Mechanism-Based Approach to the Successful Prevention of Cocaine Inhibition of the Neuronal (α3β4) Nicotinic Acetylcholine Receptor†

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ABSTRACT: The nicotinic acetylcholine receptor (nAChR) belongs to a family of five channel-forming proteins that regulate communication between the approximately 10^{12} cells of the nervous system. A minimum mechanism of inhibition of the muscle-type nAChR (*1*) by the noncompetitive inhibitors cocaine and MK-801 [(+)-dizocilpine, an anticonvulsant] indicated they bind to a regulatory site, with higher affinity for the closed-channel form than for the open-channel form, thus shifting the equilibrium toward the closed-channel form and inhibiting receptor function. The mechanism predicts that compounds that bind to this regulatory site with equal or higher affinity for the open-channel conformation than for the closed-channel conformation will prevent receptor inhibition (*1*). Does a neuronal form of the receptor behave similarly? The mechanism of inhibition of the neuronal nAChR by cocaine and MK-801 using rapid chemical kinetic techniques was investigated. The α3β4 nAChR stably expressed in HEK 293 cells was used in these investigations. Whole-cell currents originated from a major and minor nAChR isoform. Only the major isoform has been characterized. For the dominant, rapidly desensitizing isoform, the carbamoylcholine dissociation constant for the site controlling receptor activation, K_d, is 2 mM; the channel-opening equilibrium constant, Φ^{-1}, is 4; and the dominant desensitization rate constant, k_{34}, is 20 s^{-1}. Cocaine inhibits the receptor noncompetitively, with an apparent K_I of 84 and 26 μM at high and low carbamoylcholine concentrations, at which concentrations the receptor is mainly in the open- or closed-channel form, respectively. Similar results were obtained with MK-801. A combinatorially synthesized RNA ligand and a cocaine analogue alleviated cocaine inhibition of this neuronal receptor.

Ionotropic neurotransmitter receptors are integral membrane proteins that mediate signal transmission between neurons and at neuromuscular junctions (*2*). The muscle-type nicotinic acetylcholine receptor (nAChR)[1] [$(\alpha1)_2\beta1\gamma\delta$ subunit stoichiometry] is the archetypal ligand-gated ion channel (reviewed in refs *3* and *4*; ref *5*). In this laboratory, transient kinetic techniques were developed (*6*−*8*) to measure the rate and equilibrium constants associated with the activation and desensitization of ionotropic neurotransmitter receptors and the mechanism of their inhibition (*1*, *9*−*12*). Understanding the mechanism by which therapeutic agents, for example, local anesthetics, and abused drugs such as cocaine, inhibit the nAChR has been a goal for over two decades (*13*) and the subject of many publications (reviewed in ref *14*).

By using rapid chemical kinetic techniques (*6*−*8*), it was shown recently that the muscle-type nAChR has two binding sites for noncompetitive inhibitors (*12*). Only the rapidly equilibrating inhibitory site has been investigated so far. The noncompetitive inhibitors cocaine (*15*), philanthotoxin-343 (*16*), and MK-801 (*17*) bind to a regulatory site on the closed-channel conformation of the muscle-type nAChR with higher affinity than to the site on the open-channel form, thereby shifting the equilibrium toward the closed-channel form and inhibiting receptor function (*1*, *11*, *12*). The simplified reaction scheme for the muscle nAChR is shown in Figure 1.

nAChRs in the central and peripheral nervous system differ substantially in their pharmacological and structural features from the nAChR at the neuromuscular junction (ref *18*; reviewed in refs *19* and *20*). Here, we report investigations of the mechanism of action of the rat α3β4 neuronal receptor expressed in HEK 293 cells, its inhibition by the abused drug cocaine and the anticonvulsant MK-801 [(+)-dizocilpine], and prevention of this inhibition. The α3β4 acetylcholine receptor is a major subtype of the neuronal nAChR in autonomic ganglia (reviewed in ref *21*); α3-containing nAChRs are present at particularly high density in the superior cervical ganglion, pineal, and adrenal glands (reviewed in ref *21*). They are also present in the *substantia nigra*, striatum, hippocampus, *locus coeruleus*, habenulo-interpeduncular tract, and cerebellum (reviewed in ref *21*).

Cocaine is a commonly abused drug (*22*, *23*). Inhibition of dopamine reuptake via the dopamine transporter is generally believed to be the primary mechanism underlying the reinforcing properties of this drug (*24*). However, increasing evidence suggests that this alone cannot fully

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; SCG, superior cervical ganglion; PCP, phencyclidine.

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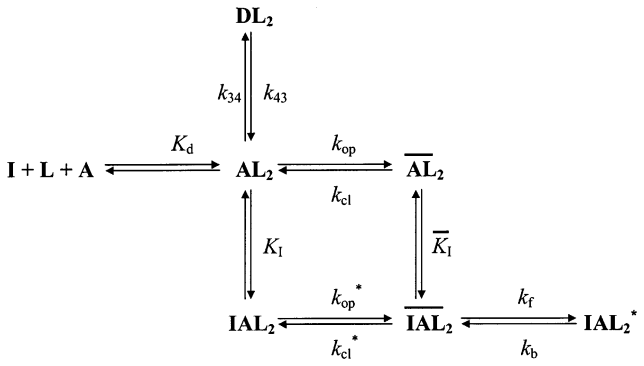

FIGURE 1: Proposed inhibition mechanism for the rapidly equilibrating inhibitor site of the muscle nAChR. A represents the nondesensitized (active) receptor, L the neurotransmitter, and I the inhibitor. AL_2 and $\overline{AL}_2$ are, respectively, the closed- and open-channel forms in the absence of an inhibitor. IAL_2 and $\overline{IAL}_2$ are, respectively, the closed- and open-channel forms in the presence of an inhibitor. DL_2 represents the desensitized (inactivated) form, and IAL_2^* an inhibited inactive form. K_d is the dissociation constant of the activating ligand. $\bar{K}_I$ and K_I are the dissociation constants of the inhibitor from the open- and closed-channel forms, respectively. k_{op} and k_{op}^* are the rate constants for channel opening in the absence and presence of inhibitor. k_{cl} and k_{cl}* are the rate constants for channel closing in the absence and presence of inhibitor. k_{34} and k_{43} are the dominant rate constants for receptor desensitization and resensitization. k_f and k_b are the rate constants associated with the relatively slow formation of an inactive state of the receptor with the inhibitor bound that can be observed at high concentrations of inhibitor and neurotransmitter (*11*, *12*).

explain the drug's complex pathophysiological effects (see, for instance, ref *25*). In addition to the muscle-type and neuronal nAChRs (*10*, *15*, *26*, *27*), cocaine also inhibits other ligand-gated (*28*−*32*) and voltage-gated (*33*, *34*) ion channels. Despite intensive efforts during the last two decades, there are no suitable medications for the treatment of cocaine inhibition (*24*, *35*). Two new approaches have recently emerged. One is based on the use of catalytic antibodies that bind to cocaine and hydrolyze it (*36*, *37*). The other approach relies on understanding the mechanism of receptor inhibition (*1*). It led to the idea of finding ligands that bind to the same site as noncompetitive inhibitors do but unlike inhibitors have a higher affinity for this site on the open-channel receptor form than the closed-channel form (*1*). In contrast to inhibitors, these ligands would not shift the channel-opening equilibrium toward the closed-channel receptor form but would displace inhibitors, thus alleviating the inhibition (*1*). Ligands that alleviate cocaine and MK-801 inhibition of the muscle nAChR were subsequently identified (*1*, *38*).

Here, we extend our previous observations on the mechanism-based prevention of cocaine inhibition of the muscle-type nAChR (*1*) to the neuronal nAChR containing the α3 and β4 subunits, using both macromolecular RNA ligands and cocaine derivatives to prevent inhibition. Some of this work was reported previously in a preliminary form (*39*).

MATERIALS AND METHODS

Chemicals. (+)-MK-801 maleate (dizocilpine maleate), (−)-ecgonine methyl ester·HCl, carbamoylcholine chloride, acetylcholine chloride, and (−)-cocaine·HCl were purchased from Sigma, and (*S*)(−)-nicotine was from Aldrich. Salts and other buffer constituents were of the highest purity available and were obtained from Sigma, Fisher Scientific, or EM Science. Cell culture media and supplements were purchased from Invitrogen/Gibco (Grand Island, NY); cell culture plastic ware was purchased from Corning Inc. (Corning, NY).

Cell Culture. The KXα3β4R2 cell line was established by Xiao et al. (*40*) by transfection of HEK 293 cells with the rat neuronal nAChR α3 and β4 subunit genes. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 0.25 mg/mL G418 (Geneticin) antibiotic, 100 units/mL penicillin G, and 0.1 mg/mL streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air, in 25 cm^2 flasks. The cells were passaged every 3 days. For whole-cell current recordings, the cells were plated on 35 mm diameter plastic dishes. At 50−70% confluence, the cells were scraped off the dish, dispersed in fresh medium until a single-cell suspension was achieved, transferred to a new dish, allowed to sediment for 30 min, and then immediately used for the electrophysiological experiments.

Whole-Cell Recordings. The whole-cell current recordings were performed with use of an Axopatch 200A integrating patch clamp amplifier and Clampex 5.5 data acquisition software (both from Axon Instruments, Foster City, CA) as described by Hamill et al. (*41*). The solution in the pipet (intracellular buffer) was 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 2 mM tetraethylammonium chloride, 25 mM HEPES/KOH (pH 7.4); the extracellular buffer was 145 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, 25 mM HEPES/NaOH (pH 7.4). Glass pipets (electrodes) were pulled on a PIP5 two-stage puller (HEKA Electronik, Lambrecht/Pfalz, Germany) and fire-polished on a MF-830 microforge (Narishige Scientific Instrument Lab., Tokyo, Japan). The electrode resistance was typically 3−4 MΩ. The series resistance was typically 4−6 MΩ. The fluctuation in the transmembrane voltage was no more than 10%. All measurements were carried out at ambient (22−24 °C) temperature and a transmembrane voltage of −60 mV. The current signal was low-pass filtered at 1 kHz using the amplifier's built-in circuitry (4-pole low-pass Bessel filter) and digitized at 200−800 Hz.

Rapid Application of Ligand Solutions. The cell-flow method used for rapid ligand application to membrane-bound proteins on cell surfaces has been described elsewhere (*6*). Briefly, a single voltage-clamped cell without processes was lifted off its substratum, placed at a distance of ca. 200 μm from the porthole of a U-tube, and perfused with neurotransmitter solution (sometimes also containing the inhibitors). The linear flow rate of solutions emerging from the flow device with a porthole 200 μm in diameter was typically ∼2 cm/s. Cells were allowed to recover for at least 2 min between each application, a time sufficient for complete resensitization (reactivation) of the receptors.

The observed rise time of the whole-cell current to its maximum observed value in the experiments described was 50 to 150 ms, depending on the agonist concentration employed and the linear flow rate used. Because desensitization of the nAChR also occurs on the millisecond time scale, the observed current amplitude was corrected for desensitization as described previously (*6*).

Most of the cells tested showed a two-exponential current decay. In some cases (such as experiments done with low concentrations of an agonist), the slower component was too slow to be resolved in the time interval of the ligand application. In other cases, the current associated with the slower component was practically fully suppressed by the inhibitor used (see Results and Discussion). Unless indicated otherwise, only the dominant, rapidly desensitizing current component was analyzed.

Fitting the Experimental Data to the Equations. All linear and nonlinear least-squares regressions and plotting of the experimental results were performed using Origin 7.0 data analysis software (Origin Lab Corp. Northampton, MA). The subroutine used can be obtained by writing to this laboratory.

RNA Aptamer II-3. Class II RNA aptamer #3 (aptamer II-3) was prepared as described by Ulrich et al. (*42*).

RESULTS AND DISCUSSION

Activation, Desensitization, and Self-Inhibition of the α3β4 nAChR Induced by Agonists. Previous investigations of the mechanism of the α3β4 nAChR were performed by using techniques with low time resolution, such as a 2 min ion flux (e.g., refs *40* and *43*) or *Xenopus laevis* oocyte voltage-clamp recordings (e.g., ref *44*). As shown previously (*45*−*47*), the measured current due to the formation of open receptor channels obtained in these experiments is a not just a measure of the concentration of open receptor channels but reflects also the rates of receptor activation and inactivation (desensitization) (*6*). The rapid chemical reaction techniques used here allow one to obtain current amplitudes that reflect the concentration of open receptor channels (*6*). This approach has previously been used in investigations of several neurotransmitter receptors [reviewed in Hess and Grewer (*8*)].

Here, for the first time, a rapid chemical reaction technique is used in investigations of the α3β4 neuronal nAChR. The trace in Figure 2A shows a representative response to a saturating (3.3 mM) concentration of carbamoylcholine, a stable analogue of acetylcholine. The observed current amplitude decreases as the concentration of activating ligand increases (Figure 2B), reflecting an accelerated desensitization of the receptor. The current decay corresponds to a desensitization of the receptor and becomes faster as the concentration of agonist increases. In most cases, two exponential functions are required to adequately fit the current decay region of the traces.

The existence of two desensitization components deserves further explanation. In one of the previous investigations of agonist effects with this cell line containing the α3β4 neuronal receptor (*48*), only a single time constant of desensitization was specified; however, the duration of the ligand application employed (∼1.5 s) was too short to resolve the desensitization processes. Moreover, the authors observed two types of cell, one with a rapid and another with a slow current decay. In a subsequent paper using the same receptor and cell line (*49*), it was mentioned that often a clear monoexponential decay was not observed. Multiple single-channel conductances were observed previously for the recombinantly expressed α3β4 nAChR (*18*, *50*, *51*).

We observed that at very high agonist concentrations (3.3−10 mM for nicotine, 10 mM for acetylcholine, and 18 and 33 mM for carbamoylcholine), the observed current decay can no longer be fitted with two exponentials; instead, three exponential terms are needed (Figure 2B) (*52*). We believe that the fastest component, with a time constant of 20−50 ms, that is observed only at such elevated agonist concentrations, may reflect inhibition of the receptor by the agonist. Such rapid inactivation of the receptor prior to the fast desensitization process has been observed previously with the muscle-type nAChR (*52*, *53*).

In Figure 2C, the concentration dependence for nicotine- and carbamoylcholine-elicited currents is shown. Because each cell contains a different number of receptors, each data point was normalized to a standard concentration of agonist (330 μM for nicotine and 3.3 mM for carbamoylcholine). The experimentally determined ratio of corrected current amplitudes elicited by 330 μM nicotine and 3.3 mM carbamoylcholine is 1.1 ± 0.1 (average of six independent measurements). Thus, both carbamoylcholine and nicotine behave as full agonists, eliciting essentially the same maximum current at saturating concentrations. This is in agreement with previous reports (*43*).

The data in Figure 2C were plotted according to eq 1 (*6*):

$$I_A = I_M R_M L^2 [L^2 + \Phi(L + K_d)^2]^{-1} \quad (1)$$

I_A is the current amplitude, L the concentration of activating ligand, I_M the current due to 1 mol of open receptor-channels, and R_M the number of moles of the receptor in the membrane. K_d is the agonist dissociation constant for the site controlling receptor activation, and Φ^{-1} is the channel-opening equilibrium constant. The value of $I_M R_M$, 2.7 nA, that gives the best fit of the data to eq 1 was obtained by reiteration (*54*).

Estimates for K_d and Φ for nicotine can be obtained from the slope and intercept, respectively, when the data are plotted according to eq 2, which is a rearranged form of eq 1.

$$(I_M R_M \cdot I_A^{-1} - 1)^{1/2} = \Phi^{1/2} + \Phi^{1/2} \cdot K_d \cdot L^{-1} \quad (2)$$

The values of Φ^{-1}, the channel-opening equilibrium constant, for nicotine and carbamoylcholine are 3.3 ± 1 and 3.8 ± 0.5, respectively. For nicotine, K_d is 73 ± 18 μM, and for carbamoylcholine, K_d is 2.1 ± 0.4 mM. The values for the carbamoylcholine dissociation constant for the site controlling receptor activation, K_d, and for the channel-opening equilibrium constant, Φ^{-1}, are close to those reported (*55*) previously for nAChRs in a rat pheochromocytoma (PC12) cell line containing α3, α5, α7, β2, and β4 subunits (*56*). Two values for Φ^{-1} for the rat α3β4 nAChR expressed in *Xenopus laevis* oocytes were calculated from the k_{cl} and k_{op} values obtained using the single-channel current-recording technique (*57*); these were 2.2 (*18*) and 120 (*58*).

We estimated the dominant desensitization rate constant, k_{34} (Figure 1), based on the concentration dependence of the observed desensitization coefficient α (Figure 2D). The data were plotted according to the following equation (*6*):

$$\alpha = k_{34} \Phi L^2 [(L + K_d)^2 \Phi + L^2]^{-1} \quad (3)$$

Since values for K_d and Φ have been determined (see above), the only parameter to be evaluated was k_{34}. The estimates for k_{34} are 15 ± 1 and 20 ± 1 s^{-1} for nicotine and carbamoylcholine, respectively. The values are somewhat

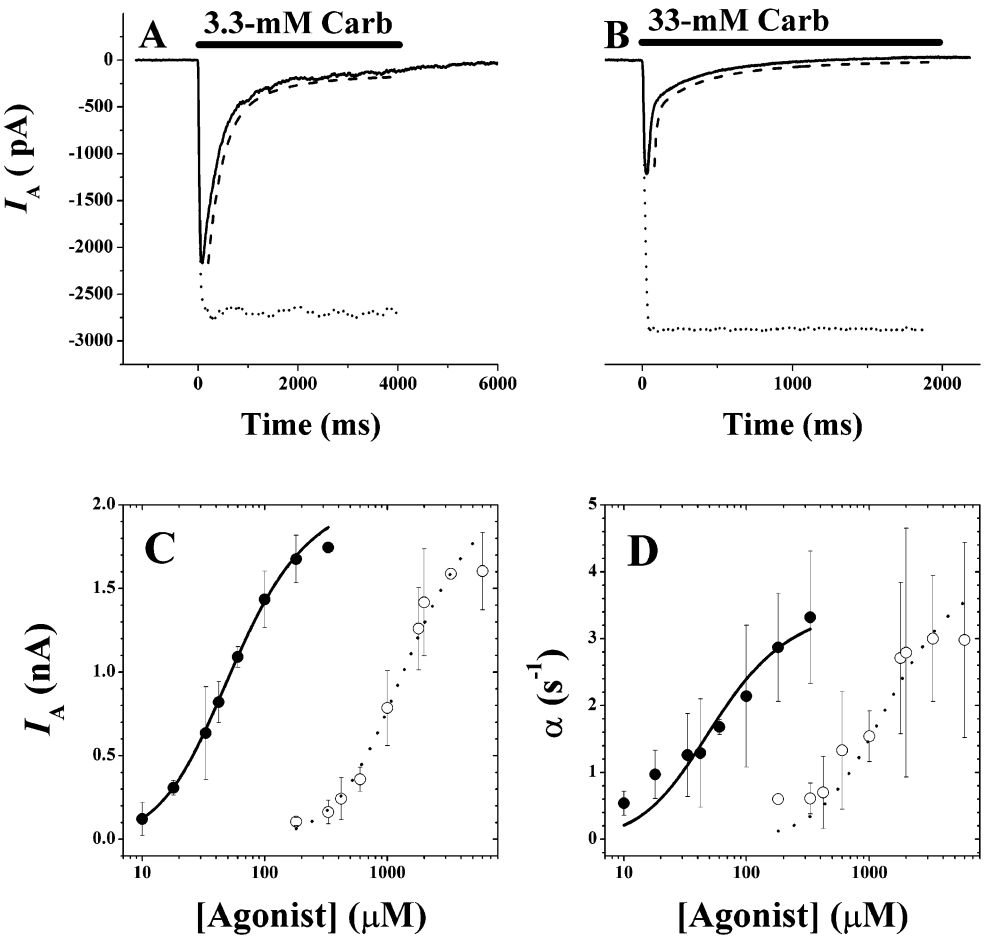

FIGURE 2: Cell-flow experiments with HEK 293 cells containing the α3β4 nAChR. The current traces were recorded with the same cell at pH 7.4, 22−24 °C, and −60 mV. The length of carbamoylcholine application is indicated by a horizontal bar. τ is the time constant(s) associated with the desensitization reaction(s), and I is the current associated with the individual time constants. The time constants (τ) and current amplitudes (I_A) are indicated as mean ± standard deviation. In panels A and B, observed currents are indicated as solid lines, and the current corrected for desensitization is given by the dotted line. The current calculated for the desensitization reaction is indicated by the dashed line; the currents associated with the different desensitization processes are indicated by I_{A1}, I_{A2}, and I_{A3}. The time constant is similarly numbered. (A) 3.3 mM carbamoylcholine. $\tau_1 = 1283 \pm 107$ ms, $I_{A1} = -449.0 \pm 0.6$ pA, $\tau_2 = 289 \pm 6$ ms, and $I_{A2} = -2274.9 \pm 1.4$ pA. (B) 33 mM carbamoylcholine. $\tau_1 = 636 \pm 18$ ms, $I_{A1} = -292.0 \pm 0.1$ pA, $\tau_2 = 198 \pm 6$ ms, and $I_{A2} = -283.7 \pm 0.1$ pA, $\tau_3 = 16.4 \pm 0.1$ ms, and $I_{A3} = -2422.1 \pm 0.1$ pA. (C) Dependence of the whole-cell current amplitudes, I_A, on the concentration of an agonist, carbamoylcholine (○) or nicotine (●). Each data point represents three to eight independent measurements (22−24 °C, − 60 mV, pH 7.4) made with two to six cells. For carbamoylcholine, all points were normalized to the response to 3.3 mM carbamoylcholine. For nicotine, all points were normalized to the response to 330 mM nicotine. The curves were scaled to each other using the experimentally determined ratio of currents elicited by 330 μM nicotine and 3.3 mM carbamoylcholine (1.1 ± 0.1). The theoretical lines drawn are based on eq 1. For nicotine, K_d is 73 ± 18 μM and Φ is 0.30 ± 0.09 and for carbamoylcholine, K_d is 2.1 ± 0.4 mM and Φ is 0.26 ± 0.04. (D) Desensitization of the α3β4 nAChR by carbamoylcholine (○) and nicotine (●). The data in panel C were used. The rate coefficient for rapid receptor desensitization, a (see eq 4), at each agonist concentration was evaluated from the decay phase of the current as described in the text. The rate constant for receptor desensitization, k_{34}, was evaluated from the concentration dependence of α. The lines through the data points were drawn using eq 4, and values for k_{34}, Φ, and K_d are 20 ± 1 s^{-1}, 0.26, and 2.1 mM, respectively, for carbamoylcholine and 15 ± 1 s^{-1}, 0.30, and 73 μM, respectively, for nicotine.

lower than those reported for the muscle-type nAChR in BC_3H1 cells, 35 s^{-1} for carbamoylcholine (*6*) and 47 s^{-1} for nicotine (*59*). This is in agreement with the observation that the neuronal α3β4 nAChR has relatively slow desensitization kinetics (*60*).

Effect of Cocaine on Closed- and Open-Channel Conformations of the α3β4 nAChR. It has been known for many years that cocaine is a noncompetitive inhibitor of the nAChR in the electric organ of certain fish (*15*, *26*). The affinity for cocaine of the nAChR in the electric organ of *Torpedo californica* and *Electrophorus electricus* is relatively high [K_I is 58 and 14 μM, respectively (*61*)]. K_I for the open-channel form of the mouse muscle-type nAChR (BC_3H1 cells) is 300 μM (*10*). The affinity for cocaine of the α3β4 nAChR expressed in *X. laevis* oocytes is higher; K_I is 17 μM (*27*). K_I for cocaine inhibition of neuronal nAChRs expressed in the PC12 cell line (as determined by 15 s $^{22}Na^+$ influx measurements) is ∼10 μM (*15*). Additional information about the mechanism of inhibition by cocaine of the rapidly desensitizing neuronal α3β4 nAChR is obtained from the rapid chemical kinetic measurements presented here.

Representative current traces for cocaine inhibition of the rat α3β4 nAChR expressed in the HEK 293 cell line are given in Figure 3A and B.

When cocaine is coapplied with the agonist, carbamoylcholine, it depresses the current amplitude. In the presence of 3.3 mM carbamoylcholine and 33 μM cocaine, the slowly desensitizing nAChR current is depressed to a much larger

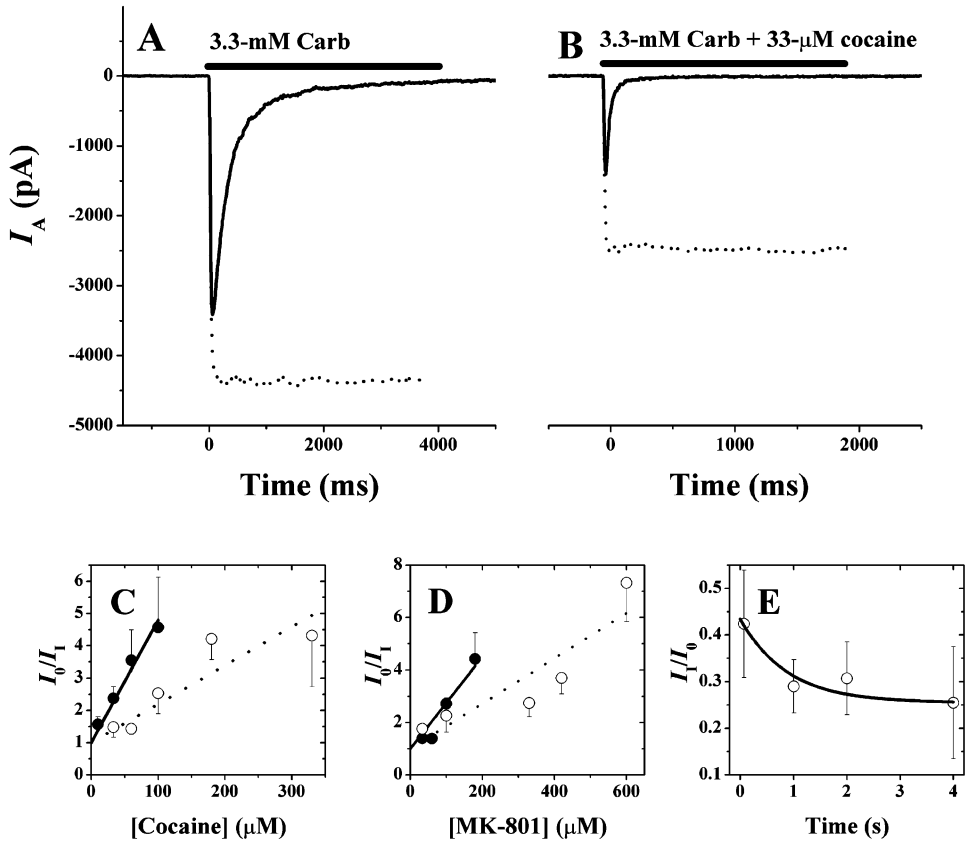

FIGURE 3: Inhibition of the α3β4 nAChR by cocaine or MK-801. The same cell was perfused with carbamoylcholine alone or with carbamoylcholine plus cocaine or carbamoylcholine plus MK-801. In the cell-flow experiments in panels A and B, the duration of the application of ligands is indicated by a horizontal bar. Conditions and designations are as in Figure 1 (22−24 °C, −60 mV, pH 7.4). The currents associated with the different desensitization processes are indicated by I_A and I_{A2}. The time constant is similarly numbered. (A) 3.3 mM carbamoylcholine alone. $\tau_1 = 936 \pm 29$ ms, $I_{A1} = -806.2 \pm 0.6$ pA, $\tau_2 = 221 \pm 3$ ms, and $I_{A2} = -3483.5 \pm 0.7$ pA. (B) 3.3 mM carbamoylcholine coapplied with 33 μM cocaine. $\tau_1 = 150 \pm 7$ ms, $I_{A1} = -231.2 \pm 0.5$ pA, $\tau_2 = 26.9 \pm 0.6$ ms, and $I_{A2} = -2237 \pm 1$ pA. Each data point in panels C−E represents three to five independent measurements done with an average of two to three cells. (C) Inhibition of the rapidly equilibrating site by cocaine. Ratios of the maximal current amplitudes in absence, I_0, and presence, I_I, of cocaine, are shown. The inhibitor, at the indicated concentrations, was coapplied with either 3.3 (○) or 0.33 (●) mM carbamoylcholine. From the current rise time, we estimated that the receptors were preincubated with the inhibitor for, on average, ∼70 ms. The best fit of the data to eq 4 gives a $\bar{K}_I$ value of 84 ± 12 μM and a K_I value of 26 ± 1 μM at high and low concentrations of carbamoylcholine, respectively. (D) Inhibition of the rapidly equilibrating site by MK-801. Conditions were as in panel C. The best fit of the data to eq 4 gives a $\bar{K}_I$ value of 116 ± 16 μM and a K_I value of 57 ± 6 μM. (E) Inhibition by MK-801 of the slowly equilibrating site. Ratios of the maximal current amplitudes in the presence, I_I, and absence, I_0, of MK-801, are plotted (○) as a function of the duration of preincubation with the inhibitor alone. The activating ligand was 1 mM carbamoylcholine, and the MK-801 concentration was kept constant (100 μM) throughout the preincubation period and the perfusion with agonist. The rate constant for the slow inhibition process, k'', was calculated to be 1.1 ± 0.5 s^{-1}, as described in the text.

extent than the rapidly desensitizing one. This is the first report of the discrimination in cocaine inhibition between rapidly and slowly decaying components (receptor isoforms). It is important to mention that in slow functional assays [such as ion flux experiments on the second-to-minute time scale (*62*) and *X. laevis* oocyte recordings on the seconds time scale (*27*)], the slower component will make the dominant contribution to the measurements.

The fast process for the inhibition of the receptor by cocaine that occurs during the mixing time of ligands (∼70 ms) is shown in Figure 3C. The inhibition measurements can be well-approximated (solid lines in Figure 3C,D) by eq 4 (*8*)

$$\frac{I_0}{I_I} = 1 + \frac{[I]}{K_{I(obs)}} \quad (4)$$

I_0 and I_I are the current amplitudes in the absence and the presence of inhibitor, respectively. [I] is the molar concentration of an inhibitor (cocaine), and $K_{I(obs)}$ is the observed dissociation constant of the inhibitor.

At a low agonist concentration (330 μM carbamoylcholine), when the ion channels are predominantly closed, $K_{I(obs)}$ reflects the affinity for cocaine of the closed-channel conformation of the receptor. At a high agonist concentration (3.3 mM carbamoylcholine), when the ion channels are predominantly open, $K_{I(obs)}$ reflects the affinity for cocaine of the open-channel conformation of the receptor ($\bar{K}_I$). The values for K_I and $\bar{K}_I$, obtained from the slope of the lines (Figure 3C), are 26 ± 1 and 84 ± 12 μM, respectively.

Since cocaine exhibits greater potency at lower concentrations of the agonist, inhibition of the receptor by this abused drug can be described by a mechanism (Figure 1) previously established for the muscle-type nAChR in BC_3H1 cells (*11*, *12*), in which an inhibitor binds to a regulatory site on the closed-channel conformation of the nAChR with higher affinity than to the site on the open-channel conformation and thereby shifts the equilibrium toward the closed-channel

form, inhibiting the receptor. For the mechanism proposed (Figure 1), the principle of microscopic reversibility [see Hammes (*63*)] requires that the ratio of $\bar{K}_I/K_I$ is equal to the ratio of Φ^*/Φ, where Φ and Φ^* are the channel-closing equilibrium constants in the absence and presence of an inhibitor, respectively. Having determined (Figure 2C) that Φ is equal to 0.26 for carbamoylcholine, we can estimate Φ^* as 0.84 in the presence of inhibitor corresponding to channel-opening equilibrium constants of 3.8 and 1.2 in the absence and presence of inhibitor, respectively.

Neuronal α3-containing nAChRs are the main mediators of fast synaptic transmission in the autonomic nervous system (*64*). This includes neurotransmission in sympathetic and parasympathetic nerves that innervate the heart; α3-knockout mice have a severe cardiac phenotype (*64*). After in vitro injection in rats, the concentration of cocaine can be as high as 20−80 μM in blood and as high as 120−350 μM in the brain (*65*, *66*). So, inhibition of a neuronal nAChR by cocaine, with K_I values of 26 and 84 μM, respectively, as reported here, can account for some physiological and toxicological effects of this abused drug, such as the inhibition of sympathetic neural activity that can affect the heart (*64*).

Effect of MK-801 on Closed- and Open-Channel Conformations of the Neuronal nAChR. Our interest in this compound was initiated by reports that it is capable of alleviating cocaine-induced behavioral sensitization and intoxication (see, i.e., ref *67*). We, therefore, investigated the effects of MK-801 on the neuronal α3β4 receptor using the same conditions as for cocaine (see above).

Like cocaine, MK-801 depresses the current amplitude when coapplied with carbamoylcholine, acetylcholine, or nicotine, in agreement with previous reports (Figure 3D) (*68*). As with cocaine, the slowly desensitizing current component (receptor isoform) is affected to a substantially greater extent than the rapidly desensitizing component. A plot of the ratio of the maximum current for the corrected rapidly desensitizing form obtained in the absence (I_0) and presence (I_I) of MK-801 as a function of the inhibitor concentration is shown in Figure 3D.

The values of $K_{I(obs)}$ obtained from the slopes of the lines (Figure 3D) using eq 4 are 116 ± 16 μM at 3.3 mM carbamoylcholine and 57 ± 6 μM at 0.33 mM carbamoylcholine. Thus, MK-801 itself is an inhibitor of the neuronal nAChR and cannot be used to alleviate inhibition of this receptor by cocaine. This is in agreement with previous results with the muscle-type nAChR (*1*, *12*).

Binding of the Inhibitors to the Neuronal α3β4 nAChR Includes an Additional Slow Process. So far, we have described what happens when the inhibitor is coapplied with agonist. However, besides this fast inhibition process, which occurs during the mixing time (~70 ms), another slower process was detected in the inhibition of the muscle-type nAChR by MK-801 (*12*). We, therefore, investigated the effect of preincubation of the neuronal α3β4 receptor with MK-801 on the current (Figure 3E). The data are plotted as I_I/I_0 for the rapidly desensitizing form versus preincubation time. Cells were perfused with MK-801 alone for the time indicated; subsequently, 1 mM carbamoylcholine was coapplied with 100 μM MK-801. About 80% of the inhibition process went to completion within the mixing time of MK-801 and the cell surface receptors (~70 ms). A second much slower process goes to completion within about 1 s. The data were fitted using eq 5 (*12*)

$$I_I/I_0 = \frac{I_{I(t=\infty)}}{I_0} + \frac{I_{I(t=0)}}{I_0}\exp(-k''t) \qquad (5)$$

$I_{I(t=\infty)}$ is the current amplitude in the presence of inhibitor after a long preincubation time; $I_{I(t=0)}$ is the initial value of the current amplitude in the presence of inhibitor (i.e., total current amplitude minus $I_{I(t=\infty)}$); and k'' is the rate constant for the slower inhibition process. The $I_{I(t=\infty)}/I_0$ term was set as 0.255 (the value with a 4 s preincubation), and $I_{I(t=0)}/I_0$ and k'' were varied by a fitting routine (see Materials and Methods). $I_{I(t=0)}/I_0$ was estimated as 0.179 ± 0.033, and k'' was estimated as 1.1 ± 0.5 s^{-1}. The results are in general agreement with those for the muscle-type nAChR, although the contribution of the slower inhibition process is less prominent with the neuronal receptor than with the muscle-type receptor (*12*).

The contribution of the slowly equilibrating regulatory site can also be seen in the inhibition of the neuronal α3β4 nAChR by cocaine. Preincubation of a cell with cocaine alone increases inhibition as compared with simple coapplication (data not shown). This result is in agreement with previous data obtained with the muscle-type nAChR (*10*) and the rat neuronal α3β4 nAChR (*27*, *69*).

Alleviation of Cocaine Inhibition of α3β4 nAChR by Aptamer II-3. Aptamers are combinatorially selected biopolymers (usually oligonucleotides) that bind various targets (*70*, *71*). The technique for selection of such aptamers, SELEX (systematic evolution of ligands by exponential enrichment) (*70*, *71*), was modified (*42*) for use with a membrane-bound protein, the *Torpedo* nAChR. Displacement of the RNA aptamers from the membrane-bound nAChR by the cocaine analogue phencyclidine (PCP) produced two classes of RNA aptamers, each with a different consensus sequence (*42*). Both classes displace cocaine from the muscle-type nAChR in BC_3H1 cells; class I aptamers bind with higher affinity to the closed- than open-channel receptor form, and as predicted from the mechanism (Figure 1) (*1*), are potent inhibitors of the nAChR. Class II aptamers bind with about equal affinity to the closed- and open-channel receptor forms, and as predicted from the mechanism (*1*), do not inhibit the receptor but do counteract receptor inhibition by cocaine (*1*). We have tested the effect of a representative class II aptamer, aptamer II-3, on the neuronal α3β4 nAChR (Figure 4).

At the concentrations used, the aptamer itself has no effect on receptor function, when preincubated or coapplied with carbamoylcholine (data not shown). However, when coapplied with carbamoylcholine and cocaine, aptamer II-3 reduces the inhibition of the receptor by cocaine (Figure 4).

Figure 4A,B shows the concentration dependence for the alleviation of cocaine inhibition of the open-channel form of the receptor by RNA aptamer II-3. The experimental data were plotted according to eq 6 (*1*)

$$\frac{I_0}{I_I} = 1 + \frac{[I]}{K_{I(obs)}}\frac{K_A}{[\text{aptamer}] + K_A} \qquad (6)$$

I_0 and I_I are the current amplitudes for the rapidly desensitizing form in the absence and presence of inhibitor, respectively. [*I*] is the molar concentration of the inhibitor (cocaine),

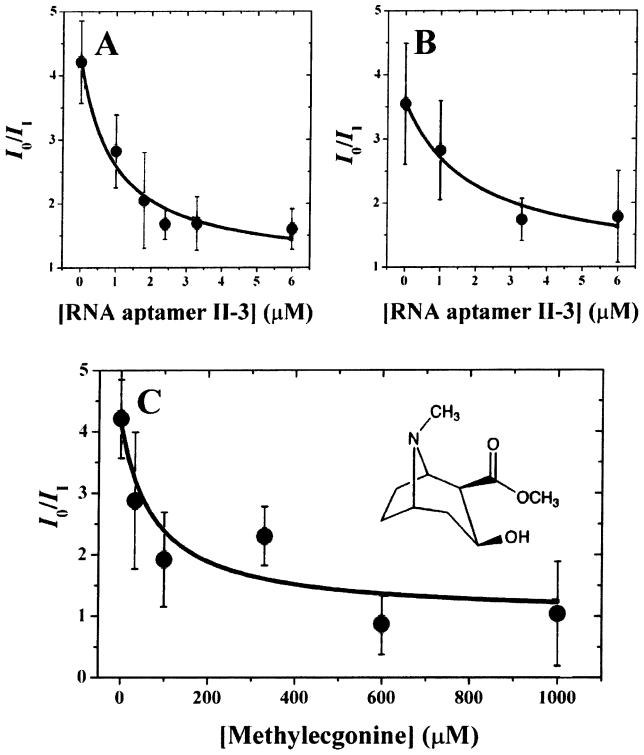

FIGURE 4: Alleviation of cocaine inhibition of the α3β4 nAChR by class II RNA aptamer 3 (A and B) and by the cocaine derivative methylecgonine (C). Each data point represents three to five independent measurements (22−24 °C, −60 mV, pH 7.4), one measurement per cell. (A) Alleviation by aptamer II-3 of cocaine inhibition of the open-channel form of the nAChR. At a constant concentration of carbamoylcholine (3.3 mM), the ratio of the maximum current amplitudes obtained in the absence, I_0, and presence, I_I, of a constant concentration of cocaine (180 μM) was determined as a function of the concentration of aptamer II-3. Using eq 4, the best fit of the data in experiments in which the ligands were coapplied with carbamoylcholine gives a K_A value of 1.0 ± 0.2 μM for the aptamer and a $\bar{K}_I$(obs) value of 56 ± 3 μM for cocaine using eq 6. (B) Alleviation by aptamer II-3 of cocaine inhibition of the closed-channel form of the nAChR. At a constant concentration of carbamoylcholine (0.33 mM), the ratio of the maximum current amplitudes obtained in the absence, I_0, and presence, I_I, of a constant concentration of cocaine (60 μM) was determined as a function of the concentration of aptamer II-3 using eq 6. The best fit of the data gives a K_A value of 2.0 ± 0.5 mM for the aptamer and a $K_{I(obs)}$ value of 23 ± 2 μM for cocaine. (C) Alleviation by methylecgonine (structure given in the figure) of inhibition of the open-channel form of the nAChR. The best fit of the data in experiments in which the ligands were coapplied with carbamoylcholine gives a K_A value of 79 ± 54 μM for methylecgonine and a $\bar{K}_{I(obs)}$ value of 58 ± 12 μM for cocaine.

and [aptamer] the molar concentration of the aptamer II-3. $K_{I(obs)}$ is the observed dissociation constant of the inhibitor, and K_A is the dissociation constant of the aptamer. Preincubation with the aptamer does not produce substantially greater alleviation of receptor inhibition than coapplication alone does (not shown).

Figure 4A,B demonstrates that the affinity of aptamer II-3 toward the closed-channel form of the neuronal α3β4 receptor is comparable to that for the open-channel form. This is what was predicted by the mechanism of nAChR inhibition (Figure 1) and demonstrated earlier for the muscle type of the receptor (*1*).

Alleviation of Cocaine Inhibition of α3β4 nAChR by Methylecgonine. Understanding the mechanism of cocaine inhibition of the muscle type nAChR (*1*, *9*−*12*) and how to test for compounds that alleviate this inhibition (*1*) led subsequently to the discovery of several low molecular weight cocaine analogues that are capable of relieving cocaine inhibition of the muscle-type nAChR (*38*). The compounds are either synthetic phenyltropane (RTI-4229-70) (*72*) or carbomethoxytropenone (RCS compounds) analogues of cocaine (*38*).

We have tested the natural cocaine metabolite methylecgonine (*73*) with the α3β4 nAChR. Methylecgonine, which does not inhibit the α3β4 nAChR at concentrations up to 1 mM (not shown), alleviates inhibition of this receptor by cocaine (see Figure 4C). The apparent affinity of methylecgonine toward the neuronal nAChR is substantially lower than for the muscle-type nAChR (*73*). Nevertheless, because the compound lacks systemic toxicity (*74*), it can be used in a high dose, which can partially overcome its low potency. Moreover, derivatives of methylecgonine, created by, for example, a combinatorial synthesis approach, might have a higher potency.

CONCLUSIONS

Both cocaine and MK801 are noncompetitive inhibitors of the neuronal α3β4 nAChR. The dissociation constants of cocaine for the neuronal receptor (K_I = ∼26 μM for the closed-channel receptor form and $\bar{K}_I$ = ∼84 μM for the open-channel receptor form), unlike those for the muscle-type nAChR, fall within the concentration range of cocaine found in the blood of cocaine-treated animals (*65*, *66*). As with the muscle-type nAChR, the neuronal α3β4 nAChR has two binding sites for the inhibitors, one equilibrating in the millisecond, the other in the second time domain. The mechanism of inhibition of the rapidly equilibrating inhibitor site of the neuronal α3β4 nAChR and the muscle-type nAChR appears the same (Figure 1). The predictions of the mechanism (Figure 1) are (i) compounds that inhibit are expected (*1*) to bind with higher affinity to the closed-channel conformation than to the open-channel conformation and thereby shift the channel-opening equilibrium to the closed-channel form and inhibit the receptor. These predictions are realized with both the inhibitors, cocaine (Figure 3C) and MK-801 (Figure 3D). (ii) Compounds that bind to the same site as the inhibitors but with equal or higher affinity for the open- than the closed-channel receptor conformation will not inhibit and are capable of displacing inhibitors that bind to this site (*1*). These predictions are realized (*1*) with both a combinatorially synthesized RNA polymer (refs *1* and *42* and Figure 4A,B) and cocaine derivatives (refs *38* and *73* and Figure 4C).

Addiction to cocaine is a serious social, medical, and economic problem in the United States and worldwide. Countless attempts have been made previously to find compounds that will displace abused drugs, such as cocaine, from their sites of action in the nervous system. So far, no compound has been found that alleviates cocaine inhibition (*24*), and currently no drugs exist in the United States for the treatment of cocaine inhibition. We have shown previously that cocaine inhibition of the muscle-type nAChR can be alleviated using combinatorially selected RNA ligands (aptamers) (*1*) and nontoxic cocaine analogues (*38*) identified by a mechanism-based approach. The easily available and

thoroughly studied muscle-type nAChRs of the *T. californica* electric organ and the BC_3H1 clonal cell line were used as model systems. However, because of their low affinity for cocaine, nAChRs at the neuromuscular junction are likely to make only a minor contribution to the pharmacological and toxicological effects of this abused drug. On the other hand, neuronal nAChRs in the central and peripheral nervous systems have a much higher affinity for cocaine than does the muscle-type nAChR (*10*). Such neuronal nAChRs, therefore, appear to be more important targets for cocaine.

Our approach for finding compounds that are potentially useful for the treatment of cocaine addiction and intoxication differs in that we first investigated the mechanism of inhibition. Using transient kinetic methods, developed in this laboratory (*6*−*8*), it is now possible to distinguish between alternative mechanisms of inhibition. Previously, this allowed us to develop an approach for successful prevention of inhibition of the muscle nAChR by the abused drug cocaine (*1*, *38*) and now of the neuronal ($\alpha3\beta4$) nAChR.

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