1, 100%).

For RCS-III-143. Major product: ^{1}H NMR (DMSO-d_6) δ 7.92−7.82 (m, 2 H), 7.76−7.57 (m, 3 H), 5.98 (d, 1 H, J = 1.6 Hz), 4.38 (s, 1 H), 3.63 (dd, 1 H, J = 4.3, 9.1 Hz), 3.25 (d, 1 H, J = 7.5 Hz), 2.57 (ddd, 1 H, J = 4.3, 7.5, 14.0 Hz), 2.12 (s, 3 H), 1.85 (m, 1 H). Minor product: ^{1}H NMR (DMSO-d_6) δ 7.92−7.82 (m, 2 H), 7.76−7.57 (m, 3 H), 5.95 (d, 1 H, J = 1.1 Hz), 4.02 (d, 1 H, J = 6.4 Hz), 3.82 (m, 1 H), 3.56 (d, 1 H, J = 1.1 Hz), 2.36 (m, 1 H), 2.24 (s, 3 H), 1.88 (m, 1 H). Mixture MS: FABMS m/z 328 (M + 1, 100%).

In the same fashion, RCS-III-202A was synthesized as an inseparable 8:3 mixture of *exo*-7-benzenesulfonyl-2-carbomethoxy-3-phenyl-2-tropen-4-one (major) and *exo*-6-benzenesulfonyl-2-carbomethoxy-3-phenyl-2-tropen-4-one (minor) cycloadducts (Figure 1B) from *N*-methyl-3-carbomethoxy-4-phenyl-5-hydroxypyridinium iodide and phenyl vinyl sulfone. Hydrolysis of RCS-III-202A (LiOH, 2:1 CH_3OH/H_2O, room temperature, 19 h) afforded RCS-III-218 (Figure 2).

For RCS-III-202A. Major product: ^{1}H NMR ($CDCl_3$) δ 7.96 (d, 2 H, J = 8.1 Hz), 7.67 (m, 1 H), 7.59 (m, 2 H), 7.34−7.27 (m, 3 H), 7.09−7.04 (m, 2 H), 4.38 (s, 1 H), 3.87 (dd, 1 H, J = 4.8, 9.7 Hz), 3.77 (d, 1 H, J = 7.5 Hz), 3.43 (s, 3 H), 2.96 (ddd, 1 H, J = 4.8, 7.5, 14.5 Hz), 2.56 (s, 3 H), 2.10 (dd, 1 H, J = 9.7, 14.5 Hz). Minor product: ^{1}H NMR ($CDCl_3$) δ 7.93 (d, 2 H, J = 8.1 Hz), 7.67 (m, 1 H), 7.59 (m, 2 H), 7.34−7.27 (m, 3 H), 7.09−7.04 (m, 2 H), 4.15 (d, 1 H, J = 6.4 Hz), 4.08 (s, 1 H), 3.55 (m, 1 H), 3.51 (s, 3 H), 2.84 (m, 1 H), 2.59 (s, 3 H), 2.37 (dd, 1 H, J = 9.1, 12.9 Hz). Mixture MS: FABMS m/z 412 (M + 1, 100%).

For RCS-III-218. Major product: ^{1}H NMR (DMSO-d_6) δ 7.86 (d, 2 H, J = 8.0 Hz), 7.73 (m, 1 H), 7.63 (m, 2 H), 7.24−7.11 (m, 5 H), 4.08 (s, 1 H), 4.03 (dd, 1 H, J = 4.3, 9.1 Hz), 3.37 (d, 1 H, J = 7.0 Hz), 2.62 (ddd, 1 H, J = 4.3, 7.5, 14.0 Hz), 2.30 (s, 3 H), 2.00 (dd, 1 H, J = 9.1, 14.0 Hz). Minor product: ^{1}H NMR (DMSO-d_6) δ 7.96 (d, 2 H, J = 8.0 Hz), 7.73 (m, 1 H), 7.63 (m, 2 H), 7.24−7.11 (m, 3 H), 3.93 (dd, 1 H, J = 7.5, 8.6 Hz), 3.75 (d, 1 H, J = 5.9 Hz), 3.70 (s, 1 H), 2.43 (m, 1 H), 2.38 (s, 3 H), 2.24 (m, 1 H). Mixture MS: FABMS m/z 404 (M + 1, 100%).

RESULTS AND DISCUSSION

To understand the predictions of an inhibition mechanism, it is necessary to examine the equations that pertain to this mechanism. A useful approach in presenting these equations is to take the ratio of the concentration of open receptor channels in the absence of an inhibitor to the concentration of open receptor channels in the presence of the inhibitor. All the symbols are described in the legend of Figure 1. In deriving eq 2 below, we have assumed that L and $I \gg R_O$ (the concentration of receptor sites in the cell membrane; L, I, and R represent molar concentrations). It is also assumed that the values of the dissociation constant K_1 of the activating ligand L are the same for all its sites on the closed-channel form of the receptor. It is further assumed the K_I values for the inhibitors are the same for all their sites on the closed-channel receptor form. The equation is based on the observation that during the time of measurements the receptor is not completely inhibited by high concentrations of the inhibitor (*22*, *25*). The dissociation constant of the inhibitor from the open-channel receptor conformation is designated as $\overline{K_I}$. Amp and Amp_I represent the maximum current amplitude corrected for receptor desensitization that occurs while the channel-activating ligand (carbamoylcholine) equilibrates with the cell surface receptors, in the absence and presence of the inhibitor, respectively.

The equation for the cyclic inhibition mechanism in Figure 1B (eq 2) is

$$\frac{\text{Amp}}{\text{Amp}_I} = \frac{(L + K_1)^2\Phi\left(1 + \frac{I_O}{K_I}\right) + L^2\left(1 + \frac{I_O}{\overline{K_I}}\right)}{[(K_1 + L)^2\Phi + L^2]\left(1 + \frac{I_O}{\overline{K_I}}\right)} \quad (2)$$

In this equation, the terms $(L + K_1)^2\Phi$ and L^2 divided by $(K_1 + L)^2\Phi + L^2$ represent the fractions of the receptor in the closed- and open-channel conformations, respectively (*20*).

The equation for the cyclic inhibition mechanism makes two predictions. When the dissociation constant of the inhibitor from the closed-channel conformation K_I is smaller than that from the open-channel form $\overline{K_I}$ (eq 2), the mechanism (Figure 1B) predicts that a compound with a higher affinity for the closed-channel form than for the open-channel form will shift the channel-opening equilibrium toward the closed-channel form and will inhibit the receptor (*25*). The mechanism also predicts that an inhibitor that binds preferentially to the closed-channel conformation of the receptor will be more effective at low concentrations of the activating ligand, where the fraction of receptors in the closed-channel conformation is larger than the fraction of the receptors in the open-channel conformation. Figure 4 illustrates this point with MK 801 [(+)-dizocilpine] (Figure 2), an inhibitor of the nAChR.

These findings are in contrast to what is predicted by the open-channel blocking mechanism (eq 3). In that mechanism, the inhibitor binds to the open-channel form of the receptor and is, therefore, expected to be more efficient at higher concentrations of L when the receptor is mainly in the open-channel conformation.

$$\frac{\text{Amp}}{\text{Amp}_I} = \frac{(L + K_1)^2\Phi + L^2\left(1 + \frac{I_O}{\overline{K_I}}\right)}{(L + K_1)^2\Phi + L^2} \quad (3)$$

A second and important prediction made by the cyclic inhibition mechanism (Figure 1B) is that an inhibitor

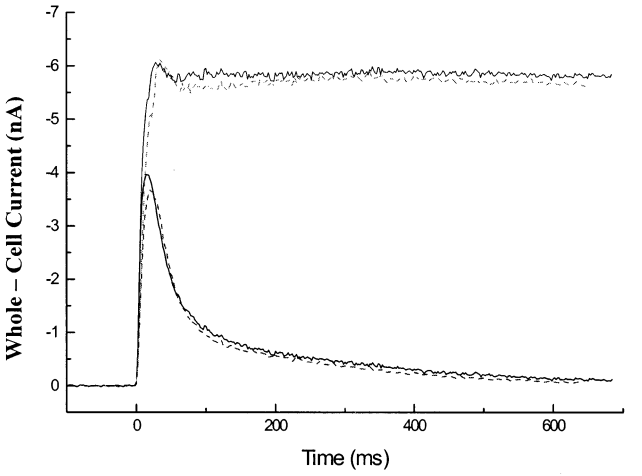

FIGURE 3: Cell-flow experiment with 100 μM carbamoylcholine (—) and 100 μM carbamoylcholine with 80 μM RCS-III-218 (- - -). The two upper lines represent the current amplitudes corrected for desensitization (*36*).

derivative that binds with equal affinity to the open- and closed-channel conformations will not inhibit the receptor (*25*). Inspection of eq 2 indicates that when the dissociation constants of the compound from the closed-channel form K_I and open-channel form $\overline{K_I}$ are the same, the ratio Amp/Amp$_I$ (eq 2) equals 1. This means that the concentration of open-receptor channels is the same in the presence and absence of this compound. If compounds that bind with equal affinity to the closed- and open-channel forms also bind to the same site as the inhibitors, they are expected to displace the inhibitors from their site and prevent receptor inhibition (Figures 4−6).

In looking at previously synthesized compounds, we found the investigations of Lerner-Marmarosh et al. (*33*) to be of special interest. These authors synthesized 16 tropane derivatives, including RTI-4229-70 (*34*) (Figure 2), and evaluated their effectiveness in antagonizing the behavioral effects of cocaine and their ability to compete with [^{3}H]mecamylamine, [^{3}H]nicotine, and [^{3}H]-3-quinuclidinylbenzylate binding to nAChRs in calf brain membranes. Of the 16 derivatives, RTI-4229-70 was the only compound that had a binding affinity 2 orders of magnitude *weaker* than those of the other cocaine analogues studied with these membranes (*33*), under conditions in which the nAChR was in the closed-channel form. It was, therefore, rejected for further evaluation in those studies. However, for this very reason, it appeared to be a candidate for testing the proposed mechanism of nAChR inhibition (Figure 1B) (*25*). The question asked in this report is as follows. Does RTI-4229-70 bind with equal affinity to the closed- and open-channel nAChR forms and thereby prevent receptor inhibition by cocaine?

Because the muscle-type nAChR is desensitized within the millisecond time domain (Figure 3) (*41*), transient kinetic techniques (*20*, *36*) suitable for investigating reactions on cell surfaces in the microsecond to millisecond time region were required for the measurements. The cell-flow technique with a time resolution of 10 ms (*36*) was used here.

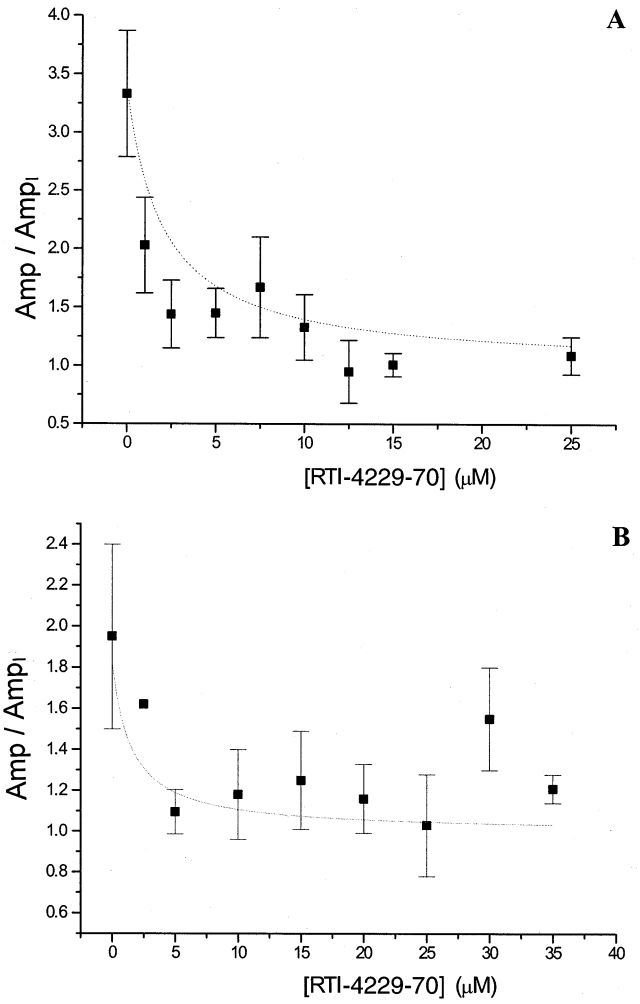

FIGURE 4: Alleviation by RTI-4229-70 of MK-801 inhibition of the nAChR, determined by using the cell-flow technique at (A) low and (B) high concentrations of carbamoylcholine. $K_{D(Alv)}$ is the dissociation constant of the compound alleviating inhibition. (A) With 100 μM carbamoylcholine and 500 μM MK-801, K_I = 210 ± 40 μM and $K_{D(Alv)}$ = 1.5 ± 0.7 μM. (B) With 500 μM carbamoylcholine and 500 μM MK-801, K_I = 608 ± 190 μM and $K_{D(Alv)}$ = 1.5 ± 1.5 μM.

Typical cell-flow experiments are shown in Figure 3. The solid line is from an experiment in which a BC_3H1 cell was equilibrated with carbamoylcholine and the resulting whole-cell current was measured using a technique developed by Hamill et al. (*21*). The current first rises as a result of opening of transmembrane channels and then falls as a result of receptor desensitization. The lines parallel to the abscissa give the current corrected for receptor desensitization that occurs while the receptors equilibrate with the activating ligand (*22*, *36*). The dashed lines represent an identical experiment in which a compound that alleviates receptor inhibition, RCS-III-218, was also present at a concentration of 80 μM. Similarly, all the compounds that were tested that alleviate (Figures 4−6) receptor inhibition have no effect on the carbamoylcholine-induced current. The compounds we investigated were RTI-4229-70 (*34*) [3β-(4-chlorophenyl)tropane-2β-carboxylic acid] and, based on its structure, four new, unsaturated, tropane derivatives, RCS-III-143, RCS-III-140A, RCS-III-218, and RCS-III-202A (Figure 2). In compounds RCS-III-218 and RCS-III-202A, the C3 benzoate group of cocaine has been replaced with an aryl

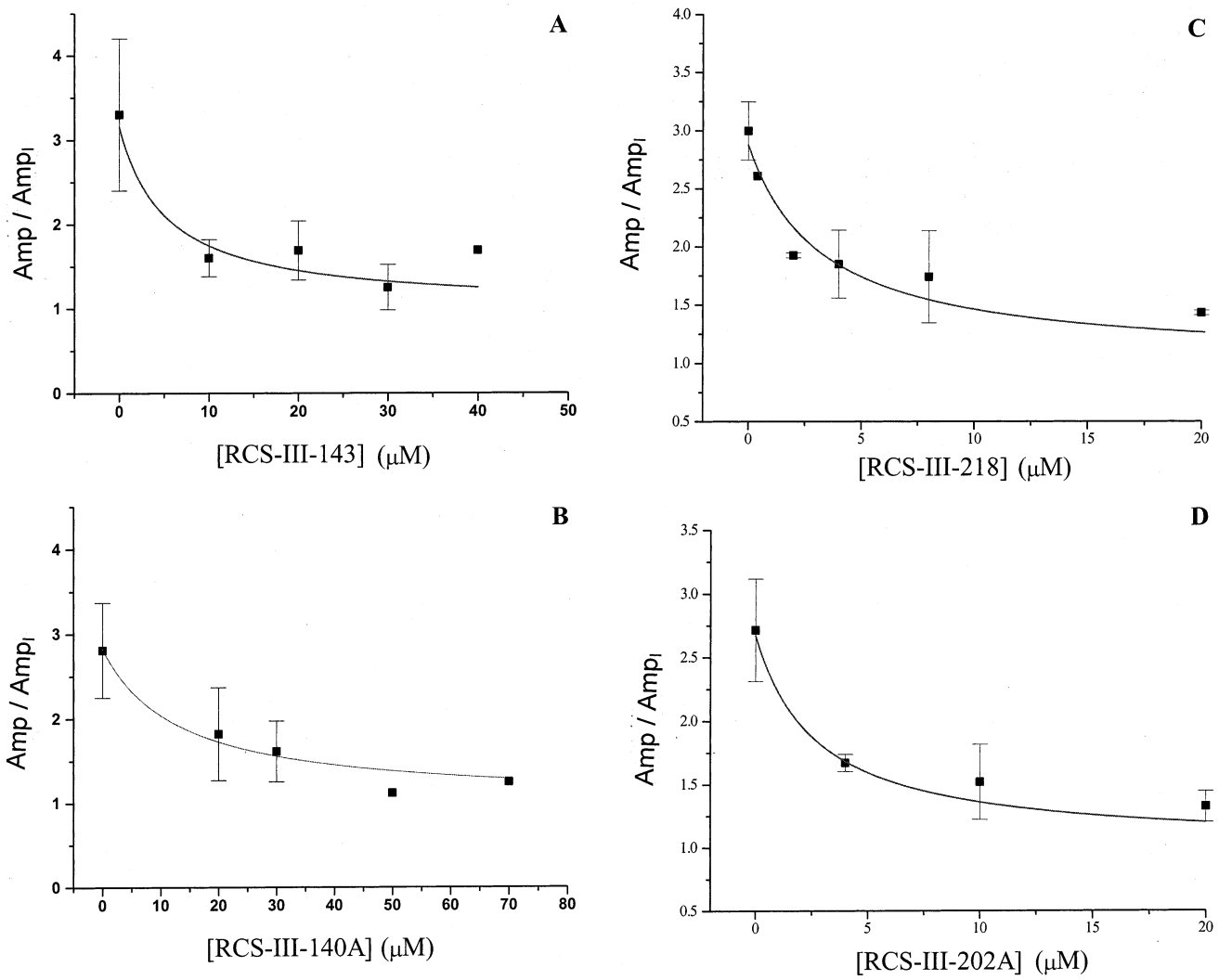

FIGURE 5: Alleviation by RCS derivatives of MK-801 inhibition of the nAChR, determined by using the cell-flow technique. Carbamoylcholine (100 μM) and MK-801 (500 μM) were present in all the experiments. (A) With RCS-III-143, $K_I = 232 \pm 96$ μM and $K_{D(Alv)} = 5 \pm 4$ μM. (B) With RCS-III-140A, $K_I = 274 \pm 84$ μM and $K_{D(Alv)} = 13 \pm 11$ μM. (C) With RCS-III-218, $K_I = 266 \pm 22$ μM and $K_{D(Alv)} = 3.3 \pm 1.0$ μM. (D) With RCS-III-202A, $K_I = 299 \pm 72$ μM and $K_{D(Alv)} = 2.8 \pm 1.2$ μM.

group. It has previously been shown (*42*) that the potency and selectivity of the interaction of cocaine with the dopamine transporter, a major target of cocaine, can be enhanced by replacing its C3 benzoate group with an aryl group. The dipolar cycloaddition approach was designed to accommodate the synthesis of chemical libraries, and represented a simple method for preparing tropanes embodying a 3-aryl substituent within an unsaturated ketocarboxylic acid/ester system. Such compounds might also be used to explore the effect of additional spatial and rotational restriction on the aryl substituent, whose binding site on the dopamine transporter is thought to be a hydrophobic barrel-shaped cavity (*43*).

Activity of the Compounds on the nAChR. None of the tropane derivatives that were tested was an inhibitor or an activator of the nAChR (Figure 3), but all were active in alleviating the inhibition by MK-801 [(+)-dizocilpine] at the rapidly equilibrating site (*22*, *24*) of the nAChR (Figures 4 and 5). RTI-4229-70 (Figure 6A), RCS-III-218 (Figure 6B), and RCS-III-140A and RCS-III-202A (Figure 6C) alleviated inhibition by cocaine of the nAChR (Table 1). RTI-4229-70 was chosen for further characterization.

Comparison of the Activities of RTI-4229-70 at Low and High Concentrations of Carbamoylcholine. RTI-4229-70 (Figure 4A), the most potent of the tropane derivatives that were tested, has the same apparent dissociation constant, $K_{D(Alv)}$, of 1.5 μM at 100 and 500 μM carbamoylcholine in relieving the inhibition of the nAChR by MK-801. As mentioned, the proposed inhibition mechanism (*25*) indicates that compounds that alleviate receptor inhibition bind with essentially equal affinity to the open- and closed-channel forms of the receptor. Therefore, these compounds do not change the channel-opening equilibrium and do not inhibit (*25*) (see eq 2). In contrast, compounds that inhibit are expected to bind with higher affinity to the closed-channel than the open-channel form and thereby shift the channel-opening equilibrium to the closed-channel form (*25*) (see eq 2). As can be seen from the experiments depicted in Figure 4, the K_I value of the inhibitor MK-801 is approximately 3 times larger at 500 μM carbamoylcholine than at 100 μM carbamoylcholine. Similar results have previously been found with cocaine (*23*). The available data indicate that at 500 μM carbamoylcholine the fraction of the nAChRs in BC_3-H1 cells in the open-channel form is approximately 3 times

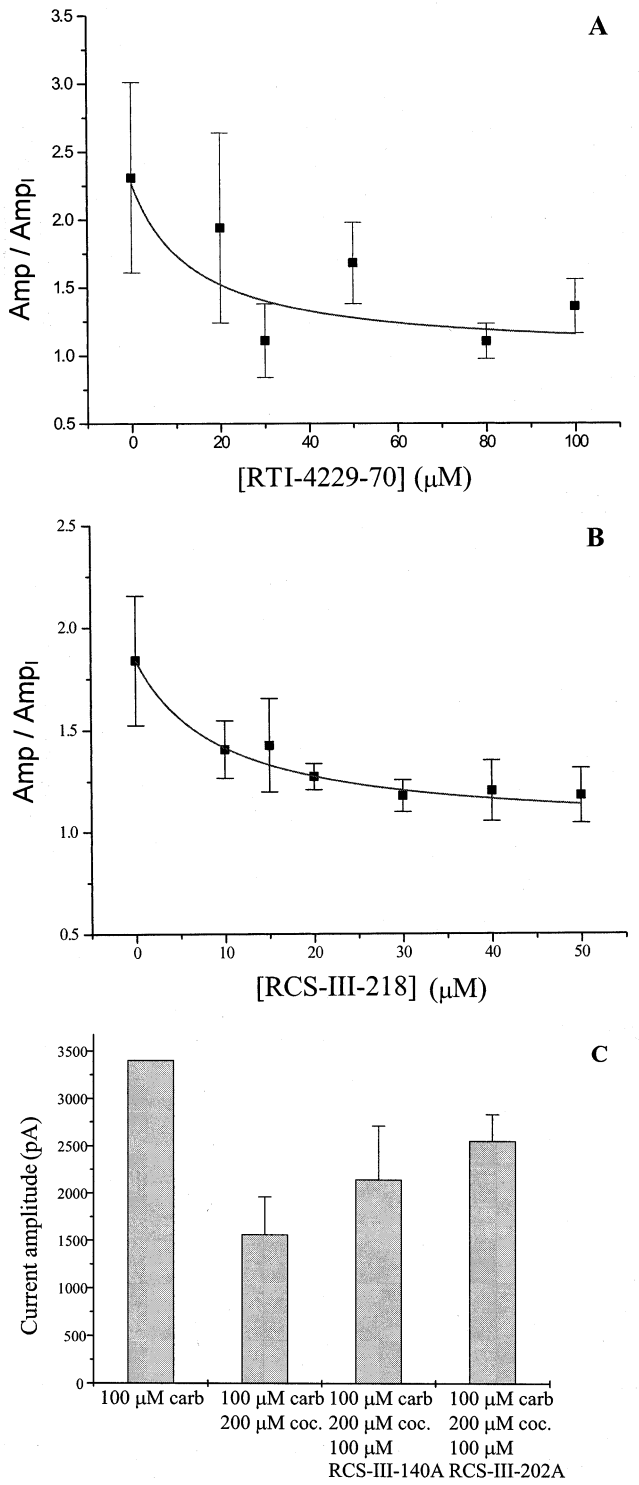

FIGURE 6: Alleviation by RCS derivatives of cocaine inhibition of the nAChR, determined by using the cell-flow technique. Carbamoylcholine (100 μM) and cocaine (200 μM) were present in the experiments in panels A−C. (A) For alleviation by RTI-4229-70 of cocaine inhibition of the nAChR, $K_I = 158 \pm 86$ μM and $K_{D(Alv)} = 14 \pm 12$ μM. (B) For alleviation by RCS-III-218, $K_I = 239 \pm 13$ μM and $K_{D(Alv)} = 11 \pm 2$ μM. (C) For alleviation by RCS-III-140A and by RCS-III-202A, at a constant concentration (100 μM) of carbamoylcholine, the current amplitude was obtained in the absence and presence of a constant concentration (200 μM) of cocaine and in the presence of 100 μM RCS-III-140A or RCS-III-202A.

greater than at 100 μM carbamoylcholine (*36*). Thus, both the inhibitors and their derivatives that prevent inhibition have properties consistent with the predictions of the inhibition mechanism (Figure 1B, eq 2).

Comparison of the Activities of RTI-4229-70, RCS-III-143, RCS-III-140A, RCS-III-218, and RCS-III-202A. Although RTI-4229-70 is slightly more potent than the RCS compounds that have been studied, the activity of these compounds in alleviating MK-801 inhibition of the nAChR is very similar (see Figures 5 and 6 and Table 1). In the case of the most active compounds, RTI-4229-70 and RCS-III-218 (Figure 2), we demonstrated that they alleviate cocaine inhibition, and also determined their apparent dissociation constant, $K_{D(Alv)}$, in this process (Figure 6A,B and Table 1). We also determined that RCS-III-140A and RCS-III-202A alleviate cocaine inhibition (Figure 6C).

In the study described in this paper, a transient kinetic technique was used to confirm predictions arising from a proposed mechanism of inhibition. Compounds that alleviate noncompetitive inhibition of a channel-forming protein were found, and it was demonstrated that analogues of noncompetitive inhibitors that are likely to bind to the same site are good candidates in such a search.

Transient kinetic techniques developed for the investigation of soluble proteins have provided essential insights into the mechanism by which enzymes accelerate and regulate the metabolism of essential cellular components and translate the nucleic acid code (*17*, *18*, *46*). The results obtained here indicate that transient kinetic techniques adapted to investigations of reactions mediated by membrane-bound proteins (*19*, *20*) may be equally useful. Because both rapidly and slowly equilibrating inhibitor-binding sites exist on the nAChR, and because the inhibitor binds to an open-channel form with a lifetime of only milliseconds, the use of transient kinetic techniques in which equilibration of the receptor with ligands is rapid compared to the ensuing steps of the reaction was essential (see Figure 3). The mechanism of inhibition of the muscle nAChR suggested not only the properties of compounds that could prevent receptor inhibition but also the approach needed to find such compounds. In previous searches for compounds that prevent receptor inhibition, candidates were tested for their ability to bind to the closed-channel form (*33*). The proposed mechanism for receptor inhibition (*22*, *24*, *25*) indicates that in a search for compounds that prevent inhibition, it is important to determine their dissociation constants from both the closed- and open-channel forms (*25*). Because the lifetimes of open-channel forms of neurotransmitter receptors lie in the millisecond time domain (see Figure 3), transient kinetic techniques are essential for these investigations.

The mechanism of action of noncompetitive inhibitors on protein-mediated reactions is only generally understood; i.e., these inhibitors do not bind to the same site as ligands that activate the protein. In the case of neurotransmitter receptors, an equilibrium between active (open-channel) and inactive (closed-channel) conformations is easily observed and measured. The observation that inhibition of the nAChR is brought about by noncompetitive inhibitors changing the channel-opening equilibrium unfavorably is new, and as we have also shown, such an effect of inhibitors can be reversed. It will be exciting to determine whether similar mechanisms pertain not only to other neurotransmitter receptor-mediated reactions but also to those mediated by other proteins. At least the effects of cocaine and MK-801 on the nAChR can

Table 1: Activity of the Cocaine Analogues Used in This Study in Alleviating the Inhibition of the nAChR by MK-801 and by Cocaine[a]

	100 μM carbamoylcholine and 500 μM MK-801		500 μM carbamoylcholine and 500 μM MK-801		100 μM carbamoylcholine and 200 μM cocaine	
compound	K_I (MK-801)[b] (μM)	$K_{D(Alv)}$ (compound) (μM)	K_I (MK-801)[1] (μM)	$K_{D(Alv)}$ (compound) (μM)	K_I (cocaine) (μM)	$K_{D(Alv)}$ (compound) (μM)
RTI-4229-70	210 ± 40	1.5 ± 0.7	608 ± 190	1.5 ± 1.5	158 ± 86	14 ± 12
RCS-III-143	232 ± 96	5 ± 4				
RCS-III-140A	274 ± 84	13 ± 11				
RCS-III-218	266 ± 22	3.3 ± 1.0			239 ± 13	11 ± 2
RCS-III-202A	299 ± 72	2.8 ± 1.2				

[a] The dissociation constants were determined as described in the text. [b] Values of the apparent dissociation constant of the inhibitor MK-801 of the nAChR were determined previously by Grewer and Hess (*22*): for the closed-channel form, K_I (MK-801) = 180 ± 36 μM, and for the open-channel form, K_I (MK-801) = 950 ± 400 μM.

be reversed by inhibitor analogues. It is to be expected that in this and possibly other protein-mediated reactions such molecules will be of clinical interest.

APPENDIX

Equation 2 is based on the mechanism in Figure 1B (*L*, *I*, and *R* represent molar concentrations).

(1) In the absence of an inhibitor, the maximum current amplitude is given by the molar concentration of $\overline{RL_2}$(open) (Figure 1B).

(2) When R_O represents the total molar concentration of receptor sites in the membrane

$$R_O = R + RL + RL_2 + \overline{RL_2}(\text{open}) = \overline{RL_2}(\text{open})\left(\frac{K_1^2\Phi}{L^2} + \frac{2K_1L\Phi}{L^2} + \frac{L^2\Phi}{L^2} + \frac{L^2}{L^2}\right)$$

$$\text{Amp} = \overline{RL_2}(\text{open}) = \frac{R_O L^2}{(K_1 + L)^2\Phi + L^2}$$

Amp and Amp_I are the maximum current amplitudes in the absence and presence of an inhibitor, respectively, obtained in cell-flow experiments and corrected for receptor desensitization. In the presence of an inhibitor, Amp_I represents the combined concentration of $\overline{RL_2}$ and $\overline{IRL_2}$ (Figure 1B).

$$R_O = \overline{RL_2}(\text{open})\left[\frac{K_1^2\Phi + 2K_1L\Phi + L^2\Phi}{L^2}\left(1 + \frac{I_O}{K_I}\right) + \frac{L^2}{L^2}\left(1 + \frac{I_O}{\overline{K_I}}\right)\right]$$

$$\text{Amp}/\text{Amp}_I = \frac{R_O L^2\left(1 + \frac{I_O}{\overline{K_I}}\right)}{K_1 + L^2\Phi\left(1 + \frac{I_O}{K_I}\right) + L^2\left(1 + \frac{I_O}{\overline{K_I}}\right)}$$

Amp/Amp_I is given by eq 2.

When the inhibitor binds only to the open-channel form and the resulting complex is inactive

$$\text{Amp}_I = \overline{RL_2}(\text{open}) = \frac{R_O L^2}{(L + K_1)^2\Phi + L^2\left(1 + \frac{I_O}{K_I}\right)}$$

and Amp/Amp_I is given by eq 3.

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