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Specific Activation of the α7 Nicotinic Acetylcholine Receptor by a Quaternary Analog of Cocaine

MICHAEL M. FRANCIS, ELAINE Y. CHENG, GREGORY A. WEILAND, and ROBERT E. OSWALD

Department of Molecular Medicine, Cornell University, Ithaca, New York

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ABSTRACT

Effects of cocaine and cocaine methiodide were evaluated on the homomeric α7 neuronal nicotinic receptor (nAChR). Whereas cocaine itself is a general nAChR noncompetitive antagonist, we report here the characterization of cocaine methiodide, a novel selective agonist for the α7 subtype of nAChR. Data from ^{125}I-α-bungarotoxin binding assays indicate that cocaine methiodide binds to α7 nAChR with a K_i value of approximately 200 nM while electrophysiology studies indicate that the addition of a methyl group at the amine moiety of cocaine changes the drug's activity profile from inhibitor to agonist. Cocaine methiodide activates α7 nAChR with an EC_{50} value of approximately 50 μM and shows comparable efficacy to ACh in oocyte experiments. While agonist effects are specific for the α7 neuronal nAChR and are not observed with heteromeric neuronal or skeletal muscle nAChR, antagonist effects are present for heteromeric nAChR combinations. Studies of PC12 cells transiently transfected with human α7 cDNA and expressing a variety of functional nicotinic receptor subtypes confirm the specificity of cocaine methiodide agonist effects. Our results indicate that a quaternary structural derivative of cocaine can be used as a specific agonist for the α7 subtype of neuronal nicotinic receptor.

Postsynaptic neuronal nicotinic acetylcholine receptors (nAChRs) mediate fast excitatory synaptic transmission at synapses in the central (primarily on interneurons) and peripheral nervous system (sympathetic and parasympathetic ganglia). A second population of neuronal nAChRs is localized to presynaptic terminals and can modulate release of a variety of transmitters. Despite the widespread distribution and varied synaptic localization of nAChRs in the nervous system, a degree of functional and pharmacological specificity is achieved as a result of the diversity of nAChR subtypes. Each subtype is composed of five subunits arranged around a central ion channel. Two families of nAChRs are likely present in the nervous system: 1) heteromeric receptors consisting of α2, α3, α4, and/or α6 subunits in combination with β2 and/or β4 subunits (other modulatory subunits such as β3 and α5 may also be present although not strictly required for function), and 2) receptors capable of functioning as homomers including α7-α9. The α10 subunit has also been cloned recently and appears to function only in combination with α9 (Elgoyhen et al., 2000).

Correlative evidence for the involvement of brain nAChRs in cognitive dysfunction associated with neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and schizophrenia has focused attention on brain nAChR subtypes as therapeutic targets (Kem, 2000; Leonard et al., 1996; Quik and Jeyarasasingam, 2000). The homomeric α7 nAChR is widely expressed in the nervous system and has received particular scrutiny because of its high calcium permeability and consequent potential for activation of second-messenger systems. The possible therapeutic application and experimental utility of drugs directed to this receptor subtype has driven the development and characterization of α7-selective agonists and antagonists (Mullen et al., 2000; de Fiebre et al., 1995). We have previously shown that cocaine inhibits heteromeric nAChR subtypes that contain the α4 and/or β4 subunits with high affinity (Francis et al., 2000). In the present study, we report the selective activation of α7 nAChR by a quaternary cocaine derivative, cocaine methiodide. Whereas cocaine inhibits α7 receptors, addition of a methyl group to the amine moiety of cocaine forms the quaternary compound cocaine methiodide, which permits high-affinity interaction with the agonist binding site and potent and specific activation of α7 receptors expressed in either *Xenopus laevis* oocytes or transiently transfected PC12 cells.

Materials and Methods

Chemicals. Cocaine and cocaine methiodide were provided by the National Institute on Drug Abuse (Bethesda, MD). Reagents for cell

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; FBS, fetal bovine serum; GFP, green fluorescent protein; MLA, methyllycaconitine; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium.

culture were purchased from Life Technologies (Rockville, MD). All other chemicals were purchased from Sigma (St. Louis, MO).

Preparation of RNA and Oocyte Injection. Rat nicotinic receptor cDNA clones were provided by Drs. Steve Heinemann (Salk Institute, La Jolla, CA) and Jim Boulter (UCLA, Los Angeles, CA). The chick α7 nAChR subunit clone was provided by Dr. Marc Ballivet (University of Geneva, Geneva, Switzerland) and the human α7 nAChR subunit clone (in the PMXT oocyte expression vector) was provided Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). 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 agarose gel electrophoresis under denaturing conditions (visualized with ethidium bromide). RNAs were diluted to a concentration of 600 ng/μl and stored frozen in ribonuclease-free water at −80°C.

Ovarian lobes were surgically removed from anesthetized adult female *X. laevis* and 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, and 1 mM $MgCl_2$, pH 7.4)]. Following 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 μg/ml streptomycin, and 5% horse serum) was changed daily. Recordings were made 2 to 7 days after injection depending on the RNAs being tested.

Two-Electrode Voltage-Clamp Recording. Two-electrode voltage-clamp recordings were made at room temperature in oocyte saline solution supplemented with 1.8 mM $CaCl_2$ and 1 μM atropine as described previously (Francis et al., 2000). All recordings were made using a Turbo Tec 01C amplifier (NPI Electronics, Tamm, Germany) at a holding potential of −50 mV unless otherwise noted. Recording electrodes were filled with 3 M of KCl and typically had resistances in the range of 0.5 to 3 MΩ.

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. 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 of slowly desensitizing receptor subtypes (e.g., skeletal muscle nAChR), solution exchange time is estimated to be in the range of 500 to 800 ms under these conditions (Vazquez and Oswald, 1999). Data were collected at a sampling rate of 100 Hz on a Compaq personal computer using pClamp 5.5.1 (Axon Instruments, Foster City, CA) and filtered at 30 Hz using the lowpass filter in the amplifier. 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 3 min. Values for EC_{50}, 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 were plotted using a nonlinear, least-squares fit of the Hill equation: Response = I_{max} $[agonist]^{nH}$ / ($[agonist]^{nH}$ + $(EC_{50})^{nH}$). IC_{50} was calculated with a nonlinear, least-squares fit of the equation: Response = $[IC_{50}]^{nH}$ / ($[cocaine]^{nH}$ + $(IC_{50})^{nH}$).

Cell Culture and Binding Assays in Stably Transfected GH_4C_1 Cells. Both nontransfected GH_4C_1 cells and GH_4C_1 cells that had been stably transfected with the rat α7 cDNA in the pcep4 vector were generously provided by Dr. Maryka Quik (The Parkinson's Institute, Sunnyvale, CA). Cells were grown in F10 medium supplemented with 8% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. For purposes of maintaining stable expression, hygromycin B (0.2 mg/ml) was periodically added to the culture medium. Treatment with this concentration of antibiotic was sufficient to kill nontransfected cells within 3 to 4 days. Cells were incubated in a 95% oxygen/5% carbon dioxide environment at 37°C.

Binding assays were performed on intact cells as previously described by Quik et al. (1996). Briefly, cells were plated on 24-well plates and grown to confluence. Immediately before the binding assay, cells were washed with Dulbecco's modified Eagle's medium (DMEM) containing 3.7 mM $NaHCO_3$ and 0.1% bovine serum albumin. Cells were incubated with the drug of interest at the appropriate concentration at 37°C for 1 h. ^{125}I-α-bungarotoxin was added to dishes to the desired concentration and the cells were incubated at 37°C for 90 min. Binding was terminated by removal of the ^{125}I-α-bungarotoxin and repeated washes with the DMEM solution. After termination of binding, cells were solubilized in 0.5 M NaOH and transferred to vials for gamma counting. Binding in the presence of methyllycaconitine (MLA, 10 μM) was defined as nonspecific binding. Experiments with nontransfected cells showed ^{125}I-α-bungarotoxin binding that was not significantly different from nonspecific binding. Nonspecific binding typically represented 10 to 20% of total radioligand binding for 1 nM ^{125}I-α-bungarotoxin (the concentration used for displacement studies). In each experiment, data representing specific binding for the displacement curves were normalized to maximal specific binding. Values for K_i were calculated from IC_{50} values for inhibition of ^{125}I-α-bungarotoxin binding by the equation of Cheng and Prusoff (1973): $K_i = IC_{50} / [1 + ([bungarotoxin] / K_d)]$.

Cell Culture and Whole-Cell Recording of Transiently Transfected PC12 Cells. Rat pheochromocytoma cells (PC12; obtained from the laboratory of either Dr. Rick Cerione or Dr. George Hess, both at Cornell University, Ithaca, NY) were maintained in DMEM supplemented with 10% (*v*/*v*) horse serum and 5% (*v*/*v*) FBS, 100 U/ml sodium penicillin G, and 100 μg/ml streptomycin sulfate in a 95% oxygen/5% carbon dioxide environment at 37°C. For experiments using HEK 293 cells, cells were cultured as above with the exception that DMEM was supplemented with 10% FBS. Cells were passaged 24 to 48 h before transfection and plated onto 35-mm dishes. Upon reaching 40 to 60% confluence, cells were cotransfected with the human α7 cDNA (provided by Dr. Roger Papke, University of Florida, Gainesville, FL) in the PCI-neo (Promega, Madison, WI) mammalian expression vector and a cDNA coding for green fluorescent protein (GFP) in the pEGFP-N1 plasmid (CLONTECH Laboratories, Palo Alto, CA) using the Superfect (QIAGEN, Valencia, CA) transfection reagent according to the manufacturer's recommendations. A total of 7 μg DNA was added to each dish in a 4:3 ratio of α7 to GFP. Transfection efficiency was evaluated by visual inspection under blue light (395 nm) 24 to 36 h after transfection using a TMD-EF epi-fluorescence attachment on a Nikon Diaphot-TMD inverted microscope (Nikon, Melville, NY).

Current responses from transfected and nontransfected PC12 cells were recorded using conventional whole-cell patch clamp recording methods. Pipettes were pulled from borosilicate glass capillary tubes (World Precision Instruments; Sarasota, FL) to a tip diameter of 2 to 3 μm with resistances in the range of 2 to 3 MΩ using a PP-83 pipette puller (Narishige, Greenvale, NY). Recording pipettes were filled with a solution containing 120 mM CsF, 10 mM CsCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4 with CsOH. Recordings were obtained using an Axopatch 200B amplifier (Axon Instruments) and filtered at a cutoff frequency of 5 kHz using the lowpass filter in the amplifier before being digitized at a sampling frequency of 20 kHz. Data were acquired on a Gateway 2000 PC using pClamp 6 software (Axon Instruments). All recordings were obtained at a holding potential of −80 mV 36 to 60 h after transfection. Two hours before recording, cells were replated at a lower density. Immediately before study, the culture medium was removed and the cells were washed gently with extracellular recording buffer (145 mM NaCl, 3 mM KCl, 2 mM

$CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4). Atropine (1 μM) was included in the recording buffer to inhibit potential muscarinic receptor responses. After identification of GFP-positive cells, the whole-cell configuration was obtained and cells were lifted off the culture dish and placed <50 μm from the 150 μm outflow of a u-tube application device (Udgaonkar and Hess, 1987). Solution exchange time was measured experimentally via two independent methods (Fig. 6A). Open-tip measurements of voltage changes in response to 500 mM CsCl indicated that a solution exchange time (10–90%) of ~1 ms can be obtained. However, because open-tip measurements reflect solution exchange at the tip of the pipette rather than across the surface of a cell, this technique may underestimate whole-cell solution exchange time. Therefore, we also measured solution exchange by u-tube application of a saturating concentration of noncompetitive inhibitor (1 mM cocaine) during the plateau phase of the whole-cell response (to 100 μM ACh) of a slowly desensitizing nAChR subtype expressed in HEK 293 cells (α3β4, kindly provided by Dr. Ken Kellar). Using this technique, solution exchange time (10–90%) was estimated to be ~3 ms. We take these values to represent lower and upper limits for solution exchange time because the open-tip response does not reflect exchange across the entire cell and solution exchange measured by the application of inhibitor may reflect a small binding component in addition to exchange. Apparent desensitization time constants were calculated from exponential fits to the rising and falling phase of the data traces employing desensitization correction as described by Udgaonkar and Hess (1987). Comparable values were obtained by fitting the falling phase of the responses with a single exponential using Clampfit 8.0 (Axon Instruments).

Results

Effects of Cocaine and Cocaine Methiodide on α7 nAChRs Expressed in *X. laevis* Oocytes. In contrast to the relatively high-affinity binding of cocaine to inhibitory sites on the α4 and β4 subunits described in previous work (K_D ~2 μM for α4β4 nAChRs; Francis et al., 2000), cocaine exhibits weaker binding to an inhibitory site associated with the α7 receptor. Whereas application of 1 mM cocaine alone has no effect (n = 3, data not shown), coapplication of 100 μM cocaine with 300 μM ACh produces qualitatively similar levels of inhibition for the rat, human, and chick isoforms of the receptor (Fig. 1A). Moreover, analysis of the concentration dependence of inhibition for rat and human α7 nAChR yields comparable IC_{50} values (55 ± 10 and 98 ± 19 μM, respectively); the rat isoform exhibits slightly higher affinity (Fig. 1B). We have previously shown for other nAChR subtypes that a brief equilibration with cocaine before agonist application increases inhibitory effects (Francis et al., 2000). However, for α7 nAChRs, no significant effects of pre-equilibration with inhibitor were observed. Cocaine inhibition of α7 receptors also exhibits voltage dependence (not shown), most consistent with binding to a channel site (as was observed for α3β4 nAChRs).

The effects of the quaternary cocaine derivative cocaine methiodide (Fig. 2A) on nAChR function were also evaluated. Surprisingly, current responses of rat α7 nAChRs to coapplication of 300 μM acetylcholine with 30 μM cocaine methiodide were on average 157 ± 5% of responses to 300 μM acetylcholine alone (Fig. 2B, top), suggesting either agonist activity or allosteric potentiation by cocaine methiodide. Application of cocaine methiodide alone also activates rapidly desensitizing responses in oocytes expressing either the rat, human, or chick isoforms of the receptor (Fig. 2B), demonstrating this compound to be an agonist for the α7 nAChR subtype. Curve fits of the Hill equation to cocaine methiodide concentration-response data for both the rat and human forms of α7 yielded Hill slopes <1 and EC_{50} values of 56 ± 12 and 47 ± 14 μM for rat and human α7 nAChRs , respectively (Fig. 2C). Inhibition by cocaine methiodide at high concentration is apparent (Fig. 2C, data point at 3 mM) and probably influences the Hill slope. Notably, however, inhibitory effects of cocaine methiodide do not appear to limit the efficacy of this compound relative to ACh. In addition, cocaine methiodide is approximately 6-fold more potent than acetylcholine (EC_{50} = 370 ± 34 and 281 ± 32 μM rat and human α7 nAChRs , respectively) for activation of α7 receptors. Accurate measurement of Hill slope and EC_{50} values for α7 agonists has previously been shown to be limited by the slow solution exchange time of the oocyte system relative to desensitization of α7 responses (Papke and Thinschmidt, 1998). Therefore, the EC_{50} values determined from our data may underestimate agonist potency. Comparison of the relative

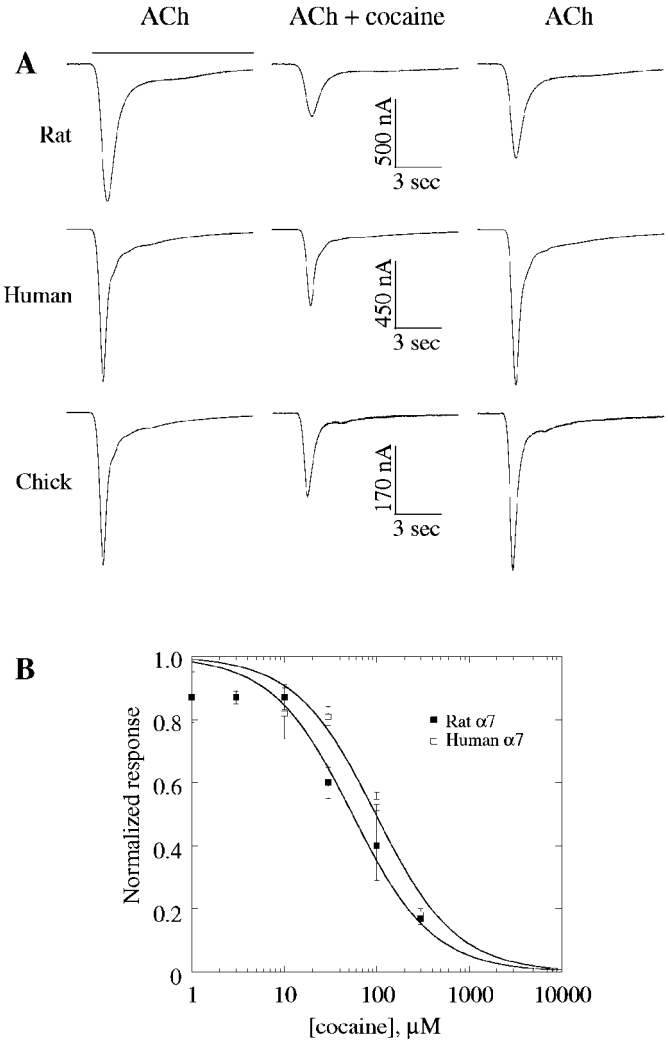

Fig. 1. Cocaine inhibits the rat, human, and chick forms of the α7 nAChR. A, responses of oocytes expressing α7 nAChRs to 300 μM ACh in the absence and presence (middle trace) of 100 μM cocaine. ACh was applied for 15 s in each case and the bar above the upper left trace shows the timing of application (in this figure and all subsequent figures). Each response is separated by a wash period of 3 min. B, concentration dependence of cocaine inhibition of rat and human α7 nAChR. Data are normalized to an initial response to 300 μM ACh. Each data point represents the mean (± S.E.M.) of three to six responses.

potency of cocaine methiodide versus ACh should be unaffected by this limitation.

Cocaine Methiodide Displaces ^{125}I-α-Bungarotoxin Binding. To confirm binding of cocaine methiodide in the vicinity of the agonist binding site, we also examined displacement of ^{125}I-α-bungarotoxin from a pituitary cell line (GH_4C_1) stably transfected with the rat α7 cDNA (Quik et al., 1996). Consistent with previous reports, our experiments indicate that this cell line shows dose-dependent binding of ^{125}I-α-bungarotoxin with a K_D value of 0.7 ± 0.2 nM, whereas nontransfected cells do not exhibit appreciable specific binding (not shown). ^{125}I-α-Bungarotoxin binding can be displaced by MLA, another specific inhibitor of α7 nAChRs, with a K_i value of 2.3 ± 0.6 nM, consistent with expression of α7 nAChRs. Moreover, cocaine methiodide displaces ^{125}I-α-bungarotoxin binding with a K_i value of 0.19 ± 0.02 μM, indicating that cocaine methiodide is a high-affinity ligand for the agonist binding site of α7 nAChRs (Fig. 3). Interestingly, the displacement curve exhibits a high degree of apparent positive cooperativity (n_H = 2.6), possibly reflecting the fact that homomeric α7 receptors may include as many as five agonist binding sites (Palma et al., 1996; Rangwala et al., 1997). Nicotine and ACh have been reported previously to inhibit ^{125}I-α-bungarotoxin binding in these cells with K_i values of 0.9 ± 0.1 and 6.2 ± 0.2 μM, respectively (Quik et al., 1996). In contrast to cocaine methiodide, cocaine does not effectively compete with ^{125}I-α-bungarotoxin binding at cocaine concentrations ranging from 0.1 to 10 μM, indicating that the presence of an additional methyl group at the amine moiety of cocaine confers affinity for the agonist binding site. ^{125}I-α-Bungarotoxin binding in the presence of 100 μM cocaine (the highest concentration tested) was 83 ± 15% of control binding (not shown), suggesting that cocaine may have low affinity for the α7 agonist binding site. However, as noted above, application of cocaine alone (up to 1 mM) elicits no detectable functional response from rat α7 receptors expressed in oocytes.

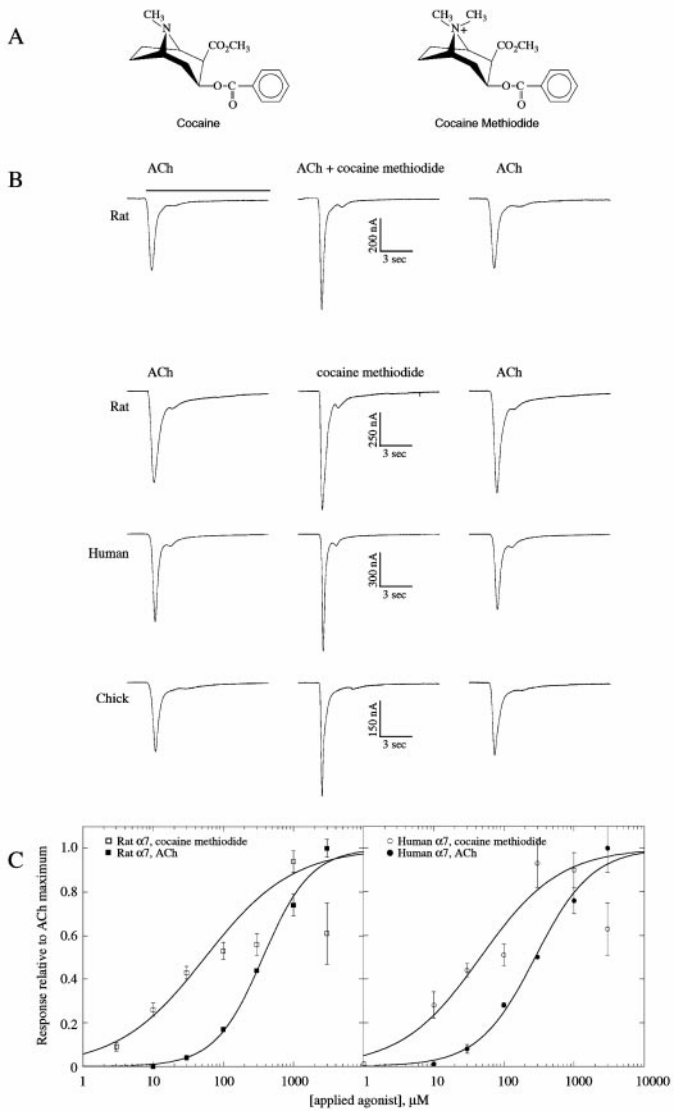

Fig. 2. Cocaine methiodide activation of the rat, human, and chick isoforms of α7 nAChR. A, structures of cocaine and cocaine methiodide. B, top, response of oocytes expressing rat α7 nAChR to 300 μM ACh in the absence and presence (middle trace) of 30 μM cocaine methiodide. Bottom, response of oocytes expressing either rat, human, or chick α7 nAChR to either 300 μM ACh (left and right traces) or 100 μM cocaine methiodide (middle trace). C, concentration dependence of cocaine methiodide and ACh activation of rat and human α7 nAChR. For both compounds, data are normalized to an initial response to 300 μM ACh and plotted relative to the maximum response to ACh. Each data point represents the mean (± S.E.M.) of 3 to 11 responses.

Effects of Cocaine Methiodide on Heteromeric nAChR Subtypes. The ability of cocaine methiodide to activate other nAChR subtypes expressed in *X. laevis* oocytes was also evaluated. Cocaine methiodide (10 μM-1 mM) was applied to oocytes expressing either the α4β2, α3β2, α3β4, or α1β1γδ subunit combinations (Fig. 4). Cocaine methiodide elicits <1% of the control response to ACh for each of these receptor types, indicating that this drug is specific for the α7 receptor among likely mammalian brain and neuromuscular junction nAChR subtypes.

Because the concentration-response curve for cocaine methiodide activation of α7 receptors shows features consistent with inhibition by agonist at high concentration, we also tested whether cocaine methiodide was an effective inhibitor of nAChR subtypes for which it exhibited little (if any) agonist activity (Fig. 5). Coapplication of 30 μM cocaine methiodide with 30 μM ACh to oocytes expressing either the α3β4 or

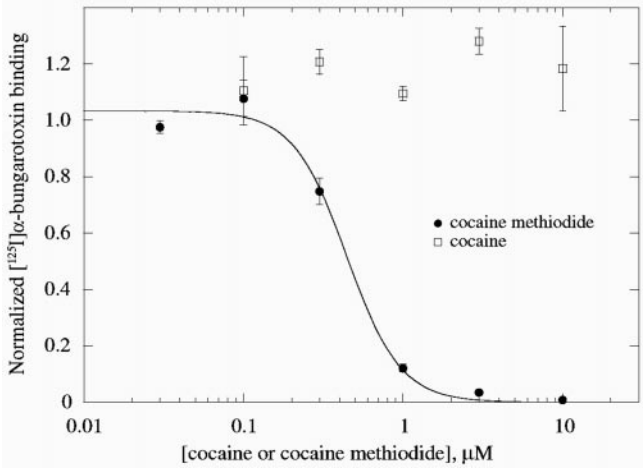

Fig. 3. Cocaine methiodide displacement of ^{125}I-α-bungarotoxin binding. Data are normalized to maximal control binding. Each data point represents the mean specific binding of at least six wells from separate experiments. Binding in the presence of 10 μM of MLA was defined as nonspecific.

α4β2 subunit combination produces similar levels of inhibition for both receptor types (47 ± 10% and 50 ± 6% respectively). In these simple coapplication experiments, the magnitude of inhibition does not differ significantly between cocaine and cocaine methiodide (we have reported previously that a short pre-equilibration with cocaine results in increased inhibition for the α3β4 nAChR subtype, Francis et al., 2000). These observations suggest that cocaine methiodide (similar to cocaine) is a general nAChR antagonist for heteromeric combinations. Moreover, given that cocaine is also a less potent inhibitor of α7 nAChRs (Fig. 1), cocaine methiodide may produce a form of α7 nAChR inhibition similar to that observed for heteromeric nAChR subtypes in concert with the agonist effects described above.

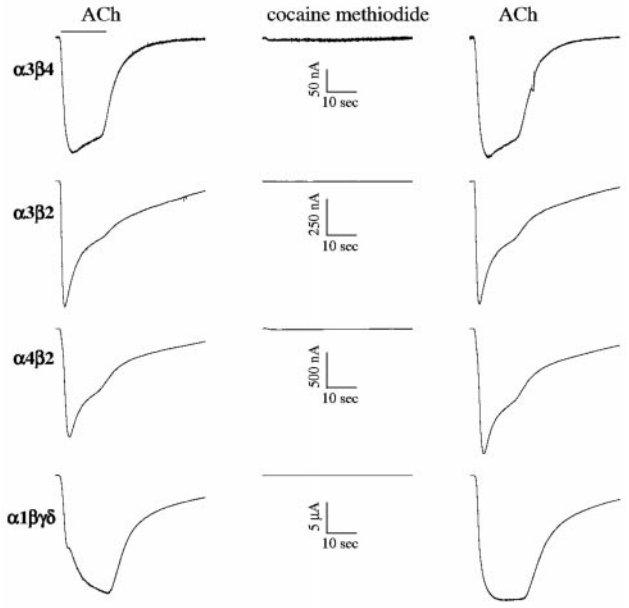

Fig. 4. Cocaine methiodide lacks agonist properties at other nAChR subunit combinations. Response of oocytes expressing either rat α3β4, α3β2, or α4β2 or mouse α1β1γδ nAChRs to either ACh (30 μM for neuronal combinations or 5 μM for neuromuscular junction nAChR; traces on left and right) or 100 μM cocaine methiodide (middle trace).

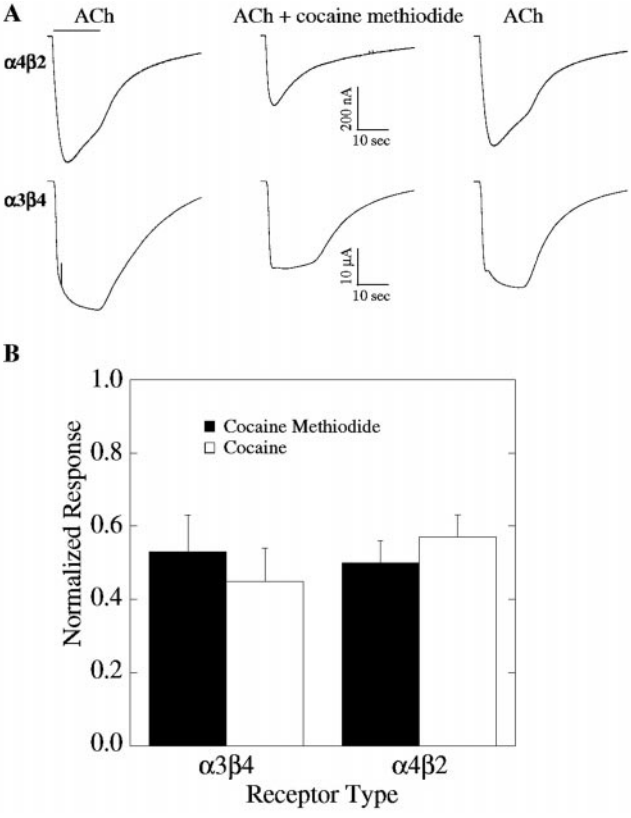

Fig. 5. Cocaine methiodide inhibits heteromeric nAChR subtypes. Responses of oocytes expressing either rat α3β4 or α4β2 nAChRs to 30 μM ACh in the absence or presence (middle trace) of 30 μM cocaine methiodide.

Cocaine Methiodide Selectively Activates α7 nAChRs in Transiently Transfected PC12 Cells. Although oocyte expression and two-electrode, voltage-clamp recording are powerful tools for evaluating drug-receptor interactions, the time resolution of the technique is limited by the requirement for perfusion of the entire surface area of the oocyte. This requirement precludes meaningful kinetic measurements for a rapidly desensitizing receptor such as α7. Whereas other receptor subtypes have been amenable to efficient heterologous expression in cultured cells, development of effective cell culture expression systems for α7 has proven more difficult. Although a few stably transfected cell lines have demonstrated significant bungarotoxin binding (Puchazc et al., 1994; Quik et al., 1996), most have not been amenable to patch clamp study (however, see Gopalakrishnan et al., 1995). The difficulties with expression seem to be a product of a functional requirement for the presence of cell-specific factors for proper post-translational processing (Cooper and Millar, 1997; Rakhilin et al., 1999; Sweileh et al., 2000) as well as α7 sequence-specific factors impacting surface expression (Dineley and Patrick, 2000). Consistent with other reports (Cooper and Millar, 1997; Rangwala et al., 1997), we have been unable to detect responses to ACh in HEK 293 cells transiently transfected with human or rat α7 cDNA. In addition, we detect only small, inconsistent responses to ACh in GH_4C_1 cells stably transfected with rat α7 cDNA, although these cells do exhibit significant bungarotoxin binding (Fig. 3). Because PC12 cells normally express nAChRs and certain variants of this cell line have been shown to express native as well as transfected α7 nAChRs (Blumenthal et al., 1997; Cooper and Millar, 1997; Rangwala et al., 1997), we decided to examine effects of cocaine methiodide on this cell line using whole-cell recording. Our initial experiments evaluated native nAChR expression in two separate PC12 cell line variants. Although both cell populations gave functional responses to ACh (not shown), we chose the variant with the lower level of intrinsic nAChR expression as the best system to evaluate effects of transfection with α7 cDNA.

Although most nontransfected (undifferentiated) PC12 cells exhibited small but measurable peak responses (273 ± 80 pA) to application of 1 mM ACh (69%, 18 of 26 cells tested; Fig. 6, A, top, and B), only a single response to 300 μM cocaine methiodide was observed (each of the drug concentrations tested should be saturating based upon the oocyte data in Fig. 2C). Cells transfected with GFP alone also showed only small ACh responses (132 ± 55 pA, in three of six cells tested) and a single response to cocaine methiodide. In contrast, nearly all GFP-positive PC12 cells cotransfected with human α7 cDNA exhibited rapid current responses to ACh (92%, 24 of 26 cells tested, Fig. 6A, center). The peak

response amplitudes of nontransfected and transfected PC12 cells for which both drugs were tested are summarized in Fig. 6B.

Of the transfected cells responding to ACh, 95% of cells tested also had fast desensitizing responses to cocaine methiodide (Fig. 6A, 21 of 22 cells tested), consistent with expression of α7 nAChR. The ACh responses of transfected cells could be divided qualitatively into two classes: those that desensitized to a steady-state level (65% of cells tested, example in Fig. 6A) and those that desensitized completely during the time course of the application (35% of cells tested, example in Fig. 7). In contrast, cocaine methiodide responses showed rapid and complete desensitization in all cells tested. Moreover, currents elicited by cocaine methiodide were inhibited completely by application of 100 nM MLA (Fig. 6A, bottom, $n = 4$).

For both classes of cells, we observed an apparent re-

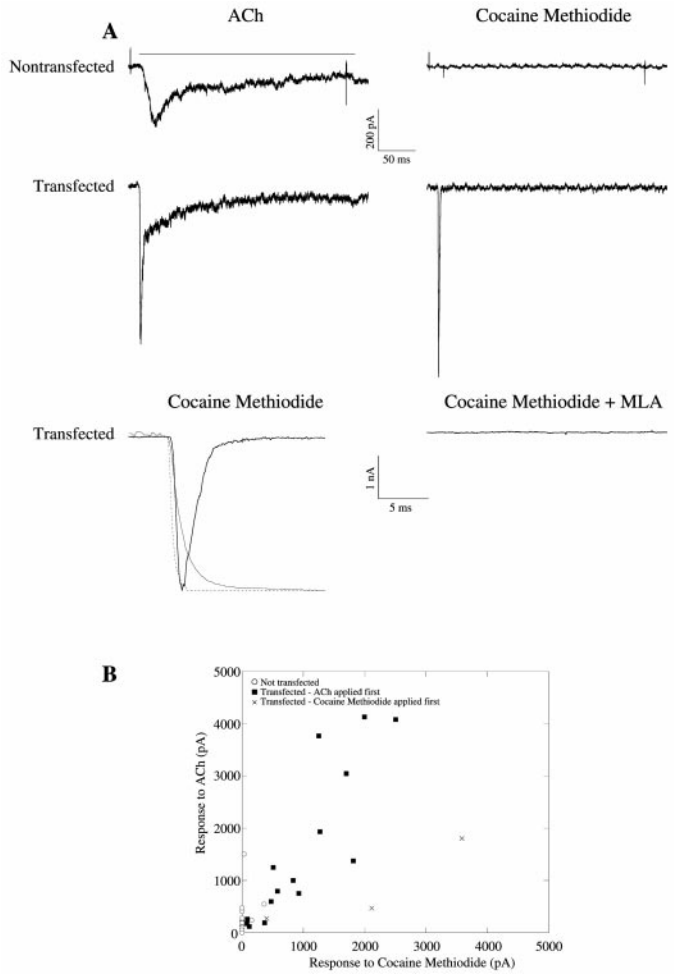

Fig. 6. Cocaine methiodide is a specific agonist for α7 nAChRs in transiently transfected PC12 cells. A, whole-cell responses of nontransfected PC12 cells (top) and PC12 cells transiently transfected with the human α7 cDNA (center and bottom) to either 1 mM ACh or 300 μM cocaine methiodide. Solutions were applied by u-tube with an exchange time (10–90%) of 1 to 3 ms. Solution exchange time (10–90%) was measured experimentally by both open-tip recording of voltage response to application of 500 mM CsCl (0.9 ms; bottom, dashed line) and u-tube application of a saturating concentration of inhibitor during the plateau phase of the whole-cell response of a slowly desensitizing nAChR subtype (2.9 ms; thin black line). The baseline noise apparent in the whole-cell solution exchange trace reflects open-channel noise before application of inhibitor. We take these measures to reflect the lower and upper limit of whole-cell solution exchange time. The trace representing the open-tip response was recorded using the same methods as the whole-cell recording and therefore could be superimposed in the time domain. The trace representing whole-cell solution exchange by application of inhibitor was necessarily acquired using a different experimental protocol and therefore was aligned according to the initial falling phase of the open-tip response. Responses to cocaine methiodide were completely inhibited by application of 100 nM MLA (lower right). B, scatterplot summarizing the peak responses of all transfected and nontransfected cells for which responses to both cocaine methiodide (300 μM) and ACh (1 mM) were recorded. Because some current rundown was observed between responses, transfected cells are classified according to the order of drug application. Because no significant differences between cells transfected with GFP alone and nontransfected cells were observed, these two classes are grouped together in the figure. Cells which had peak responses of >5 nA to cocaine methiodide or ACh were not included.

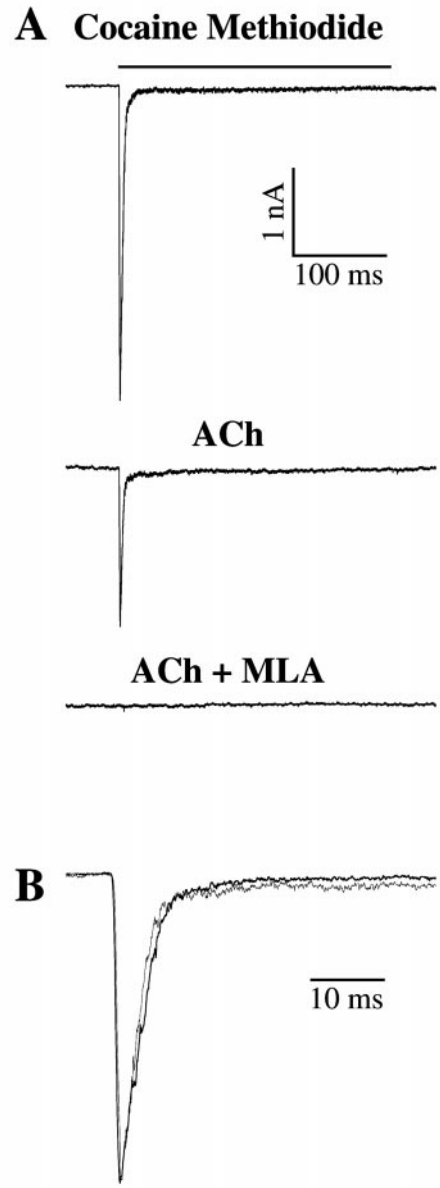

Fig. 7. ACh responses of some transfected cells desensitize rapidly with kinetics similar to cocaine methiodide responses. A, whole-cell responses to either 300 μM cocaine methiodide (top), 1 mM ACh (center), or ACh in the presence of 100 nM MLA are shown (bottom). B, the ACh (thin line) and cocaine methiodide (thicker line) responses can be superimposed when scaled to the same peak amplitude (to correct for the effects of receptor rundown). Although considerable cell-to-cell variability was observed, rundown between consecutive applications of different agonists was on average 50% (due to time required for switching solutions, ~8 min).

sponse-rise time to cocaine methiodide of ~1 ms and an apparent desensitization time constant (τ_d) in the range of 1 to 2 ms. With our application system, we observed solution exchange (10–90%) within 1 to 3 ms (Fig. 6A, bottom and legend). Thus, the peak of the response to cocaine methiodide occurs before complete whole-cell solution exchange (Fig. 6A, bottom, thin black line), indicating that desensitization probably occurs on a more rapid time scale than whole-cell agonist application in this system. This effect impacts our measurements in several ways. At the peak of the response, the maximal agonist concentration is probably not achieved across the entire surface area of the cell (Papke and Thinschmidt, 1998). In addition, rapid desensitization relative to solution exchange limits our ability to make accurate and independent measures of the activation and desensitization rates. Therefore, the apparent activation and inactivation rates may be underestimates of the rates that would be observed with a uniform step change in agonist concentration across the entire surface area of the cell.

For cells that exhibited incomplete desensitization to ACh (Fig. 6, middle trace), responses to cocaine methiodide desensitized more rapidly than responses to ACh; this process could be fit by a single exponential with an average τ_d value of 1.4 ± 0.2 ms. For cells in which only a rapidly desensitizing response component to ACh was evident, τ_d of the ACh response did not differ significantly from the same measure for cocaine methiodide (Fig. 7) in either cell type, again consistent with specific activation of α7 nAChRs by cocaine methiodide. Furthermore, ACh responses that desensitized completely were also sensitive to MLA (Fig. 7A). These data suggest that only α7-containing nAChRs are expressed in significant numbers as functional receptors in the subpopulation of cells exhibiting only a rapidly desensitizing response to ACh.

Discussion

The present study describes the conversion of cocaine from an antagonist of neuronal nAChR subunit combinations to a specific agonist of the α7 nAChR by the addition of a methyl group, converting the amine group from tertiary to quaternary. Cocaine methiodide inhibits binding of ^{125}I-α-bungarotoxin to rat α7 receptors (Fig. 3), whereas cocaine has no detectable effect on ^{125}I-α-bungarotoxin binding at a concentration ≤10 μM. Cocaine methiodide activates the rat and human isoforms of the α7 receptor with nearly 6-fold greater potency than acetylcholine (Fig. 2) and is specific for the α7 subtype in both oocyte expression studies (Fig. 4) and in studies of transiently transfected PC12 cells (Fig. 6), indicating that cocaine derivatives could have importance as subtype-selective agonists of the α7 receptor. Although cocaine methiodide does seem to exhibit some inhibitory effects at a high concentration, low affinity for an antagonist site (presumably the same site to which cocaine binds) allows cocaine methiodide to have full efficacy (compared with ACh) at the α7 receptor. Finally, we show that transient transfection of α7 nAChRs into PC12 cells used in combination with an α7-specific agonist provides a convenient and effective method to study α7 nAChR function.

The desensitization time (1–2 ms) we report in our studies of PC12 cells transfected with α7 nAChR is faster than that reported previously for responses of human α7 nAChRs, stably expressed in HEK 293 cells, to nicotine (~12 ms, Delbono et al., 1997). A few studies have reported longer τ_d value in cultured neurons as well (ranging from 8 to 27 ms; Zorumski et al., 1992; Alkondon and Albuquerque, 1993; Zhang et al., 1994), but these measures may reflect low-level activation of other nAChR subtypes in addition to α7. The very rapid desensitization we observe in our experiments does not seem to be caused by differences in application rate as the open-tip solution exchange time in this study [~1 ms (10–90%), Fig. 6A, bottom] is comparable with the faster solution exchange times (as measured by open-tip recording) reported in previous studies. Moreover, using the same application system as described in the present study, we observe kinetic properties for another rapidly desensitizing receptor type, the homomeric glutamate receptor GluR6, that are comparable with literature values for that receptor (τ_d ~ 8 ms at saturating agonist concentration; B. G. Kornreich and R. E. Oswald, unpublished observations). Secondary inhibition by cocaine methiodide could result in more rapid apparent desensitization. However, for cells that exhibit only a completely desensitizing response component to ACh (suggesting the presence of only α7-containing receptors), the desensitization time of the ACh response does not differ significantly from that of the response to cocaine methiodide. Because the choice of expression system has been shown previously to influence single-channel properties for other nAChR subtypes (Lewis et al., 1997), we cannot rule out differences in desensitization as a function of host cell type.

PC12 cells have been shown previously to express α3, α5, α7, β2, and β4 nAChR subunits (Rogers et al., 1992; Henderson et al., 1994; Fanger et al., 1995). We cannot exclude the possibility that other nAChR subunits combine with α7 subunits to form heteromeric receptors in our transfected cells. However, a previous report has concluded that native α7 nAChRs in PC12 cells are homomeric (Drisdel and Green, 2000). In our cells, we observe rapidly desensitizing responses (compared with ACh responses in the same cell), to an α7-specific agonist, which are inhibited by MLA. If α7-containing heteromeric combinations are present, they are not distinguishable on the basis of obvious differences in kinetics or MLA sensitivity in our experiments.

Previous studies of quaternary local anesthetic nAChR inhibitors have indicated that quaternization affects the state dependence of inhibition, in large part limiting inhibitory effects to the open-channel form of the receptor (Neher and Steinbach, 1978). In contrast, quaternization of cocaine confers affinity for the agonist binding site of the α7 nAChR. Although many quaternary amines exhibit some degree of affinity for the acetylcholine binding site, the efficacy and specificity of cocaine methiodide agonist effects for the α7 nAChR are novel. Because cocaine (pK_a = 8.5) is largely protonated at physiological pH (>90%), the tertiary amine group effectively contains a partial positive charge. Voltage-dependent inhibition by cocaine suggests that the charged species is responsible for at least some of the nAChR inhibitory effects observed upon coapplication with ACh in our experiments (Fig. 1). Given the fact that we do not observe any agonist effects with application of even 1 mM cocaine, it seems unlikely that the presence of an additional methyl group in cocaine methiodide confers high affinity for the α7 acetylcholine binding site solely as a function of charge.

Other factors such as steric constraints and hydrogen bonding differences are also likely to be important.

The backbone ring structure of cocaine (Fig. 2A) does share certain general features with other nAChR agonists such as (+)-anatoxin-A (Swanson et al., 1986) and epibatidine (Badio and Daly, 1994). However, although both epibatidine and (+)-anatoxin-A are potent activators of α7 nAChRs, neither compound exhibits specificity for this subtype over other nAChR subunit combinations. Among α7-selective agonists, AR-R 17779 (Mullen et al., 2000) and GTS-21 (de Fiebre et al., 1995) have been reported to show limited efficacy at heteromeric nAChR subtypes while the activity of cocaine methiodide appears specific for activation of α7 receptors. Moreover, despite the appearance of inhibition at high concentrations of cocaine methiodide, the drug is fully efficacious for α7 (compared with ACh), whereas the efficacy of GTS-21 is more limited. The primary metabolite of GTS-21, 4-hydroxy-GTS-21 (Meyer et al., 1998), has been reported to show increased efficacy at human α7 nAChR. Finally, the endogenous compound choline has also been shown to be a specific agonist for the α7 nAChR (Mandelzys et al., 1995; Papke et al., 1996) but exhibits much lower potency.

Cocaine methiodide is a quaternary amine, which makes direct clinical application of this compound unlikely because of limited brain access. However, if affinity for the α7 agonist binding site results from purely steric constraints conferred by the presence of an additional methyl group, cocaine derivatives mimicking this density may have increased therapeutic potential. A second priority for this line of development will certainly be the identification of analogs that retain affinity for α7 nAChR but lack affinity for other sites of cocaine action, such as monoamine transporters and voltage-gated sodium channels. Quaternization of cocaine does reduce affinity for the dopamine transporter (Abraham et al., 1992). The reported K_i value for cocaine methiodide binding to the dopamine transporter is in the range of 8 μM at physiological pH (Xu and Reith, 1996), roughly 40-fold lower affinity than was observed for cocaine methiodide binding to the acetylcholine site of α7 nAChR (K_i = 190 nM; Fig. 3) in this study. Because functional effects on both the transporter and α7 require higher drug concentrations than are typically measured in binding studies, direct functional comparisons will ultimately be required.

Although the agonist effects of cocaine methiodide seem specific for α7 among nAChR subtypes, antagonist activity at other nAChR subtypes and the potential for effects at other sites of cocaine action in the nervous system remain to be addressed experimentally. Nonetheless, cocaine methiodide is a promising lead compound for the development of α7-selective drugs. Furthermore, direct activation of α7 nAChRs by agonists such as cocaine methiodide should prove to be a powerful tool in functional studies that, to date, have, for the most part, relied on sensitivity to specific inhibitors of α7 nAChRs to evaluate α7-mediated effects.

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Address correspondence to: Robert E. Oswald, Department of Molecular Medicine, C3 167 Veterinary Medical Center, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. E-mail: reo1@cornell.edu