0026-895X/92/040736-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:736-742

Interactions of Cocaine with Primary and Secondary Recognition Sites on Muscarinic Receptors

DONNA D. FLYNN, ASMITA A. VAISHNAV, and DEBORAH C. MASH

Departments of Molecular and Cellular Pharmacology (D.D.F., A.A.V., D.C.M.) and Neurology (D.C.M.), University of Miami School of Medicine, Miami, Florida 33101

Received September 26, 1991; Accepted January 2, 1992

SUMMARY

Several lines of evidence have suggested that muscarinic receptors may possess more than one ligand binding site. In this study, the interactions of cocaine with primary and secondary (allosteric) sites on muscarinic receptors in membrane homogenates from post-mortem human brainstem were examined. (−)-Cocaine inhibited the binding of the tritiated muscarinic antagonists *N*-methylscopolamine (NMS) and pirenzepine to an apparent single class of sites, with K_i values of 200–300 μM. The binding of the muscarinic agonist [^{3}H]oxotremorine-M was inhibited with a similar K_i value (200 μM). (+)-Cocaine, although not the naturally occurring stereoisomer, was 10–20-fold more potent than (−)-cocaine in competing for binding to the primary muscarinic recognition site. The binding of cocaine was unaffected by guanine nucleotides or *N*-ethylmaleimide, consistent with its purported action as a competitive antagonist. Cocaine was not selective for muscarinic receptor subtypes. Rosenthal analysis of the [^{3}H]NMS saturation binding data in the presence of increasing concentrations of either (−)-cocaine or (+)-cocaine indicated that both isomers produced an apparent competitive-like reduction in the [^{3}H]NMS affinity. Schild regression analysis of the saturation binding data resulted in curvilinear plots suggestive of cooperative or allosteric interactions of (−)-cocaine with the [^{3}H]NMS-labeled receptors. The effects of (−)-cocaine on the kinetics of [^{3}H]NMS binding were consistent with an allosteric interaction with the receptor. Increasing concentrations of cocaine markedly slowed the rate of [^{3}H]NMS dissociation from the primary recognition site. The allosteric modulation of [^{3}H]NMS binding by (−)-cocaine was abolished with increasing ionic strength. Taken together, these data demonstrate that (−)-cocaine interacts with primary and allosteric recognition sites on muscarinic receptors.

Cocaine has been demonstrated to inhibit competitively, at low micromolar concentrations, binding of [^{3}H]QNB to muscarinic receptors assayed in heart and brain membranes (1). Cocaine was also effective, at similar concentrations, in blocking methacholine-induced inhibition of atrial contractions. These interactions of cocaine with muscarinic receptors are not surprising, given its structural similarity to atropine, a potent muscarinic antagonist. Most other neurotransmitter receptors, with the exception of the haloperidol-sensitive σ receptor, have relatively low or no affinity for (±)-cocaine (2, 3). It has been postulated that, whereas the antimuscarinic actions of cocaine may not contribute to the reinforcing properties of cocaine (2), they may be associated with the syndrome of excited delirium (4) and the cardiotoxic actions of cocaine (1).

There are a number of lines of evidence that suggest that muscarinic receptors possess more than one ligand binding site. Early functional studies (5) demonstrated that gallamine, a cardioselective muscarinic antagonist, did not bind competitively with carbachol at atrial muscarinic receptors. Subsequent studies demonstrated that gallamine allosterically modulated muscarinic receptor affinity in equilibrium binding assays (6, 7). Dissociation kinetic experiments demonstrated that gallamine also slowed the off-rate of the competitive antagonists [^{3}H]NMS and [^{3}H]QNB from cardiac muscarinic receptors, through its interaction with an allosteric site on the muscarinic receptor (8, 9). These complex binding behaviors exhibited by gallamine and other diverse classes of compounds suggested that a distinct site, on or closely associated with the muscarinic receptor, interacts with the primary ligand binding site in an allosteric fashion. Recent studies elucidating the molecular structure of muscarinic receptors have confirmed the existence of several aspartic acid residues that appear to be differentially involved in ligand binding (10). These aspartic acid residues are conserved in other members of the large family of membrane-spanning G protein-coupled receptors (11). Mutagenesis of residue 71 from aspartic acid to asparagine resulted in a significant modification in the allosteric interactions of galla-

This work was supported in part by National Institutes of Health Grants NS19065 and DA06227 and National Institutes of Health Biomedical Research Support Grant S07 RR-05363.

ABBREVIATIONS: QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; G protein, guanine nucleotide-binding protein; Oxo-M, oxotremorine-M; Gpp(NH)p, guanylyl-5′-imidodiphosphate; NEM, *N*-ethylmaleimide.

mine and methoctramine with the M1 muscarinic receptor (12), suggesting a possible role of the conserved aspartic acid residues in the interaction of allosteric modulators with muscarinic receptors.

Allosteric interactions at cardiac, neuronal, and smooth muscle muscarinic receptors have been reported for a number of chemically distinct classes of drugs. These include the cardioselective muscarinic antagonists (13, 14), antiarrhythmic agents (15), ganglionic and neuromuscular blockers (8, 16), acetylcholinesterase inhibitors and reactivators (17, 18), and K^+ and Ca^{2+} channel blockers (19, 20). In this report, we present evidence that demonstrates that, in addition to its competitive interaction at the primary muscarinic ligand binding site, cocaine is a member of the diverse class of drugs that interacts also with a second, allosteric, site on muscarinic receptors.

Experimental Procedures

Materials. Radioisotopes were obtained from the New England Nuclear Division of DuPont (Boston, MA), at the following specific activities: [^{3}H]NMS, 71.3–85.0; [^{3}H]pirenzepine, 87.0; and [^{3}H]Oxo-M, 87.5 Ci/mmol. Chemicals, including (−)-cocaine, were obtained from Sigma Chemical Co. (St. Louis, MO), with the exception of Gpp(NH)p, tetralithium salt, which was from Boehringer Mannheim (Indianapolis, IN), and (±)-QNB, which was from Research Biochemicals Inc. (Natick, MA). (+)-Cocaine was provided by the National Institute on Drug Abuse (Bethesda, MD).

Membrane preparation. Human brain tissue was obtained at routine autopsy from accident victims (males; age range, 20–25 years; autolysis times, 8–15 hr). Samples of the brainstem or frontal cortex were dissected and homogenized with a Brinkmann (Westburg, NY) Polytron, in 10 volumes of ice-cold 50 mM sodium phosphate buffer, 10 mM Na_3EDTA (pH 7.4), containing 0.1 mM phenylmethylsulfonyl fluoride. After a 30-min incubation on ice, membranes were collected by centrifugation at 27,000 × *g* for 10 min at 4°. Membrane pellets were resuspended in assay buffer at a concentration of 50 mg of original brain tissue/ml.

Competition and saturation binding assays. Competition binding assays between increasing concentrations of cocaine (1.0 M to 0.01 μM) and ^{3}H-labeled muscarinic ligands were performed in either 50 mM $NaPO_4$, 1 mM EDTA ([^{3}H]NMS at 0.5 nM), or 20 mM Tris·HCl, 1 mM $MnCl_2$ ([^{3}H]pirenzepine at 3 nM and [^{3}H]Oxo-M at 5 nM), pH 7.4, buffer, at 25° for 1–2 hr, as previously described (18). In some assays, Gpp(NH)p or NEM was included at a final concentration of 0.2 mM or 0.1 mM, respectively. Saturation isotherms were generated by incubating [^{3}H]NMS (0.5–5.0 nM) with various concentrations of cocaine for 60 min at 25°. Binding was terminated by rapid vacuum filtration over Whatman 934AH glass fiber filters. Filters were washed three times with ice-cold assay buffer, dried at 50°, and counted for radioactivity in 4 ml of Cytoscint (Isolab, Irvine CA), at an efficiency of 42%.

Association/dissociation kinetics. The rate of association of 1.0 nM [^{3}H]NMS to muscarinic receptors in brainstem homogenates was measured in the presence and absence of cocaine, at 25°. Binding was stopped at various times by immediate collection of labeled membranes on filters by vacuum filtration, as described above. After equilibration of membranes with 1.0 nM [^{3}H]NMS (60 min), dissociation was measured after addition of 10 μM (±)-QNB. Aliquots were removed at various times and filtered as described above.

Data analysis. Competition binding data were fitted to a one- or two-site model using the iterative, nonlinear, least-squares, regression analysis of the RS-1 program (BBN Software Products Corp., Cambridge, MA). The points in each figure, with the exception of the kinetic experiments, are means of three to six determinations from two to four experiments. Standard deviations for these determinations are not shown, because they averaged 1–4% of the means. Fifty-percent inhibitory concentrations (IC_{50} values) and the proportion of sites in each affinity state were derived from the binding curves. Dissociation constants for cocaine or carbachol were calculated from the IC_{50} values, using the equation of Cheng and Prusoff (21). To determine whether the data were significantly better fit to a one- or two-site model, the residual sum of the squares of the respective fits were compared by a partial *F* test. Data from saturation experiments were transformed by the method of Rosenthal (22), to determine the K_d and B_{max} values. Significant differences in K_d and B_{max} values were determined using a one-way analysis of variance and Scheffe's test. Association and dissociation rate constants were calculated by least squares linear regressions of the data. Schild regressions (23) were generated from the competition or saturation binding data.

Results

The competition binding curves between (−)-cocaine and ^{3}H-labeled muscarinic ligands in human brainstem membranes are shown in Fig. 1. Cocaine inhibited the binding of all three muscarinic ligands to an apparent single class of sites, with similar IC_{50} values (3–5 × 10^{-4} M). There was no effect of 0.2 mM Gpp(NH)p or 0.1 mM NEM, known uncouplers of high affinity agonist binding to G protein-coupled receptors, on the binding of cocaine to the labeled sites (data not shown). These data suggest that (−)-cocaine is a competitive antagonist at the primary muscarinic binding site. The similar K_i values for the inhibition by cocaine of [^{3}H]pirenzepine (which at 3 nM labels 33% of the M1 sites and <1% of the M2 sites) binding in the cortex (M1-rich region) and of [^{3}H]NMS and [^{3}H]Oxo-M binding in the brainstem (>80% M2 sites) suggest that cocaine is not selective for M1 or M2 receptor subtypes.

The competition between (+)- or (−)-cocaine and 0.5 nM [^{3}H]NMS for binding to the primary ligand recognition on muscarinic receptors in human brainstem membranes is shown in Fig. 2. (+)-Cocaine, which is not a naturally occurring isomer of the coca plant, was 17-fold more potent than the endogenous (−)-isomer of cocaine in inhibiting [^{3}H]NMS binding. The K_i values for the (+)- and (−)-isomers were calculated from the IC_{50} values, using the Cheng-Prusoff equation, and were 14.9 and 250 μM, respectively.

Rosenthal plots of the saturation binding isotherms for [^{3}H]NMS binding to human brainstem membranes, in the presence of increasing concentrations of the (−)- (Fig. 3A) or (+)-isomer (Fig. 3B) of cocaine, are presented in Fig. 3. These data indicate that both isomers of cocaine produce an apparent competitive-like reduction in the affinity of muscarinic binding sites for [^{3}H]NMS. Table 1 presents a summary of the K_d and B_{max} values calculated from the saturation binding isotherms. There was no statistically significant alteration (analysis of variance) in the maximal number of [^{3}H]NMS binding sites in the presence of either cocaine isomer. However, Schild regression analysis (Fig. 4; see Table 1 for K_d shift values) of the saturation binding data resulted in a curvilinear plot for (−)-cocaine, which deviated significantly from the theoretical curve for a competitive interaction between (−)-cocaine and [^{3}H]NMS. The resulting slope for (+)-cocaine (0.9) was not significantly different from a slope of 1.0, which describes a curve for a competitive interaction between (+)-cocaine and [^{3}H]NMS. Whether there was a further affinity shift with (+)-cocaine could not be studied, because at higher concentrations of (+)-cocaine the ratio of total to nonspecific binding of [^{3}H]NMS was too low to allow accurate assessment of binding. The inverse logarithm of the *x*-intercepts of the Schild regressions

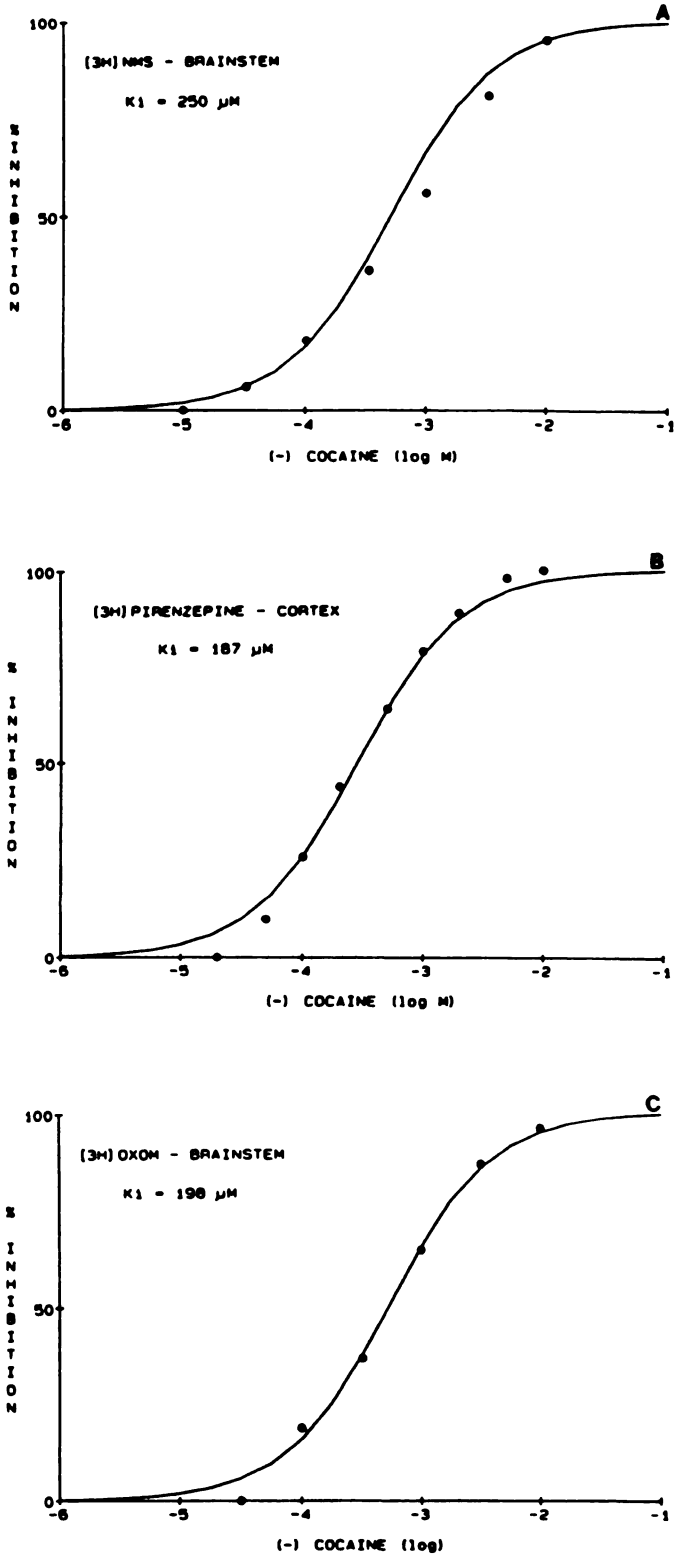

Fig. 1. Competition between (−)-cocaine and ^{3}H-muscarinic ligands for muscarinic receptors in human brainstem (A and C) or cortical (B) membrane homogenates. Assays were performed in either 50 mM $NaPO_4$, 1 mM EDTA ([^{3}H]NMS at 0.5 nM), or 20 mM Tris·HCl ([^{3}H] pirenzepine at 3 nM and [^{3}H]Oxo-M at 5 nM), pH 7.4, buffer, at 25° for 1–2 hr. The data points are averages of triplicate determinations from two to four independent experiments. The *curves* represent the best fit (determined by partial *F* test; see Experimental Procedures) of a one-site model to the data points.

for (+)- and (−)-cocaine (7 and 100 μM, respectively) were in close agreement with the inhibitory constants (K_i values) calculated from the [^{3}H]NMS competition binding experiments (Fig. 2) (23).

The effects of increasing concentrations of (−)-cocaine on the competition binding curves for the muscarinic agonist

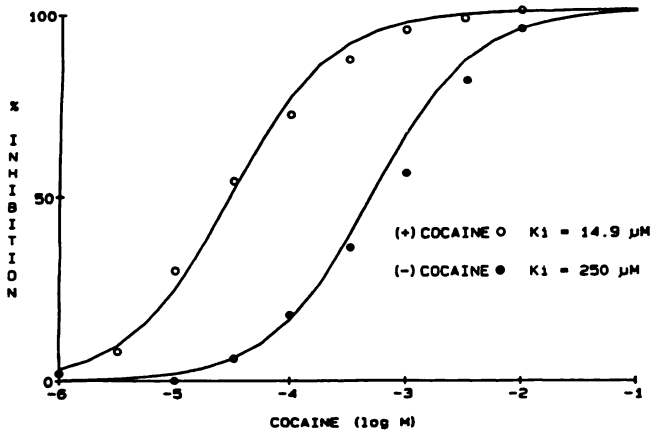

Fig. 2. Competition between (+)- or (−)-cocaine and [^{3}H]NMS for binding to muscarinic receptors in human brainstem membranes. The experiment was performed and the data were analyzed as described for Fig. 1.

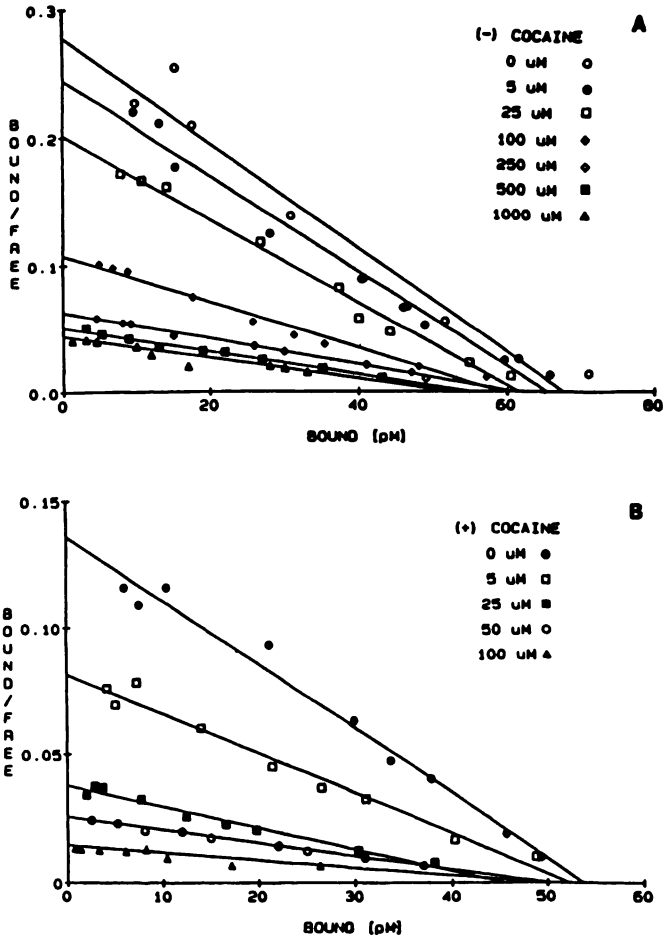

Fig. 3. Saturation isotherms (Rosenthal plots) of [^{3}H]NMS binding in the absence or presence of (−)- or (+)-cocaine in human brainstem membranes. The data points are derived from the means of triplicate determinations from a single representative experiment. The K_d and B_{max} values (see Table 1) were derived from the least-squares linear regressions of the data ($r > 0.9$ in all cases).

TABLE 1

Effects of (+)- and (−)-cocaine on B_{max} and K_d values for [^{3}H]NMS binding to human brainstem membranes

Values are means ± standard errors of three independent experiments.

Cocaine concentration	K_d	B_{max}	K_d shift
μM	nM	pmol/g of tissue	
(−)-Cocaine			
0	0.27 ± 0.01	66.6 ± 1.2	
5	0.31 ± 0.06	61.8 ± 2.6	1.1
25	0.40 ± 0.08*	63.6 ± 2.1	1.5
100	0.53 ± 0.02*	57.6 ± 1.4	2.0
250	0.77 ± 0.21*	55.2 ± 5.8	2.9
500	0.96 ± 0.14*	53.8 ± 1.0	3.6
1000	1.10 ± 0.12*	52.8 ± 0.7	4.1
(+)-Cocaine			
0	0.29 ± 0.01	53.7 ± 3.1	
5	0.51 ± 0.03*	52.2 ± 2.9	1.8
25	1.2 ± 0.02*	45.6 ± 5.2	4.1
50	2.0 ± 0.11*	50.2 ± 4.9	6.9
100	3.4 ± 0.09*	50.0 ± 2.7	11.7

* Significantly different from control (absence of cocaine) value, $p < 0.05$ (analysis of variance).

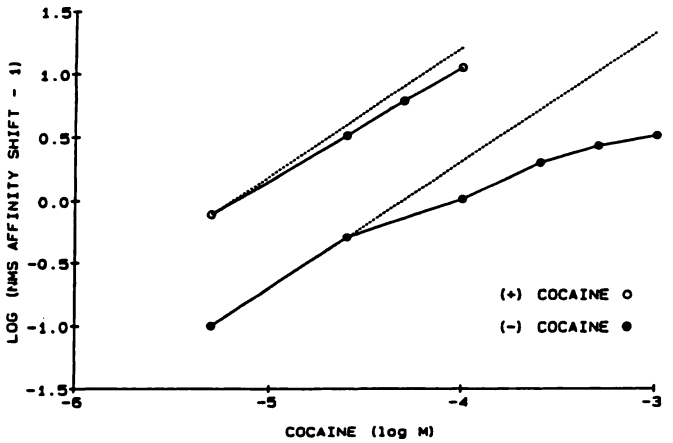

Fig. 4. Schild regressions of the effects of (−)- or (+)-cocaine on [^{3}H]NMS binding to muscarinic receptors in the human brainstem. Plots are derived from the saturation binding data shown in Table 1. – – –, Theoretical curves (slope of 1.0) for a competitive interaction between cocaine and [^{3}H]NMS.

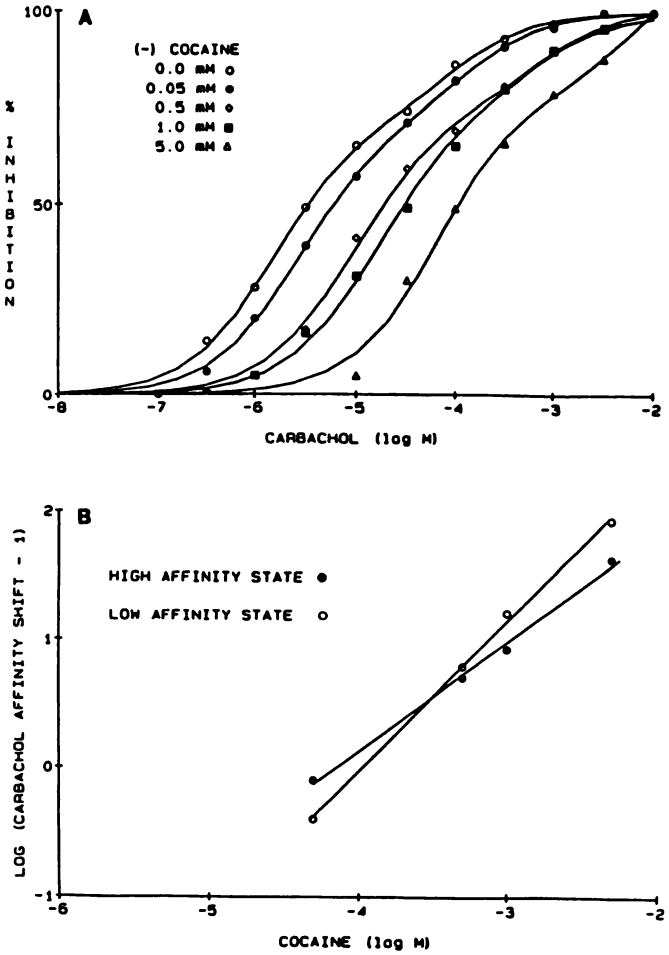

Fig. 5. Effects of (−)-cocaine on the competition binding of carbachol and [^{3}H]NMS to brainstem muscarinic receptors. A, The binding *curves* represent the best two-site fit (determined by partial *F* test) for the data points (means of triplicates from two separate experiments) generated for each concentration of cocaine. B, Schild plot derived from saturation binding data shown in A. Carbachol affinities (K_i values) were calculated from the IC_{50} values obtained from the binding curves using the Cheng-Prusoff equation and included the correction for alterations in the K_d values for [^{3}H]NMS with increasing (−)-cocaine concentrations. The shifts in carbachol affinities in the absence and presence of (−)-cocaine were calculated separately for the high and low affinity states of the receptor. *Lines* represent the least-squares linear regressions of the data ($r = 0.99$ for both lines).

carbachol and [^{3}H]NMS are shown in Fig. 5. The binding curves were shifted to the right in a parallel fashion as the concentration of (−)-cocaine was increased from 50 to 5000 μM. In the human brainstem, carbachol binds to two populations, i.e., high and low affinity agonist states, of the labeled receptors. At the concentration range studied, cocaine did not decrease the maximal shift in carbachol affinity for either agonist affinity state of the [^{3}H]NMS-labeled receptors. The resulting Schild plots (fig. 5B), derived separately for the high and low affinity states, were linear, with slopes not significantly different from 1.0. These results are indicative of an apparent competitive type of antagonism by cocaine of the binding of muscarinic agonists to high and low affinity states of the receptor.

The slowing of the rates of [^{3}H]NMS association to and dissociation from muscarinic receptors in the brainstem by increasing concentrations of (−)-cocaine is demonstrated in Fig. 6. At 1 mM (−)-cocaine, the rate of [^{3}H]NMS association was slowed by 20-fold and the dissociation rate decreased by 10-fold. The dissociation constants (K_d values) (data not shown) calculated from the kinetic data increased with increasing cocaine concentrations and agreed closely with those derived from the equilibrium saturation binding assays, differing by less than a factor of 2 for all cocaine concentrations tested. Similar effects of (−)-cocaine on the rates of [^{3}H]NMS association and dissociation were observed with cortical membranes (data not shown). These results provide evidence for a cooperative, or allosteric, interaction of (−)-cocaine with the muscarinic receptor and are consistent with the apparent negative heterotropic cooperativity seen in the saturation binding assays. There was no effect of (+)-cocaine on the rates of [^{3}H]NMS association or dissociation, consistent with the apparent lack of any observable cooperativity in the saturation binding data. Also, the dissociation rates of [^{3}H]QNB, [^{3}H]pirenzepine, and [^{3}H]Oxo-M from brainstem muscarinic receptors were unaffected by either (−)- or (+)-cocaine. These findings are consistent with the demonstration that tropate ligands with a

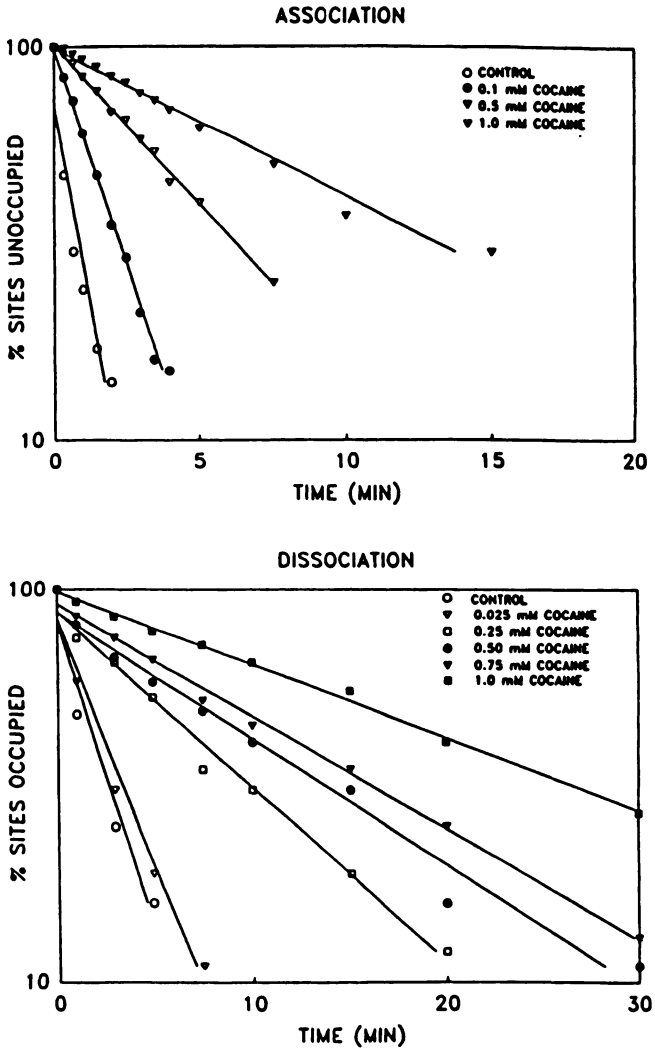

Fig. 6. Effects of (−)-cocaine on rates of association and dissociation of [^{3}H]NMS to and from brainstem muscarinic receptors at 25°. The data points represent the means of single determinations from three (association) or five (dissociation) independent experiments. Error bars are not shown because the symbols are larger than the associated error. *Lines* represent least-squares linear regressions of the data ($r > 0.95$ for all lines).

charged quaternary amine group, such as NMS, are more sensitive to allosteric modulation than ligands with a benzilate and/or tertiary amine moiety (9). Moreover, different muscarinic ligands have been suggested to induce different ligand-receptor complex conformations (24), which may be differentially sensitive to allosteric modulation.

Allosteric interactions at the muscarinic receptor have been shown to be very sensitive to changes in ionic strength (25). Therefore, we investigated the effects of NaCl on the ability of (−)-cocaine to slow the rate of [^{3}H]NMS dissociation from the receptor (Fig. 7). Concentrations between 0.1 M (data not shown) and 0.5 M NaCl abolished the efficacy of (−)-cocaine as an allosteric effector of the muscarinic receptor primary recognition site.

Discussion

The results of the present study demonstrate that (−)-cocaine acts as an apparent competitive antagonist of the binding

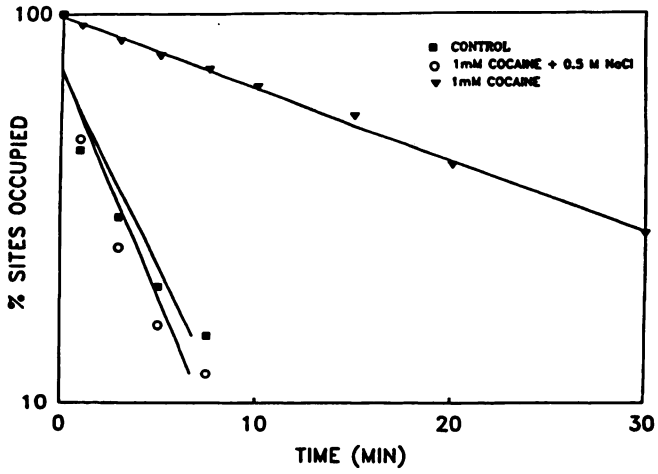

Fig. 7. Effect of 0.5 M NaCl on the allosteric slowing of [^{3}H]NMS dissociation from brainstem muscarinic receptors. Data points are the means of single determinations from three separate experiments. Data analysis was as described for Fig. 6.

of [^{3}H]NMS, [^{3}H]pirenzepine, and [^{3}H]Oxo-M to the primary ligand recognition site on muscarinic receptors in the human brainstem. The (−)-cocaine inhibitory constants (K_i values) for these ligands in both the cortex and brainstem (predominantly M1- and M2-rich brain regions, respectively) were similar, suggesting that (−)-cocaine is not selective for the M1 or M2 muscarinic receptor subtypes. The lack of effect of Gpp(NH)p or NEM on the binding of (−)-cocaine also suggests that (−)-cocaine does not recognize agonist affinity states of the receptor and that it acts as an antagonist at the primary ligand binding site. The (+)-cocaine isomer, which is not a natural constituent of the extract of the coca plant, also demonstrates non-subtype-selective affinity for the primary muscarinic binding site and is 10–20-fold more potent than (−)-cocaine. An earlier study demonstrated that (−)-cocaine also competitively antagonizes the binding of [^{3}H]QNB to muscarinic receptors in rat heart, hippocampus, and medulla, with K_i values of 19, 24, and 40 μM, respectively (1). We have obtained similar K_i values for (−)-cocaine binding to [^{3}H]QNB-labeled receptors in the human brainstem and cortex (7–10 μM) (data not shown). QNB has relatively high affinity (K_d values of 5–10 pM) (26) for muscarinic receptors, compared with other muscarinic antagonists (high picomolar to low nanomolar values) (27). The high affinity of QNB for muscarinic receptors and the relatively lower K_i values of cocaine for [^{3}H]QNB-labeled receptors, compared with [^{3}H]NMS-labeled receptors, may reflect the high lipophilicity of both of these compounds and their interaction with a hydrophobic domain on or near the receptor that is inaccessible to other muscarinic ligands.

The binding of cocaine to a secondary or allosteric site on the muscarinic receptor was evident from both equilibrium and kinetic binding experiments. Both (−)- and (+)-cocaine produced concentration-dependent increases in the K_d value of NMS, consistent with a competitive-type interaction with the muscarinic receptor. However, Schild analysis of the saturation binding data resulted in a curvilinear plot for (−)-cocaine, suggesting cooperative interactions of this isomer with the NMS-labeled receptor. In contrast, a similar Schild plot for (+)-cocaine was linear, with a slope close to unity, consistent with a competitive interaction with the muscarinic receptor. Whether there is stereoselectivity in the binding of ligands to

the secondary, allosteric, site on the muscarinic receptor, or whether allosterism of the (+)-isomer of cocaine is not detectable because of the inability to measure the effects of (+)-cocaine at concentrations higher than 10^{-4}, is not clear. Stereoselectivity of the interaction of ligands has been demonstrated for the primary muscarinic recognition site but not the allosteric site (28). For example, some phenylalkylamines, such as verapamil, have been shown to interact with both primary binding and allosteric sites on the muscarinic receptor, with the opposite stereoselectivity (29). It is possible that stereoselectivity of the interaction of ligands, particularly lipophilic ligands like cocaine and verapamil, with the primary and/or secondary sites on the muscarinic receptor could reflect steric constraints of the receptor at or near the recognition site where ligand binding occurs (30).

Cocaine, like other local anesthetics, prevents nerve conduction in nerve fibers by reversibly blocking membrane sodium channels and preventing the transient rise in sodium conductance necessary for the generation of the action potential (31). Cocaine binding to a receptor site on the membrane-bound sodium channel is strongly voltage dependent (32). Some evidence suggests that muscarinic receptors in the heart and brain are coupled to Na^+ channels (33). Whether the allosteric modulation of NMS binding to muscarinic receptors reflects such an interaction between muscarinic receptors and the Na^+ channel remains to be elucidated. Evidence to date, however, suggests that the primary muscarinic and allosteric binding sites are on the same receptor protein, because the allosteric effects of other allosteric modulators, such as gallamine and tetrahydroaminoacridine, remain even in solution (6, 34). Site-directed mutagenesis studies have demonstrated that at least four distinct aspartic acid residues are involved in muscarinic receptor-ligand interactions (10) and that at least three of these aspartates are differentially involved in modulating the effects of muscarinic allosteric ligands (12). These data provide further evidence for more than one ligand binding site on the muscarinic receptor.

Although previous studies have demonstrated that all cardioselective muscarinic antagonists also interact with a second, allosteric, site on the receptor, all allosteric antagonists at the muscarinic receptor are not cardioselective. In this study, we have demonstrated that cocaine interacts nonselectively with brain and heart muscarinic receptors at both the primary recognition site and the secondary, allosteric, effector site. Whereas cocaine toxicity is associated with plasma cocaine levels in the mid-micromolar range (35), the specific interaction of cocaine with primary and secondary sites on muscarinic receptors occurs at higher micromolar concentrations. Whether the antimuscarinic actions of cocaine described here contribute to the adverse cardiac and central nervous system manifestations of the drug remains to be determined.

References

1. Sharkey, J., M. C. Ritz, J. A. Schenden, R. C. Hanson, and M. J. Kuhar. Cocaine inhibits muscarinic cholinergic receptors in heart and brain. *J. Pharmacol. Exp. Ther.* **246**:1048–1052 (1988).
2. Ritz, M. C., R. J. Lamb, S. R. Goldberg, and M. J. Kuhar. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science (Washington D. C.)* **237**:1219–1223 (1987).
3. Sharkey, J., K. A. Glen, S. Wolfe, and M. J. Kuhar. Cocaine binding at *sigma* receptors. *Eur. J. Pharmacol.* **149**:171–174 (1988).
4. Wetli, C. V., and D. A. Fishbain. Cocaine-induced psychosis and sudden death in recreational cocaine users. *J. Forensic Sci.* **30**:873–880 (1985).
5. Clark, A. L., and F. Mitchelson. The inhibitory effect of gallamine on muscarinic receptors. *Br. J. Pharmacol.* **58**:323–331 (1976).
6. Stockton, J. M., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Modification of binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.* **23**:551–557 (1983).
7. Ellis, J., and R. H. Lenox. Characterization of the interactions of gallamine with muscarinic receptors from brain. *Biochem. Pharmacol.* **34**:2214–2217 (1985).
8. Dunlap, J., and J. H. Brown. Heterogeneity of binding sites on cardiac muscarinic receptors induced by the neuromuscular blocking agents gallamine and pancuronium. *Mol. Pharmacol.* **24**:15–22 (1983).
9. Lee, N. H., and E. E. El-Fakahany. Influence of ligand choice on the apparent binding profile of gallamine to cardiac muscarinic receptors: identification of three main types of gallamine-muscarinic receptor interactions. *J. Pharmacol. Exp. Ther.* **246**:829–838 (1988).
10. Fraser, C. M., C.-D. Wang, D. A. Robinson, J. D. Gocayne, and J. C. Venter. Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**:840–847 (1989).
11. Lameh, J., R. I. Cone, S. Maeda, M. Philip, M. Corbani, L. Nadasdi, J. Ramachandran, G. M. Smith, and W. Sadee. Structure and function of G protein coupled receptors. *Pharm. Rev.* **7**:1213–1221 (1990).
12. Lee, N. H., and E. E. El-Fakahany. Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.* **256**:468–479 (1991).
13. Giraldo, E., R. Micheletti, E. Montagna, A. Giachetti, M. A. Vigano, H. Ladinsky, and C. Melchiorre. Binding and functional characterization of the cardioselective muscarinic antagonist methoctramine. *J. Pharmacol. Exp. Ther.* **244**:1016–1020 (1988).
14. Roffel, A. F., C. R. S. Elzinga, H. Meurs, and J. Zaagsma. Allosteric interactions of three muscarine antagonists at bovine tracheal smooth muscle and cardiac M2 receptors. *Eur. J. Pharmacol.* **172**:61–70 (1989).
15. Cohen-Armon, M., Y. I. Henis, Y. Kloog, and M. Sokolovsky. Interactions of quinidine and lidocaine with rat brain and heart muscarinic receptors. *Biochem. Biophys. Res. Commun.* **127**:326–332 (1985).
16. Eglen, R. M., A. D. Michel, C. M. Cornett, E. A. Kunysz, and R. L. Whiting. The interaction of hexamethonium with muscarinic receptor subtypes *in vitro*. *Br. J. Pharmacol.* **98**:499–506 (1989).
17. Kloog, Y., and M. Sokolovsky. Bisquaternary pyridinium oximes as allosteric inhibitors of rat brain muscarinic receptors. *Mol. Pharmacol.* **27**:418–428 (1985).
18. Flynn, D. D., and D. C. Mash. Multiple *in vitro* interactions with and differential *in vivo* regulation of muscarinic receptor subtypes by tetrahydroaminoacridine. *J. Pharmacol. Exp. Ther.* **250**:573–581 (1989).
19. Waelbroeck, M., P. Robberecht, P. De Neef, and J. Christophe. Effects of verapamil on the binding properties of rat heart muscarinic receptors: evidence for an allosteric site. *Biochem. Biophys. Res. Commun.* **121**:340–345 (1984).
20. Lai, W. S., V. Ramkumar, and E. E. El-Fakahany. Possible allosteric interaction of 4-aminopyridine with rat brain muscarinic acetylcholine receptors. *J. Neurochem.* **44**:1936–1942 (1985).
21. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
22. Rosenthal, H. E. Graphical method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**:525–532 (1967).
23. Ehlert, F. J. Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol. Pharmacol.* **33**:187–194 (1988).
24. Kloog, Y., Y. Egozi, and M. Sokolovsky. Characterization of muscarinic acetylcholine receptors from mouse brain: evidence for regional heterogeneity and isomerization. *Mol. Pharmacol.* **15**:545–558 (1979).
25. Birdsall, N. J. M., E. C. Hulme, W. Kromer, and J. M. Stockton. A second drug-binding site on muscarinic receptors. *Fed. Proc.* **46**:2525–2527 (1987).
26. Flynn, D. D., and L. T. Potter. Effect of solubilization on the distinct binding properties of muscarine receptors from rabbit hippocampus and brainstem. *Mol. Pharmacol.* **30**:193–199 (1986).
27. Nathanson, N. M. Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10**:195–236 (1987).
28. Aronstam, R. S., D. J. Triggle, and M. E. Eldefrawi. Structural and stereochemical requirements for muscarinic receptor binding. *Mol. Pharmacol.* **15**:227–234 (1979).
29. Gerry, R. H., B. Rauch, R. A. Colvin, P. N. Adler, and F. C. Messineo. Verapamil interaction with the muscarinic receptor: stereoselectivity at two sites. *Biochem. Pharmacol.* **36**:2951–2956 (1987).
30. Zobrist, R. H., K. M. Giacomini, W. L. Nelson, and J. C. Giacomini. The interaction of phenylalkylamine calcium channel blockers with the 1,4-dihydropyridine binding site. *J. Mol. Cell Cardiol.* **18**:963–974 (1986).

31. Strichartz, G. R., and J. M. Ritchie. The action of local anesthetics on ion channels of excitable tissues. *Handb. Exp. Pharmacol.* **81**:21–52 (1987).
32. Wang, G. K. Cocaine-induced closures of single batrachotoxin-activated Na^+ channels in planar lipid bilayers. *J. Gen. Physiol.* **92**:747–765 (1988).
33. Cohen-Armon, M., H. Garty, and M. Sokolovsky. G-protein mediates voltage regulation of agonist binding to muscarinic receptors: effects on receptor-Na^+ channel interaction. *Biochemistry* **27**:368–374 (1988).
34. Potter, L. T., C. A. Ferrendelli, H. E. Hanchett, M. A. Hollifield, and M. V. Lorenzi. Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M1 muscarine receptors. *Mol. Pharmacol.* **35**:652–660 (1989).
35. Mittleman, R. E., and C. V. Wetli. Death caused by recreational cocaine use. *J. Am. Med. Assoc.* **252**:1889–1893 (1984).

Send reprint requests to: Donna D. Flynn, Ph.D., Department of Molecular and Cellular Pharmacology (R-189), University of Miami School of Medicine, P.O. Box 016189, Miami, FL 33101.