

Subtype-Selective Inhibition of Neuronal Nicotinic Acetylcholine Receptors by Cocaine Is Determined by the $\alpha 4$ and $\beta 4$ Subunits

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels of the central and peripheral nervous system that regulate synaptic activity from both pre- and postsynaptic sites. Nicotine binding to brain nAChRs is thought to underlie the induction of behavioral addiction to nicotine, probably as a result of desensitizing/inhibitory effects. Here, another commonly abused drug, cocaine, is shown to selectively inhibit particular nAChR subtypes with a potency in the low micromolar range by interacting with separate sites associated with the $\alpha 4$ and $\beta 4$ nAChR subunits. Chimeric receptor subunits and site-directed mutants were used to localize sequence determinants of cocaine affinity to: 1) a region of $\alpha 4$ located between residues 128 and 267 and 2) a site within the pore-lining M2 domain of $\beta 4$ that includes the 13' phenylalanine residue. The voltage dependence for inhibition associated with each site is

consistent with these results. Analysis of the effects of incorporation of mutant and chimeric subunits also permitted identification of sequence elements important in receptor activation. For $\alpha 3$ -containing receptors, a region or regions contained within the N-terminal extracellular domain of neuronal β subunits influence the time course of responses to acetylcholine. Conversely, the 13' residue of the $\beta 4$ subunit M2 region is important in determining acetylcholine potency, indicating a role for this residue in agonist binding/gating processes. In summary, the present work describes sequence elements critical in both cocaine inhibition and acetylcholine activation of nAChRs and indicates that nAChRs may provide a site of interaction for the effects of nicotine and cocaine in the nervous system.

Although cocaine binds to a variety of nervous system sites, its potency for reinforcing effects are best correlated with binding to the dopamine transporter (Ritz et al., 1987). The reinforcing effects of cocaine are attenuated in dopamine transporter and dopamine receptor knockout mice (Xu et al., 1994; Giros et al., 1996). In general, these findings are consistent with an obligatory role of the dopaminergic system in mediating the reinforcing properties of cocaine (see, however, Rocha et al., 1998). Thus, the functional effects and structural basis for cocaine interactions with neurotransmitter transporters (for dopamine and serotonin in particular) have justifiably received considerable attention. In contrast, evaluation of the potential for and functional relevance of cocaine effects on other critical elements in synaptic function has lagged behind. Central and peripheral effects of cocaine independent of binding to the dopamine transporter probably contribute to its addictive potential and toxicity profile.

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Although cocaine is an effective inhibitor of voltage-gated sodium channels (resulting in its local anesthetic activity) and the structural basis for this interaction has been well characterized (Ragsdale et al., 1994; Li et al., 1999), few studies have examined interactions between cocaine and ligand-gated ion channels (LGICs) of the nervous system. This fact is perhaps somewhat surprising, because much of our current understanding of the pore structure and gating kinetics of an entire family of structurally related LGIC subtypes has arisen from the use of quaternary local anesthetic derivatives as structural probes of the neuromuscular junction subtype of nicotinic acetylcholine receptor (nAChR). Cocaine shares key structural features with other local anesthetics (specifically an ionizable amine group and a hydrophobic or aromatic moiety) and, like other local anesthetics, noncompetitively inhibits muscle nAChR (Swanson and Albuquerque, 1987; Niu et al., 1995). However, little information is available about the potential for inhibition of nervous system nAChRs by the cocaine concentrations normally associated with abuse and/or toxicity.

Neuronal nAChRs are pentameric LGICs that function

either as homomers ($\alpha 7$ – $\alpha 9$) or as heteromeric combinations of α ($\alpha 2$ – $\alpha 4$, $\alpha 6$) and β ($\beta 2$ and $\beta 4$) subunits. Brain nAChR subtypes have been implicated in a variety of central nervous system (CNS) processes, including presynaptic regulation of neurotransmitter release (for review, see Wonnacott, 1997). Moreover, activation and/or subsequent desensitization of CNS nAChR subtypes is believed to underlie behavioral addiction to nicotine (for review, see Dani and Heinemann, 1996). Additionally, neuronal nAChR subtypes of the peripheral nervous system are critical elements for transmission in sympathetic and parasympathetic ganglia. A few recent studies have begun to characterize interactions between central effects of cocaine and nicotine (Lerner-Marmarosh et al., 1995; Damaj et al., 1999); however, a clear picture of the effects of cocaine on the diverse subtypes of neuronal nAChRs has yet to take shape. The goals of the present study are to assess the potential for subtype-specific effects of cocaine on neuronal nAChRs and evaluate the underlying structural determinants.

Materials and Methods

Chemicals. Acetylcholine stock solutions were made daily and diluted to the working concentration in oocyte saline solution. Cocaine hydrochloride was provided by the National Institute on Drug Abuse and concentrated stock solutions were stored frozen in aliquots. All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Production of Chimeric cDNAs and Mutagenesis. Rat nAChR cDNA clones were provided by Drs. Steve Heinemann and Jim Boulter (Salk Institute, La Jolla, CA). The production and functional evaluation of chimeric β subunits has been described previously (Papke et al., 1993). For these chimeras ($\beta 2_{228}/\beta 4$ and $\beta 4_{230}/\beta 2$), the numeric subscript refers to the number of N-terminal amino acids contributed by a given β subunit. Chimeric nicotinic receptor α subunit cDNAs were created using homologous restriction sites conserved between α subunit sequences. Specifically, a *Bst*XI site located in a stretch of conserved sequence that codes for the extracellular loop region between M2 and M3 was used to create chimeric subunits that exchange sequence between $\alpha 3$ and $\alpha 4$ ($\alpha 3_{267}/\alpha 4$ and $\alpha 4_{267}/\alpha 3$). Similarly, a homologous *Pst*I site located immediately after sequence coding for the first of the two cysteines in the N-terminal extracellular domain (which form the cysteine loop characteristic of the nicotinic gene family) was used to create chimeric subunits which exchange sequence between $\alpha 2$ and $\alpha 4$ ($\alpha 2_{128}/\alpha 4$ and $\alpha 4_{128}/\alpha 2$). In each case, the numeric subscript refers to the amino acid immediately preceding the site at which the coding sequence is exchanged. α Subunit amino acids are numbered according to the system adopted for the muscle α subunit, in which the vicinal cysteine residues characteristic of nicotinic receptor α subunits are numbered 192 and 193. Restriction fragments were isolated from an agarose gel using the GeneClean III kit (Bio 101, Vista, CA) and subsequently ligated by homologous end ligation. After bacterial transformation, chimeric DNAs were evaluated by restriction digest and subsequent DNA sequencing.

Single mutations were introduced using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, antiparallel mutagenic oligonucleotides were synthesized containing the base(s) coding for the mutation(s) of interest flanked by 10 to 15 bases of wild-type sequence. During consecutive rounds of temperature cycling, the mutant oligonucleotides prime extension of each of the strands of the parental clone by *Pfu* DNA polymerase. After amplification, parental (methylated) DNAs were digested by *Dpn*I and the remaining DNAs were transfected into competent bacteria. Mutant cDNAs were later confirmed by DNA sequencing.

Sequence alignments were made using ClustalW 1.7 on the BCM

server (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>) and shaded for presentation using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Preparation of RNA and Oocyte Injection. After linearization and purification of cloned cDNAs, RNA transcripts were generated using the mMessage mMachine in vitro RNA transcription kit (Ambion, Austin, TX). Resultant RNA transcripts were evaluated by UV spectroscopy and denaturing agarose gel electrophoresis (visualized with ethidium bromide). RNAs were diluted to a concentration of 600 ng/ μ l and stored frozen in ribonuclease-free water at -80°C .

Ovarian lobes were surgically removed from anesthetized adult female *Xenopus laevis* frogs and then cut open to expose the oocytes. The ovarian tissue was then treated with collagenase for about 2 h at room temperature (1 mg/ml in oocyte saline solution: 82.5 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4 , 15 mM HEPES, 1 mM MgCl_2 , pH 7.4). After harvest, healthy stage-5 oocytes were isolated and injected with 50 nl each of a mixture of the appropriate subunit RNAs. Sterile oocyte storage medium (oocyte saline solution supplemented with 1.8 mM CaCl_2 , 5 U/ml penicillin, 5 μ g/ml streptomycin, and 5% horse serum) was changed daily. Recordings were made 2 to 7 days after injection depending on the RNAs being tested.

Electrophysiology. Two-electrode voltage-clamp recordings were made at room temperature in oocyte saline solution supplemented with 1.8 mM CaCl_2 and 1 μ M atropine. All recordings were made using a Turbo Tec 01C amplifier (Adams & List, Westbury, NY) at a holding potential of -50 mV unless otherwise noted. Recording electrodes were filled with 3 M KCl and typically had resistances in the range of 0.5 to 3 M Ω .

Oocyte recording solution was perfused at a rate of 5 ml/min through a Lucite recording chamber via a large bore pipette (1.5 mm diameter) placed about 0.5 mm above the oocyte. Agonist and antagonist solutions were applied by loading a loop near the terminus of one arm of the perfusion line. Constant perfusion was maintained by switching to the other arm of the perfusion line during loading of the drug loop. Antagonist solution can be pre-equilibrated by switching to a second reservoir before loading of the drug loop. Perfusion of oocyte saline solution from an independent reservoir at a rate of 2 ml/min maintained bulk flow through the recording chamber at all times. Based on the rise time of current responses, solution exchange times in the range of 500 to 800 ms are achieved under these conditions. Data were collected at a sampling rate of 100 Hz on a Gateway (N. Sioux City, SD) personal computer using Clampex 7 (Axon Instruments; Foster City, CA). From the time of each drug application, 2 min of data were acquired.

Each experimental response was normalized to an initial control response to agonist alone. A second control application of agonist alone subsequent to the experimental application permitted assessment of inhibition time course and receptor rundown. Each drug application was separated by a wash period of approximately 4 min. Values for EC_{50} , the Hill coefficient, and IC_{50} were estimated from curve fits to normalized data using Kaleidagraph 3.08 (Abelbeck/Synergy Software; Reading, PA). Data for receptor activation by acetylcholine were plotted using a nonlinear least-squares fit to the Hill equation:

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC}_{50})^n}$$

For voltage-dependence experiments, e-folding voltages were calculated from exponential fits to data of the form $\Lambda-1$, where Λ represents the ratio of control response to response in the presence of inhibitor (Ascher et al., 1979). The IC_{50} value was calculated with a nonlinear, least-squares fit to the equation:

$$\text{Response} = \frac{[\text{IC}_{50}]^n}{[\text{cocaine}]^n + (\text{IC}_{50})^n}$$

Because the inhibition is noncompetitive, IC_{50} was taken to be K_i and the ΔG° was calculated as $R\text{Tln}K_i$, where R is 1.987 kcal/mol and

T is the absolute temperature in Kelvin. The standard deviation for ΔG° ($\sigma_{\Delta G^\circ}$) was calculated from the expression:

$$\sigma_{\Delta G^\circ} = \sqrt{\left(\frac{RT}{K_1}\right)^2 \sigma_{K_1}^2}$$

and the error in $\Delta\Delta G^\circ$ from:

$$\sigma_{\Delta\Delta G^\circ} = \sqrt{\sigma_{\Delta G_1^\circ}^2 + \sigma_{\Delta G_2^\circ}^2}$$

where $\Delta\Delta G^\circ$ is $\Delta G_1^\circ - \Delta G_2^\circ$.

Initial experiments in which cocaine was coapplied with acetylcholine exhibited variability in terms of degree of inhibition by cocaine. Presumably, this variability was related to nonuniformities in perfusion of the entire surface area of the oocyte. Therefore, in later experiments, including all those described in this article, cocaine was pre-equilibrated with the oocyte for a period of about 30 s before application of agonist. This protocol allowed more reliable quantification of the degree of inhibition by cocaine. Moreover, the IC_{50} value for cocaine inhibition of the $\alpha 3\beta 4$ subunit combination was approximately $17 \pm 2 \mu M$ with coapplication, compared with $6 \pm 1 \mu M$ using a 30-s preincubation.

Results

Cocaine Inhibition of nAChR Subtypes. Because the oocyte expression system permits regulated expression of nAChRs of known subunit composition, it is possible to asso-

ciate pharmacological effects with specific subunit types. The effect of application of $10 \mu M$ cocaine with $30 \mu M$ ACh on a number of nAChR subunit combinations is shown in Fig. 1A, whereas inhibition curves for the various subunit combinations are shown in Fig. 1B (IC_{50} values are summarized in Table 1). The relative efficacy of the $30 \mu M$ agonist concentration in the absence of cocaine ranged from 41 to 58% of the maximal current (I_{max}) for all of the wild-type receptor combinations tested with the exception of $\alpha 2\beta 2$ and $\alpha 2\beta 4$ (12 and 18%, respectively; Table 2). Because of the lower potency for activation of $\alpha 2\beta 2$ and $\alpha 2\beta 4$ receptors, the effects of cocaine were also tested in combination with an agonist concentration near the EC_{50} value for these receptor subtypes ($130 \mu M$ ACh). No significant difference in cocaine IC_{50} value was detected for either subunit combination (data not shown).

The effects of cocaine were evaluated initially on nAChRs formed from the $\alpha 3$ subunit in combination with either the $\beta 2$ or $\beta 4$ subunit. Nicotinic receptors, including the $\alpha 3$ subunit, predominate in the peripheral nervous system, where they function to mediate transmission in the autonomic ganglia (Xu et al., 1999). Recent reports have described functional roles for brain $\alpha 3$ -containing nAChRs as well (Luo et al., 1998; Quick et al., 1999). Cocaine inhibits $\alpha 3\beta 4$ receptors with an IC_{50} value of approximately $6 \mu M$. The role of the β subunit in mediating the inhibitory effects of cocaine was

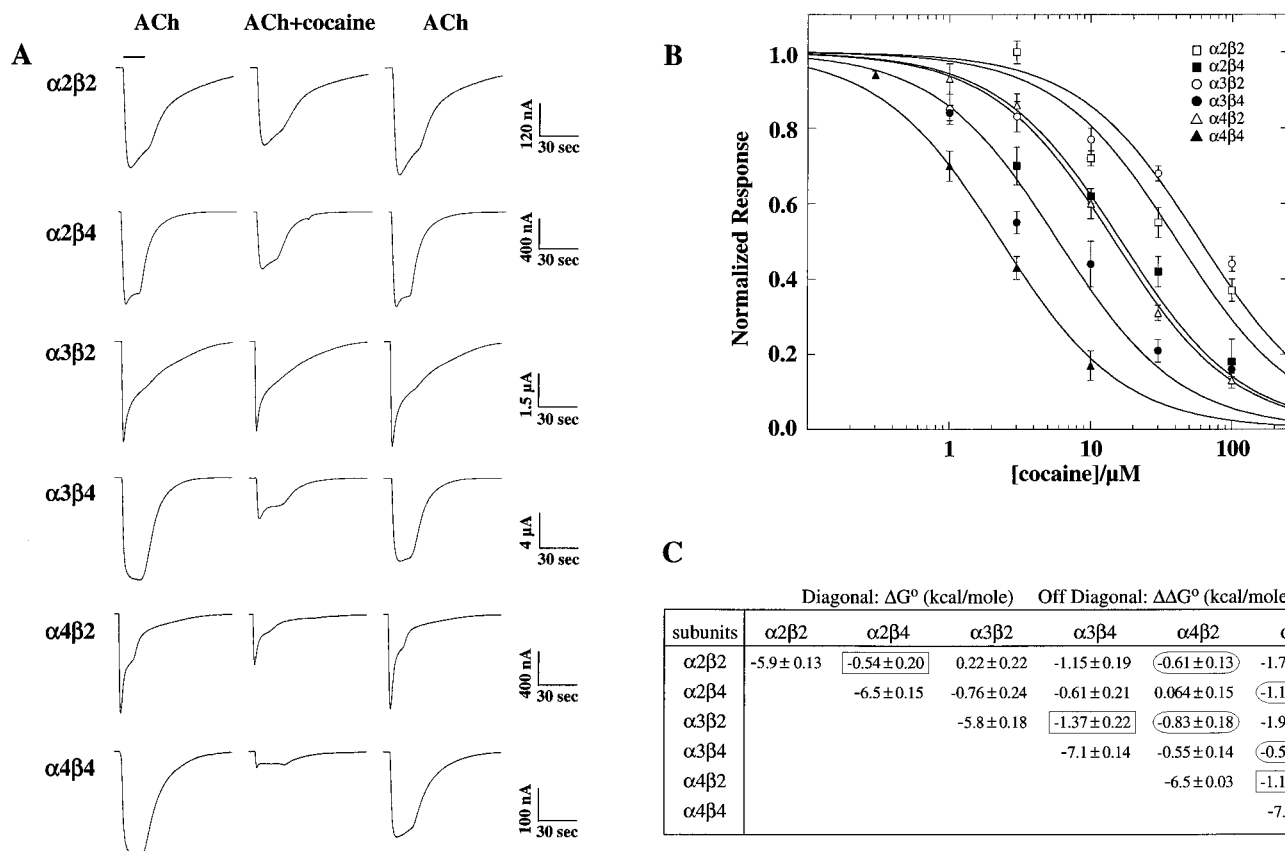


Fig. 1. Cocaine inhibition of nAChR subtypes. A, responses of neuronal nAChRs to $30 \mu M$ ACh in the absence and presence (middle trace) of $10 \mu M$ cocaine. ACh was applied for 15 s in each case, and the bar above the first trace (upper left) shows the timing of the application. In this figure and subsequent figures, the trace at left is the response to a control application of ACh alone, the middle trace is the response to application of ACh with cocaine, and the trace at right is the response to a second control application of ACh representing recovery from inhibition. Each application is separated by approximately 4 min. B, concentration dependence of cocaine inhibition of nAChR. Each data point represents the mean response of at least four oocytes to the coapplication of $30 \mu M$ ACh with varying concentrations of cocaine relative to $30 \mu M$ ACh alone. C, ΔG° and $\Delta\Delta G^\circ$ values associated with cocaine binding to nAChR subunit combinations. Boxed values indicate $\Delta\Delta G^\circ$ values for comparisons of $\beta 2$ - versus $\beta 4$ -containing receptors, and circles indicate $\Delta\Delta G^\circ$ values for $\alpha 4$ - versus $\alpha 3$ - and $\alpha 2$ -containing receptors.

evaluated by comparison with expression of the $\beta 2$ subunit in combination with $\alpha 3$. The IC_{50} value for cocaine inhibition of $\alpha 3\beta 2$ nAChRs is about 10-fold higher (60 μ M) than observed for $\alpha 3\beta 4$ receptors, indicating lower apparent affinity for this subunit combination. Based on the results for $\alpha 3$ -containing receptors, it seems likely that a high-affinity site for cocaine binding is determined by the presence of the $\beta 4$ subunit, whereas any site(s) associated with the $\beta 2$ and $\alpha 3$ subunits are relatively low-affinity.

Because the most widespread nAChR subunit combination in the brain with a high affinity for nicotine contains the $\alpha 4$ and $\beta 2$ subunits (Flores et al., 1992), potential effects of cocaine on this subunit combination are also of interest. Expression of the $\alpha 4$ subunit with the $\beta 2$ subunit increases apparent cocaine affinity about 4-fold compared with $\alpha 3\beta 2$ nAChRs (Fig. 1B), indicating that in the case of the $\alpha 4\beta 2$ subunit combination, the presence of the $\alpha 4$ subunit increases cocaine binding affinity. In contrast, $\alpha 2\beta 2$ receptors exhibit a lower apparent cocaine affinity, similar to that of $\alpha 3\beta 2$ receptors. Consistent with the results described above for the $\beta 4$ subunit, $\alpha 2\beta 4$ receptors exhibit higher apparent cocaine affinity than $\alpha 2\beta 2$ receptors. Pairing of the $\beta 4$ subunit with the $\alpha 4$ subunit further increases apparent cocaine affinity. Cocaine inhibits $\alpha 4\beta 4$ receptors with an IC_{50} value of about 2 μ M, suggesting that sites on each of the $\alpha 4$ and $\beta 4$ subunits determine apparent affinity for this subunit combination.

Calculation of differences in free energy change ($\Delta\Delta G^\circ$) associated with cocaine binding across subunit combinations permitted comparison of the effects of individual subunits

(Fig. 1C). In general, this analysis was consistent with the hypothesis that differences in apparent affinity were caused by independent contributions of the α and β subunits. However, cocaine binding to the $\alpha 2\beta 4$ subunit combination has a lower ΔG° value than would be predicted from the other measurements, suggesting a more complicated interaction with this subunit combination. The $\Delta\Delta G^\circ$ value associated with the presence of $\alpha 4$ is consistent for comparisons across all subunit combinations tested with the exception of $\alpha 2\beta 4$ (mean of -0.67 ± 0.27 kcal/mol for $\alpha 4\beta 2$ versus $\alpha 3\beta 2$, $\alpha 4\beta 2$ versus $\alpha 2\beta 2$, and $\alpha 4\beta 4$ versus $\alpha 3\beta 4$ compared with -1.17 ± 0.17 for $\alpha 4\beta 4$ versus $\alpha 2\beta 4$). Likewise, the $\Delta\Delta G^\circ$ associated with the presence of $\beta 4$ is consistent across subunit combinations with the exception of $\alpha 2\beta 4$ (average of -1.24 kcal/mol for $\alpha 3\beta 4$ versus $\alpha 3\beta 2$ and $\alpha 4\beta 4$ versus $\alpha 4\beta 2$ compared with -0.54 ± 0.20 for $\alpha 2\beta 4$ versus $\alpha 2\beta 2$). Moreover, the $\Delta\Delta G^\circ$ value between the subunit combination with the lowest apparent affinity ($\alpha 3\beta 2$) and the receptor type with the highest apparent affinity ($\alpha 4\beta 4$) is about -1.9 ± 0.2 kcal/mol, consistent with additive rather than synergistic effects of the two subunits.

Contribution of β Subunit to Inhibition by Cocaine.

Individual nicotinic receptor subunits share a conserved membrane topology composed of an N-terminal extracellular segment followed by four hydrophobic transmembrane domains. Comparison of the $\beta 2$ and $\beta 4$ subunit amino acid sequences reveals a number of differences in the N-terminal putative extracellular domains of the two subunits (Fig. 2A). In contrast, only a single amino acid differs between the pore-lining second transmembrane (M2) domains of the two subunits. To distinguish between these two potential regions of cocaine interaction (N terminus versus membrane-spanning domains), the effects of cocaine were tested on receptors incorporating chimeric β subunits in which all sequence N-terminal to M1 is exchanged between $\beta 2$ and $\beta 4$ (Fig. 2B). Functional effects of these exchanges on the kinetics of inhibition by neuronal bungarotoxin have been evaluated previously (Papke et al., 1993).

Receptors incorporating the $\alpha 3$ subunit with β subunits that exchange the N-terminal domain between $\beta 2$ and $\beta 4$ are not appreciably different from wild-type receptors in terms of cocaine sensitivity (Fig. 2, C and D; Table 1), indicating that the site of cocaine interaction with the β subunit is located C-terminal to M1. As measured from the application of 30 μ M ACh with a range of cocaine concentrations, the IC_{50} values for cocaine inhibition of wild-type $\alpha 3\beta 4$ receptors and chimeric $\alpha 3\beta 2_{228}/\beta 4$ receptors are nearly identical (6 μ M for each), whereas wild-type $\alpha 3\beta 2$ receptors and chimeric $\alpha 3\beta 4_{230}/\beta 2$ receptors show comparable IC_{50} values (60 and 35 μ M, respectively). The mean response of the chimeric receptors to 30 μ M acetylcholine alone was 32% of I_{max} for $\alpha 3\beta 2_{228}/\beta 4$ receptors but only 3% of I_{max} for $\alpha 3\beta 4_{230}/\beta 2$ receptors. Therefore, the effects of cocaine were also evaluated at an agonist concentration closer to the EC_{50} value for $\alpha 3\beta 4_{230}/\beta 2$ receptors (250 μ M). No significant difference in the cocaine IC_{50} value was detected.

Although exchange of the N-terminal domain between $\beta 2$ and $\beta 4$ has little effect on cocaine sensitivity, the exchange does seem to have reciprocal effects on response kinetics (Fig. 2C). Wild-type $\alpha 3\beta 2$ and chimeric $\alpha 3\beta 2_{228}/\beta 4$ receptors show qualitatively similar rapid response profiles during the time course of the agonist application (15 s), whereas wild-type

TABLE 1

IC_{50} values for cocaine inhibition of neuronal nAChRs

Receptor Type	IC_{50} μ M
$\alpha 3\beta 2$	60 ± 18
$\alpha 2\beta 2$	41 ± 9
$\alpha 4\beta 2$	15 ± 1
$\alpha 2\beta 4$	16 ± 4
$\alpha 3\beta 4$	6 ± 1
$\alpha 4\beta 4$	2
$\alpha 3\beta 2_{228}/\beta 4$	6 ± 2
$\alpha 3\beta 4_{230}/\beta 2$	35 ± 8
$\alpha 3\beta 4_{13'V}$	50 ± 10
$\alpha 3\beta 2_{13'F}$	17 ± 1
$\alpha 3_{267}/\alpha 4\beta 2$	44 ± 17
$\alpha 4_{267}/\alpha 3\beta 2$	16 ± 2
$\alpha 2_{128}/\alpha 4\beta 2$	15 ± 3
$\alpha 4_{128}/\alpha 2\beta 2$	36 ± 16

TABLE 2

Activation of neuronal nAChRs by acetylcholine

Receptor Type	EC_{50} μ M	Hill Coefficient	30 μ M/1 mM
$\alpha 3\beta 2$	70 ± 14	0.63 ± 0.09	0.43
$\alpha 2\beta 2$	131 ± 39	2 ^a	0.12
$\alpha 4\beta 2$	53 ± 18	0.81 ± 0.17	0.41
$\alpha 2\beta 4$	137 ± 15	1.08 ± 0.08	0.18
$\alpha 3\beta 4$	43 ± 4	1.02 ± 0.09	0.45
$\alpha 4\beta 4$	25 ± 2	0.91 ± 0.07	0.58
$\alpha 3\beta 2_{228}/\beta 4$	125 ± 30	0.68 ± 0.12	0.32
$\alpha 3\beta 4_{230}/\beta 2$	231 ± 2	1.82 ± 0.02	0.03
$\alpha 3\beta 4_{13'V}$	276 ± 17	1.59 ± 0.15	0.04
$\alpha 3\beta 2_{13'F}$	117 ± 17	0.80 ± 0.10	0.31
$\alpha 3_{267}/\alpha 4\beta 2$	169 ± 31	0.92 ± 0.15	0.23
$\alpha 4_{267}/\alpha 3\beta 2$	101 ± 19	0.55 ± 0.07	0.44
$\alpha 2_{128}/\alpha 4\beta 2$	36 ± 16	0.77 ± 0.21	0.52
$\alpha 4_{128}/\alpha 2\beta 2$	223 ± 56	0.84 ± 0.18	0.23

^a Constrained for curve fit.

$\alpha 3\beta 4$ and $\alpha 3\beta 4_{230}/\beta 2$ receptors show more protracted maximal responses. Certain difficulties, such as temporal averaging associated with physical limitations in achieving rapid and homogeneous solution exchange over the surface of the oocyte membrane, are intrinsic to the oocyte system and can complicate reliable quantification of receptor desensitization. Although this caveat should be kept in mind, for this relatively moderate agonist concentration (30 μM), the observed changes in response kinetics with incorporation of the chimeric β subunits are most consistent with altered receptor desensitization properties. Previous studies have described differences in steady-state dose-response relationships (Cohen et al., 1995) and single-channel kinetics (Papke and Heinemann, 1991) between $\alpha 3\beta 2$ and $\alpha 3\beta 4$ receptors. Our observations extend the studies of these subunit combinations by demonstrating that sequence elements contributing to desensitization kinetics are contained within the N-terminal domain of neuronal β subunits.

Owing to the high sequence homology in the M2 domains of

receptor subunits in the nicotinic superfamily, a common nomenclature has been adopted that designates the approximately 20 amino acids that comprise an element of the channel pore (Charnet et al., 1990). Homologous amino acid positions in M2 are designated 1' to 20' from the intracellular to extracellular extent of the membrane-spanning domain. Because the $\beta 2$ and $\beta 4$ subunits differ by only a single residue located at the 13' position (V in $\beta 2$; F in $\beta 4$) and this substitution has been implicated previously as a determinant of affinity for another noncompetitive inhibitor of neuronal nAChRs (Substance P; Stafford et al., 1998), the effects of reciprocal mutation of this residue on cocaine sensitivity were evaluated (Fig. 3, A and B; Table 1). Expression of the $\beta 2_{13'F}$ and $\beta 4_{13'V}$ subunits with the $\alpha 3$ subunit results in functional nAChRs that exhibit altered sensitivities to inhibition by cocaine. Specifically, $\alpha 3\beta 4_{13'V}$ mutant receptors show a decreased sensitivity to inhibition by cocaine to a level comparable with that of $\alpha 3\beta 2$ receptors (IC_{50} values of 50 μM). Conversely, $\alpha 3\beta 2_{13'F}$ receptors show an almost 4-fold

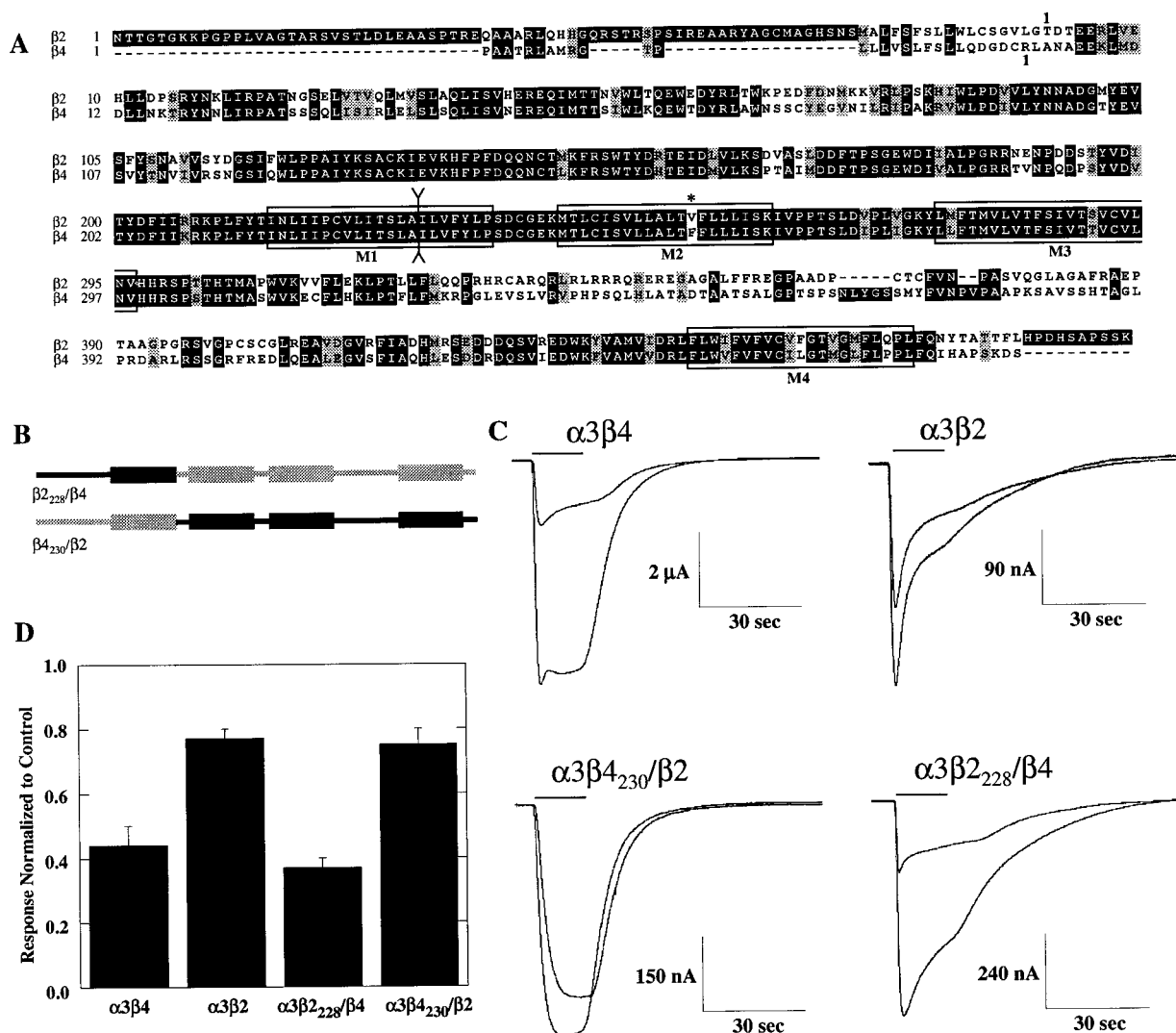


Fig. 2. Cocaine inhibition of β subunit chimeras. **A**, sequence alignment of nAChR β subunits. Identical amino acids are shaded black. Conservative differences are in gray. The four putative transmembrane domains are boxed. *, 13' position. The position of the break point in the chimeras is shown. **B**, schematic depicting structure of β subunit chimeras. In this figure and in Fig. 6, the thicker bars represent putative transmembrane regions. **C**, responses of neuronal nAChRs to an application of 30 μM ACh in the absence and presence (smaller amplitude trace) of 10 μM cocaine. Note the kinetic differences in the control responses. The length of the agonist application is shown by the bar above the traces. **D**, the mean response of nAChR subtypes to the coapplication of 30 μM ACh with 10 μM cocaine. Each column represents the mean ($\pm$ S.E.) of at least four oocytes.

increase in sensitivity to cocaine inhibition ($IC_{50} \sim 17 \mu M$). However, in this case, the valine to phenylalanine exchange at the 13' site does not fully recapitulate the cocaine affinity of wild-type $\alpha 3\beta 4$ receptors. Notably, substitution of valine for phenylalanine in the $\beta 4$ subunit also produces an approximately 6-fold increase in the EC_{50} value for activation by acetylcholine (Fig. 4A) such that $30 \mu M$ ACh only produces 4% of the maximal current (versus 45% for $\alpha 3\beta 4$). Again, for $\alpha 3\beta 4_{13'V}$ receptors, the inhibitory effects of cocaine were evaluated at a higher agonist concentration ($300 \mu M$) without any significant effect on IC_{50} values.

A similar effect on acetylcholine potency was noted above for $\alpha 3\beta 4_{230}/\beta 2$ (5.4-fold increase in EC_{50}) receptors suggesting that the 13' position is responsible for much of the effect on agonist potency associated with incorporation of the chimeric β subunit. Reciprocal mutation of the 13' residue of the $\beta 2$ subunit produces more modest effects (1.7-fold) on agonist potency (Fig. 4B; Table 2), indicating that this residue may not serve functionally homologous roles across the two classes of β subunit. From these data, it is not possible to distinguish between effects of this substitution on agonist binding and channel gating. However, this functional distinction between the 13' position of $\beta 2$ and $\beta 4$ may underlie our inability to achieve fully reciprocal effects on cocaine inhibition with mutation of the 13' residue. Alternatively, other sequence elements, such as adjacent transmembrane do-

main, may also directly or indirectly contribute to the binding site for cocaine.

Contribution of α Subunit to Inhibition by Cocaine.

The N-terminal domain of nicotinic receptor α subunits includes at least three stretches of amino acids previously demonstrated to contribute to agonist binding (loops A–C; for review, see Changeux and Edelman, 1998). Thus, it is possible that cocaine inhibits $\alpha 4\beta 2$ receptors by a competitive mechanism involving amino acids in the N terminus of the $\alpha 4$ subunit. Alternatively, cocaine may inhibit $\alpha 4\beta 2$ receptors by a noncompetitive mechanism involving binding to either extracellular or pore sites (including the N and C termini, transmembrane domains, and extracellular loop region between M2 and M3).

Initial experiments evaluated the mechanism of cocaine inhibition. For a competitive inhibitor, application of the IC_{50} concentration of cocaine would be expected to produce little inhibition in the presence of saturating agonist concentration. Coapplication of $30 \mu M$ cocaine (a concentration near the IC_{50} value for $\alpha 4\beta 2$ receptors as measured in the presence of $30 \mu M$ ACh; Table 1) with a range of ACh concentrations ($30 \mu M$ – 1 mM; Fig. 5) indicates that cocaine is a noncompetitive inhibitor of this receptor subtype. Application of cocaine in the presence of a higher ACh concentration (3 mM)

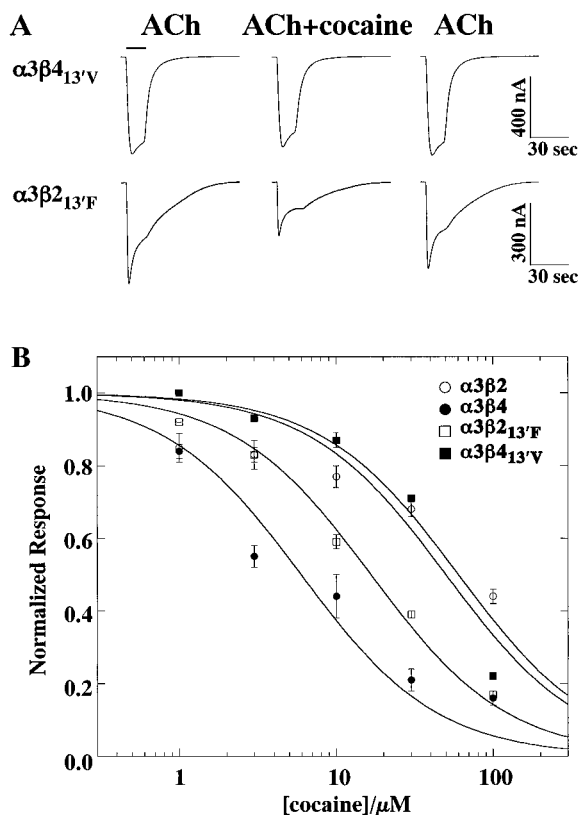


Fig. 3. Cocaine inhibition of 13' β subunit mutant nAChRs. A, responses of 13' mutant receptors to $30 \mu M$ ACh in the absence and presence (middle trace) of $10 \mu M$ cocaine. Each application is separated by approximately 4 min and the bar above the first trace shows the timing of the agonist application. B, concentration dependence of cocaine inhibition of nAChR. Each data point represents the mean response ($\pm$ S.E.) of at least four oocytes to the coapplication of $30 \mu M$ ACh with varying concentrations of cocaine and is normalized to a $30 \mu M$ ACh control response.

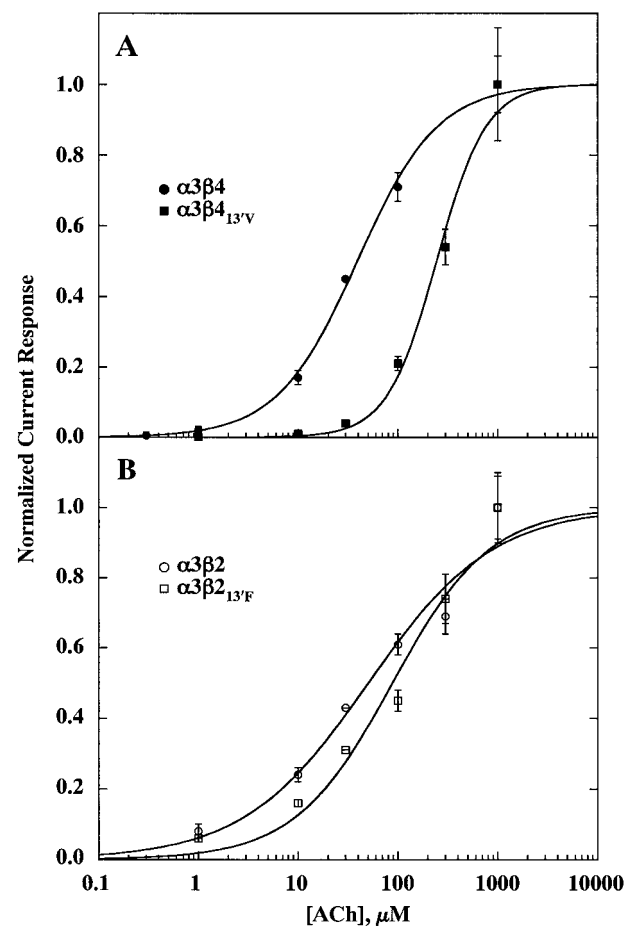


Fig. 4. Effects of mutation of β subunit 13' residue on activation by acetylcholine. A, concentration-response data for acetylcholine activation of $\alpha 3\beta 4$ and $\alpha 3\beta 4_{13'V}$ receptors expressed relative to a control application of $30 \mu M$ ACh. B, concentration-response data for acetylcholine activation of $\alpha 3\beta 2$ and $\alpha 3\beta 2_{13'F}$ receptors expressed relative to the ACh maximum. Each data point represents the mean ($\pm$ S.E.) of three to eight responses.

also produces significant inhibition (45%), consistent with noncompetitive inhibition.

To localize structural determinants of cocaine affinity on the $\alpha 4$ subunit, a similar strategy to the one described above was used. Because $\alpha 4\beta 2$ receptors exhibit a higher apparent affinity for cocaine than do $\alpha 2\beta 2$ or $\alpha 3\beta 2$ receptors, a series of chimeric subunits that exchange increasing amounts of N-terminal sequence between the $\alpha 4$ subunit and either the $\alpha 2$ or $\alpha 3$ subunits were created. The effects of these exchanges on cocaine inhibition were evaluated. The chimeric $\alpha 3_{267}/\alpha 4$ and $\alpha 4_{267}/\alpha 3$ subunits exchange sequence at a conserved *Bst*XI restriction site located at the approximate midpoint of the subunits in a region of sequence coding for the extracellular loop between M2 and M3 (Fig. 6, A and B, top). The chimeric $\alpha 4_{128}/\alpha 2$ and $\alpha 2_{128}/\alpha 4$ subunits exchange sequence at a conserved *Pst*I restriction site located in a region coding for the cysteine loop of the N-terminal extracellular domain (Fig. 6, A and B, bottom). Application of 30 μ M cocaine to receptors formed from the coinjection of the $\beta 2$ subunit with either the $\alpha 3_{267}/\alpha 4$ or $\alpha 4_{267}/\alpha 3$ chimeric subunits indicates that a site of high-affinity cocaine binding is associated with the N-terminal half of the $\alpha 4$ subunit (Fig. 6, C and D; IC_{50} values summarized in Table 1). Conversely, application of the same concentration of cocaine to receptors formed from $\beta 2$ with either the $\alpha 2_{128}/\alpha 4$ or $\alpha 4_{128}/\alpha 2$ chimeric subunits indicates that a high-affinity cocaine binding site is associated with sequence C-terminal to the initial cysteine residue of the cysteine loop in the mature $\alpha 4$ protein (Fig. 6, C and D; Table 1). For the $\alpha 2_{128}/\alpha 4\beta 2$ and $\alpha 4_{267}/\alpha 3\beta 2$ subunit combinations, application of 30 μ M ACh is sufficient to elicit 44 and 52% of the maximum current, respectively, whereas in the case of $\alpha 3_{267}/\alpha 4\beta 2$ and $\alpha 4_{128}/\alpha 2\beta 2$ receptors, 30 μ M ACh elicits only 23% of the maximum current. Again, evaluation of cocaine inhibition at higher agonist concentrations (170 μ M for $\alpha 3_{267}/\alpha 4\beta 2$ and 220 μ M for $\alpha 4_{128}/\alpha 2\beta 2$) yielded no significant change in estimated IC_{50} values. Taken together, these results localize a determinant of cocaine affinity to a

portion of the $\alpha 4$ subunit that includes approximately 90 amino acids of the N-terminal extracellular domain with M1 and M2. Because $\alpha 2\beta 2$ and $\alpha 3\beta 2$ receptors show similarly mild sensitivity to inhibition by cocaine and $\alpha 4\beta 2$ nAChRs exhibit a higher apparent affinity (Fig. 1), it seems reasonable to hypothesize that sequence determinants on the α subunits are conserved between $\alpha 2$ and $\alpha 3$, whereas they are nonconserved in $\alpha 4$. Using this logic in the context of the high degree of sequence identity that is preserved across the transmembrane domains of α subunits, it is possible to identify a stretch of approximately 50 amino acids immediately preceding M1 that likely determine cocaine affinity in $\alpha 4$ (Fig. 6A). However, reciprocal mutation between $\alpha 4$ and either $\alpha 2$ or $\alpha 3$ of eight different nonconserved residues considered to be the most likely candidates for mediating cocaine binding in this region produced receptors that differed only slightly in cocaine sensitivity, suggesting that cocaine binding may involve multiple amino acids. Given the complex folding that is presumably required for formation of the agonist binding pocket in this region, regulation of cocaine affinity for the $\alpha 4$ subunit may be a distributed phenomenon requiring contributions from multiple residues in the stretch of sequence identified by the subunit chimeras. Alternatively, the data do not rule out a contribution from the few nonconserved residues in M1 and M2.

Voltage Dependence of Cocaine Inhibition. As a second approach to localizing determinants of cocaine affinity, we sought to evaluate the disposition of cocaine binding sites with respect to the membrane electric field. The effects of changes in holding potential on inhibition were tested for the $\alpha 3\beta 4$, $\alpha 3\beta 2_{13F}$, and $\alpha 4\beta 2$ subunit combinations (Fig. 7). These subunit combinations were chosen to ensure that only a single class of binding site was evaluated in each case. For example, in the case of $\alpha 3\beta 4$ receptors, any effects of holding potential on inhibition are likely associated primarily with a site on the $\beta 4$ subunit because $\alpha 3\beta 2$ receptors show little inhibition by cocaine at the concentration used in these experiments (10 μ M; Fig. 1). Similarly, in the case of $\alpha 4\beta 2$ receptors, it is likely that any observed effects of holding potential are primarily associated with the $\alpha 4$ subunit because the $\beta 2$ subunit shows only low cocaine affinity.

Consistent with binding to a site in the channel pore, inhibition of $\alpha 3\beta 4$ receptors by cocaine shows voltage dependence. Application of 10 μ M cocaine at a standard holding potential of -50 mV results in approximately 56% inhibition (Fig. 1). Application of the same concentration of cocaine at a holding potential of -100 mV results in about 80% inhibition, whereas raising the holding potential to -20 mV decreases the observed inhibition to about 30% of control. Inhibition appears linear over the range of holding potentials tested, with an e-fold change in inhibition for a 34-mV change in holding potential. Inhibition of the $\alpha 3\beta 2_{13F}$ mutant also exhibits appreciable voltage dependence (e-folding voltage of 45 mV, data not shown).

In contrast to the voltage dependence of inhibition observed for $\alpha 3\beta 4$ receptors, inhibition of $\alpha 4\beta 2$ receptors by cocaine exhibits a much weaker voltage dependence across the range of potentials tested. Specifically, application of 20 μ M cocaine to $\alpha 4\beta 2$ receptors (a concentration approximately equipotent to the 10 μ M concentration used for $\alpha 3\beta 4$ receptors) results in about 70% inhibition at -100 mV, whereas

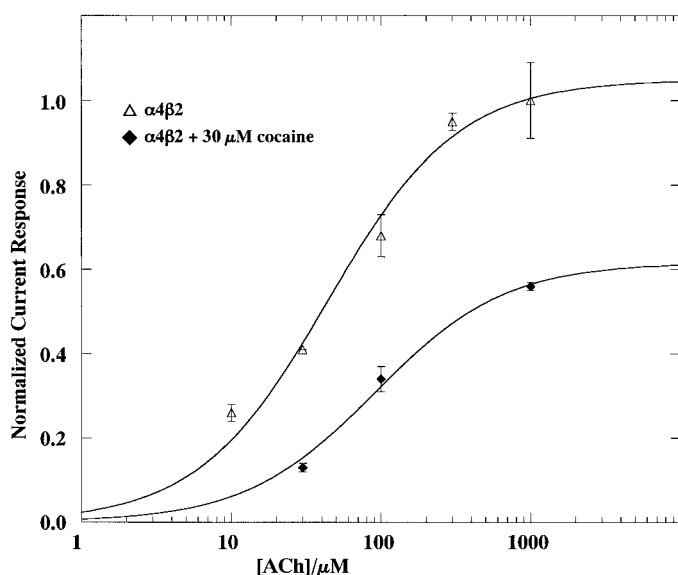


Fig. 5. Cocaine inhibition of $\alpha 4\beta 2$ receptors as a function of agonist concentration. Acetylcholine (10 μ M–1 mM) was applied in the absence and presence of 30 μ M cocaine. Each data point is expressed relative to the ACh maximum and represents the mean ($\pm$ S.E.) of three to six responses.

50% inhibition is observed at a holding potential of -20 mV (e-folding voltage of 90 mV).

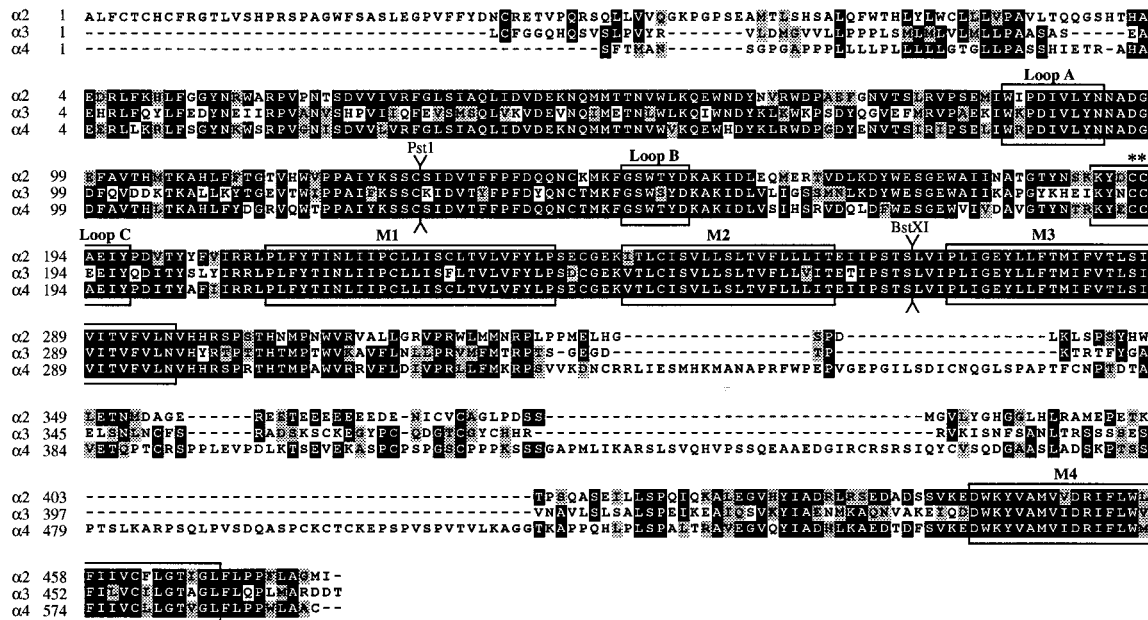
Differences in the voltage dependence of cocaine inhibition across the two receptor types suggest that cocaine inhibition arises via mechanistically distinct processes. In the case of $\alpha 3\beta 4$ receptors, the data are consistent with binding to a channel site. However, the voltage-dependence data argue against a similar mechanism for inhibition of $\alpha 4\beta 2$ nAChRs. Given the high degree of sequence identity in the M2 regions of $\alpha 2$ – $\alpha 4$ (97%, Fig. 6A), the voltage-dependence data are

most consistent with the presence of an extracellular binding site for cocaine associated with the $\alpha 4$ subunit.

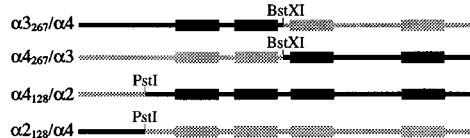
Discussion

Although the addictive properties of cocaine are likely mediated via interactions with neurotransmitter transporters, our data indicate that cocaine can also inhibit members of the nicotinic family of nervous system LGICs. Serum concentrations of cocaine can rise to a peak near $1 \mu\text{M}$ within 30 min

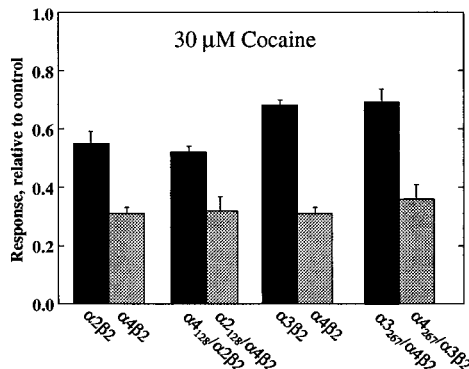
A



B



D



C

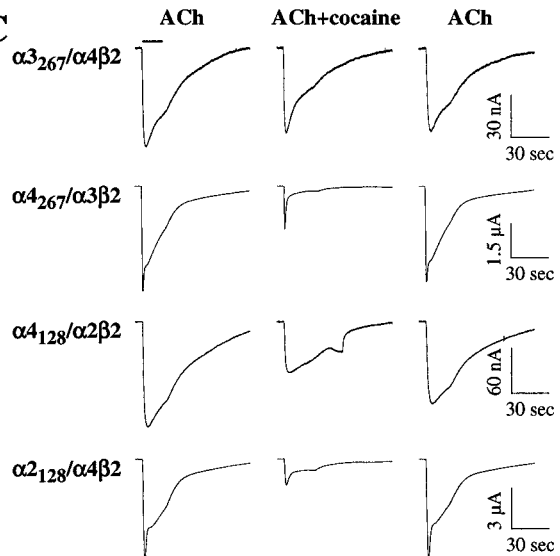


Fig. 6. Cocaine inhibition of α subunit chimeras. **A**, sequence alignment of nAChR α subunits. Identical amino acids are shaded black. Conservative differences are in gray. The three principle loops believed to participate in agonist binding and the four putative transmembrane domains are denoted. The conserved restriction sites for PstI and BstXI used in constructing the $\alpha 2/4$ and $\alpha 3/4$ subunit chimeras are also shown. Numbering is relative to the vicinal cysteines (192 and 193) indicated by the asterisk. **B**, schematic depicting structure of α subunit chimeras. Chimeric subunits were created using homologous restriction sites shared between either $\alpha 2$ and $\alpha 4$ (PstI) or $\alpha 3$ and $\alpha 4$ (BstXI). **C**, responses of α subunit chimeric receptors to $30 \mu\text{M}$ ACh in the absence and presence (middle trace) of $30 \mu\text{M}$ cocaine. The bar above the first trace shows the timing of the agonist application. **D**, the mean response of nAChR subtypes to the coapplication of ACh with cocaine. Each column represents the mean relative response ($\pm$ S.E.) of four or more oocytes to the application of $30 \mu\text{M}$ ACh with $30 \mu\text{M}$ cocaine.

after nasal insufflation (Jeffcoat et al., 1989) and brain concentrations are likely to be higher because of the lipophilic nature of the drug (Nayak et al., 1976). Our data indicate that cocaine inhibits particular nAChR subtypes with an affinity comparable with that displayed for the open/inactivated state of voltage-gated sodium channels ($\sim 10 \mu\text{M}$; Wright et al., 1997), suggesting the possibility of neuronal nAChR-mediated cocaine effects. Cocaine potency shows considerable variability across nAChR subtypes, with IC_{50} values ranging between $2 \mu\text{M}$ ($\alpha 4\beta 4$) and $60 \mu\text{M}$ ($\alpha 3\beta 2$). Although inhibition of the $\alpha 3\beta 4$ subunit combination is voltage-dependent and affected by mutation of the 13' residue of the pore-lining M2 domain, inhibition of the $\alpha 4\beta 2$ subtype shows only very weak voltage dependence and is probably associated with N-terminal sequence immediately preceding M1 (Fig. 8).

The present study also identified β subunit residues important in the processes of receptor desensitization (Fig. 2) and activation (Fig. 4). Although the observed effects on receptor desensitization are associated with the N-terminal portion of the β subunit, the most significant changes in receptor activation properties are associated with mutation of the 13' residue of the $\beta 4$ subunit. The processes of desensitization and binding/gating do not seem to be determined by common sequence elements because mutation of the 13' site does not yield appreciable differences in response time course (Fig. 3). A number of previous studies have illustrated effects of mutation of pore residues on channel gating. Most notably, the 9' position has also been implicated in the gating/binding process (Revah et al., 1991; Filatov and White, 1995; Labarca et al., 1995; Kearney et al., 1996). The observation that mutation of the 13' position has differential effects on receptor activation across the $\beta 2$ and $\beta 4$ subunits emphasizes the idea that sequence homologues in the pore region are not necessarily functional homologues.

Inhibition of $\alpha 3\beta 4$ Receptors May Involve Open-Channel Block. Receptors incorporating the $\alpha 3$ subunit with chimeric subunits that pair N-terminal $\beta 2$ sequence with $\beta 4$ sequence C-terminal to M1 show sensitivity to cocaine inhibition comparable with wild-type $\alpha 3\beta 4$ receptors

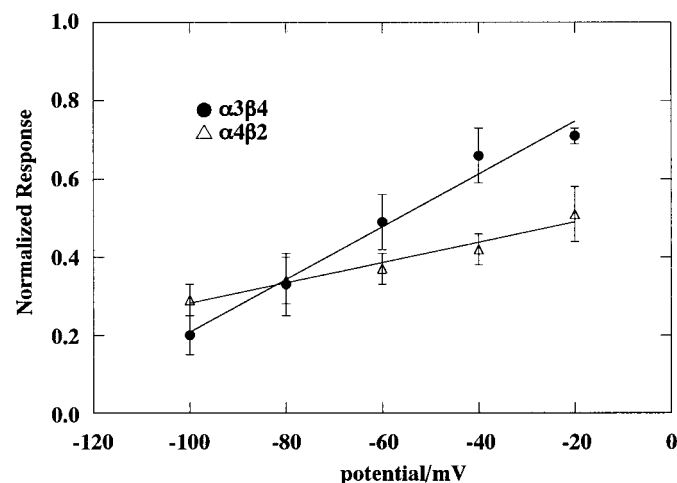


Fig. 7. Voltage dependence of cocaine inhibition of $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs. Acetylcholine ($30 \mu\text{M}$) was applied in the absence and presence of either $10 \mu\text{M}$ ($\alpha 3\beta 4$) or $20 \mu\text{M}$ ($\alpha 4\beta 2$) cocaine after a brief equilibration period at the specified holding potential. Each data point represents the mean ($\pm$ S.E.) response of four or more cells.

whereas the reciprocal chimeric receptors (N-terminal $\beta 4$ sequence with C-terminal $\beta 2$ sequence) are inhibited by cocaine with a potency similar to wild-type $\alpha 3\beta 2$ receptors. This result localizes the site of high-affinity cocaine binding to the C-terminal portion of the $\beta 4$ subunit beyond M1. Within this region, exchange of the 13' site between $\beta 2$ and $\beta 4$ has reciprocal effects on the concentration dependence of cocaine inhibition, implicating the 13' position as a determinant of cocaine binding affinity in $\alpha 3\beta 4$ receptors. Consistent with binding to a site in the ion channel pore, cocaine inhibition of $\alpha 3\beta 4$ receptors exhibits voltage dependence. Given this observation and the dependence on sequence in the M2 region, cocaine may act as a conventional open-channel blocker on the $\alpha 3\beta 4$ subtype of nAChR. However, the observation that cocaine has a lower potency at $\alpha 3\beta 2_{13'F}$ receptors compared with wild-type $\alpha 3\beta 4$ receptors or chimeric $\alpha 3\beta 2_{228}/\beta 4$ receptors may indicate that other sequence elements not contained within the M2 domain can contribute to the disposition of the cocaine binding site. Residues in the adjacent membrane-spanning domains or connecting loops could contribute directly to a cocaine-binding site or otherwise affect the exposure of the 13' residue to the channel pore. Additionally, because the functional role of the 13' position in receptor activation does not seem to be maintained across classes of β subunit (Fig. 4), exposure of this residue may differ during gating of the two receptor subtypes.

Inhibition of both the dopamine transporter and sodium channels by cocaine involves binding to phenylalanine and/or

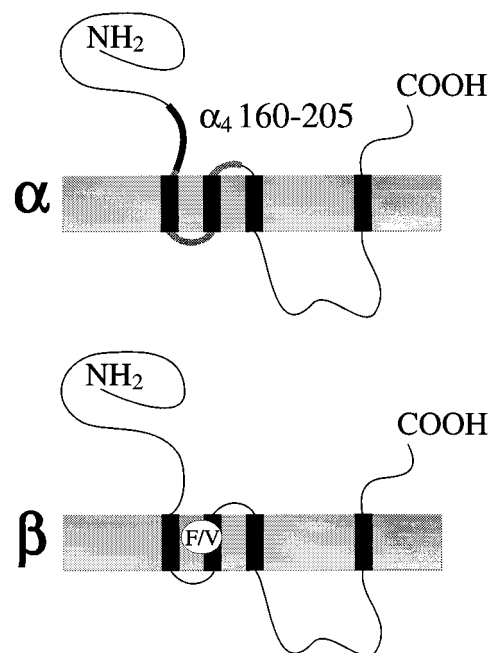


Fig. 8. Putative membrane topology of nAChR subunits and regions involved in determining cocaine affinity. In these studies, a stretch of sequence immediately preceding and inclusive of M1 and M2 of the $\alpha 4$ subunit (top, gray) is shown to contribute to determination of cocaine affinity. Based on the degree of sequence conservation in the membrane spanning domains of the α subunits, a smaller region preceding M1 (top, black) probably contains the cocaine binding site in $\alpha 4$. A second site associated with the presence of a phenylalanine residue (F) at the 13' position of the pore-lining M2 segment of the $\beta 4$ subunit (bottom) also can determine cocaine affinity. The lower affinity $\beta 2$ subunit has a valine residue (V) at this position.

other aromatic residues (Ragsdale et al., 1994; Lin et al., 1999), suggesting a common mechanism of action across classes of protein. The importance of cation- π interactions between charged groups and aromatics in receptor-ligand interactions and protein structure has recently emerged (for review, see Dougherty, 1996). Our data may indicate that the protonated amine group of cocaine interacts with the aromatic ring of phenylalanine. In this case, voltage dependence might arise from the essentially electrostatic nature of the cation- π interaction. Alternatively, the phenyl ring of cocaine could interact with the aromatic group of the 13' phenylalanine. In this case, the amine moiety may project deeper into the pore. The size of cocaine is estimated to be on the order of 6×12 Å. Given the partially helical nature of nAChR subunit M2 regions (Akabas et al., 1994; Unwin, 1995), the protonated amine group of cocaine may project as deep as the 6' position (~ 5.5 Å/turn). In either scenario, selectivity for $\beta 4$ -containing receptors arises from the presence of phenylalanine at the 13' site, a feature that distinguishes cocaine inhibition of $\beta 4$ -containing neuronal nAChRs from both quaternary local anesthetic inhibition of muscle nAChR (Charnet et al., 1990) and inhibition of $\alpha 3\beta 4$ receptors by mecamylamine (Webster et al., 1999), both of which involve binding to the 6' and 10' positions. As the human and rat forms of the nAChR $\beta 4$ subunit are the only subunits in the nicotinic superfamily that include a phenylalanine at this position, inhibition of nAChRs by this mechanism is restricted to receptors containing the $\beta 4$ subunit. A third possibility that seems less likely is that the 13' site contributes allosterically to a cocaine binding site located distal from the pore.

Potential Allosteric Inhibition of $\alpha 4\beta 2$ Receptors.

The α subunit chimeras localize a determinant of cocaine affinity to a region that includes a portion of the N-terminal extracellular segment with M1 and M2. On the basis of sequence identity alone, contributions from a large number of residues in this region, particularly residues in the membrane-spanning domains, can be eliminated (Fig. 6A). By concentrating on residues that differ between $\alpha 4$ and the other α subunits, a likely determinant of cocaine affinity can be further localized to a relatively nonconserved stretch of 50 amino acids immediately preceding M1. A region containing the vicinal cysteine pair thought to be a component of the acetylcholine binding site (loop C) is also contained within this stretch of amino acids, suggesting that cocaine may act in the vicinity of the agonist binding site. Consistent with a previous study (Lerner-Marmarosh et al., 1995), our results indicate that cocaine is not competitive with ACh over a 100-fold range in ACh concentration ($30 \mu\text{M}$ – 3 mM ; Fig. 5). Therefore, cocaine probably binds to an allosteric site on $\alpha 4\beta 2$ receptors and inhibits activation in a noncompetitive fashion.

Possible Functional Relevance of Cocaine Effects on nAChRs. Nicotinic receptors containing the $\alpha 4$ and $\beta 2$ subunits are the predominant form of high-affinity nicotine receptors in the brain and the activation and/or subsequent desensitization of this subunit combination by nicotine is believed to be responsible for the addictive properties of the drug (Peng et al., 1994; Dani and Heinemann, 1996; Picciotto et al., 1998). Thus, this receptor type could provide a site of interaction for cocaine and nicotine effects. Synergistic effects between nicotine and the noncompetitive inhibitor

mecamylamine on receptor turnover have been described previously (Peng et al., 1994).

Because nicotine is a desensitizing agonist with residual inhibitory properties on nAChRs expressed in oocytes (de Fiebre et al., 1995; Fenster et al., 1997), the physiological agonist acetylcholine was used in these studies to examine the inhibitory properties of cocaine independent of those of nicotine. However, another recent study has examined cocaine antagonism of both behavioral measures of nicotine-elicited effects, such as antinociception, and nicotine activation of heterologously expressed receptor subtypes, demonstrating significant inhibition in both cases (Damaj et al., 1999).

A few recent reports have demonstrated the functional significance of $\alpha 3\beta 4$ -containing nAChRs in the CNS. Functional studies employing an $\alpha 3\beta 4$ -selective conotoxin have demonstrated the importance of $\alpha 3\beta 4$ -containing nAChRs in the medial habenula (Quick et al., 1999) and in mediating nicotine-elicited release of noradrenaline in the hippocampus (Luo et al., 1998). Moreover, nicotine-elicited hippocampal noradrenaline release is inhibited by cocaine with an IC_{50} value of $< 1 \mu\text{M}$ (Hennings et al., 1999). Although these native subunit combinations are likely to be more complex than the simple pair-wise combinations used in our studies, these results are consistent with our data and underscore the possibility of central effects associated with inhibition of neuronal nAChRs.

In light of the growing appreciation that nicotinic receptors play a modulatory role centrally via regulation of the release of other neurotransmitters and activation of certain classes of interneurons, interactions between cocaine and nAChRs could be of considerable importance. Although the euphoric effects of cocaine are probably mediated by the dopaminergic system, given the widespread involvement of nAChRs in the peripheral nervous system (Xu et al., 1999), nAChR inhibition could also be contributory to peripherally mediated toxic effects of cocaine. Although additional studies in situ are obviously required, the present results indicate that neuronal nAChRs constitute an additional site of potential cocaine action in the nervous system that should be incorporated into conceptual models of the drug's pharmacology.

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