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In vivo effects of 3-iodocytisine: Pharmacological and genetic analysis of hypothermia and evaluation of chronic treatment on nicotinic binding sites

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ABSTRACT

Several cytisine derivatives have been developed in the search for more selective drugs at nicotinic acetylcholine receptors (nAChR). Binding experiments in transfected cell lines showed that the iodination of cytisine in the position 3 of the pyridone ring increased potency at α7-nAChR and to a lesser extent at the α4β2 subtypes, both of which are widely expressed in the brain. However, no *in vivo* studies have been published on this compound. Inhibition curves presented here using wild type, β2, and β4-null mutant mice confirm that 3-IC binds to α4β2*, α7* and α3β4* receptors with higher affinity than cytisine (asterisk indicates the receptor may contain additional subunits, Lukas et al., 1999). Intraperitoneal injection of 3-iodocytisine (3-IC) induced considerable dose-dependent hypothermia in DBA/2J and C57BL/6J mice. This response was blocked by mecamylamine and partially inhibited by hexamethonium. β4-null mice displayed significantly less 3-IC-induced hypothermia than wild-type mice, β2-null mice were somewhat less affected than wild types, while responses of α7*-null mice were similar to wild types. Mice treated chronically with 3-IC display a marked increase in α7* and α4β2* binding sites determined by radioligand binding in membrane preparations from cerebral cortex and hippocampus. Quantitative autoradiographic analysis of 28 brain regions of mice treated with 3-IC was consistent with the membrane binding, detecting an increase of cytisine-sensitive [^{125}I]epibatidine binding sites, while cytisine-resistant [^{125}I]epibatidine sites were unchanged. [^{125}I]α-Bungarotoxin binding sites also exhibited up-regulation. These results give a first evaluation of *in vivo* consequences of 3-IC as a potent agonist with marked effects on mice.

1. Introduction

Nicotinic acetylcholine receptors (nAChR) constitute a family of ligand-gated ion channels assembled in neurons as pentameric receptors. While mRNA encoding nine nAChR subunits (α2–α7 and β2–β4) is expressed in mammalian brain, the major neuronal subtypes in the central nervous system are homomeric α7*-nAChR and heteromeric α4β2*-nAChR (Dani and Bertrand, 2007). nAChR mediate several simple and complex behavioral and physiological responses (Picciotto, 2003). nAChR also mediates short-term and long-term responses to nicotine and similar drugs indicating a role in nicotine tolerance and dependence (Dani and DeBiasi, 2001). nAChRs have also been implicated in several human neurological and psychiatric disorders. Single point mutations in either the α4 or β2 nAChR subunits have been identified as the cause of several different Autosomal Dominant Nocturnal Frontal Lobe Epilepsies (Hogg and Bertrand, 2004). Although the evidence is not as obvious, nAChR also appear to be involved in the etiology of Alzheimer's and Parkinson's diseases as well as in psychopathologies such as anxiety disorders, Tourette's syndrome and schizophrenia (Bourin et al., 2003; Leonard et al., 2001). Because of the relevance of nicotinic physiology to health issues, nAChR ligands are envisioned as potential therapeutic agents for Parkinson's disease (Quik, 2004), obsessive–compulsive disorder (Salin-Pascual and Basanez-Villa, 2003) and schizophrenia (Harris et al., 2004).

Specific and potent nicotinic ligands would facilitate investigation of the multiplicity of *in vivo* effects modulated by nAChR.

Cytisine is a plant alkaloid with higher affinity for neural α4β2-nAChR than nicotine (Pabreza et al., 1991). It is a partial agonist at β2*-nAChR but a full agonist at β4*-nAChR (Luetje and Patrick, 1991). Due to its semi-rigid structure cytisine has been used as

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a template for the preparation of new nicotinic receptor ligands. A group of cytisine derivatives has, indeed, been developed (Imming et al., 2001; Slater et al., 2003). Varenicline, which has recently been approved for smoking cessation, is such an analog (Lam and Patel, 2007). An electrophysiological characterization of the effects of cytisine and its bromo derivatives, in particular 3-bromocytisine, showed a potent effect on inward currents in ACh-activated neurons in cat petrosal ganglion neurons in culture (Varas et al., 2006). The C3-halogenated derivatives of cytisine, and particularly 3-iodocytisine (3-IC) display higher binding affinity than cytisine determined either α7* and α4β2* receptors in rat brain with K_i values of 115 nM and 0.17 nM compared to 8 μM and 1.2 nM, respectively (Abin-Carriquiry et al., 2006). Similarly, for human α7 and α4β2 expressed in *Xenopus* oocytes 3-IC has K_i values of 7.0 and 0.7 nM compared to 30 μM and 0.6 nM for cytisine respectively (Slater et al., 2003). 3-IC is also more potent that cytisine in stimulating [^{3}H]noradrenaline release from rat hippocampal slices and for [^{3}H]dopamine release from rat striatal synaptosomes with EC_{50} values of 0.22 μM and 0.011 μM for 3-IC compared to 7.4 μM and 0.28 μM for cytisine, respectively (Abin-Carriquiry et al., 2006). Similarly, in *Xenopus* oocytes expressing human α7-nAChR, the EC_{50} value for 3-IC was 1.5 μM, compared to 83 μM for cytisine, while EC_{50} values for the high and low agonist sensitive forms of α4β2-nAChR were 0.8 nM and 86 nM for 3-IC and 5 nM and 2 μM for cytisine (Slater et al., 2003; Abin-Carriquiry et al., 2006). Beyond these *in vitro* analyses, 3-IC has never been tested in an *in vivo* model. Here, we describe the acute effect of 3-IC in the modulation of body temperature in mice including evaluation of the effect of some nicotinic antagonists and deletion of either the α7, β2 or β4 nAChR genes on the hypothermic response. Finally we expand the effect of chronic 3-IC treatment on nAChR binding sites in mouse brain.

2. Materials

[^{125}I]Epibatidine (2200 Ci/mmol) was purchased from Perkin–Elmer Life Science, Boston, MA and [^{125}I]α-bungarotoxin (2000 Ci/mmol) from GE healthcare. A85380, cytisine, mecamylamine, hexamethonium, scopolamine, nicotine, chloral hydrate, pentobarbital, polyethyleneimine (PEI) and bovine serum albumin (BSA) fraction V were purchased from Sigma Chemical Company, St. Louis, MO. 4-(2-Hydroxyethyl)-piperazineethanesulfonic acid (HEPES) half-sodium salt was from Roche Diagnostics Corporation, Indianapolis, IN.

3. Methods

3.1. Synthesis of 3-IC

Cytisine was purified from *Sophora secundiflora* seeds using standard methodology. Monohalogenated cytisine derivatives were prepared by treating cytisine with iodine monochloride. The iodinated isomers were separated by column chromatography on silica gel, crystallized to homogeneity and characterized by ^{1}H and ^{13}C NMR and HREIMS as reported before (Slater et al., 2003).

3.2. Mice

DBA/2J and C57BL/6J mice were bred at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO and housed five per cage prior to testing or surgery and chronic nicotine treatment.

$\alpha7^{-/-}$ (Orr-Urtreger et al., 1997), $\beta2^{-/-}$ (Picciotto et al., 1995) and $\beta4^{-/-}$ (Xu et al., 1999) and wild-type littermates were generated by mating heterozygotes for each mutation. Each of these null mutant mice has been backcrossed with C57BL/6JJ mice for at least 10 generations at the time of the experiments. Mice were weaned at 25 days of age and housed with like-sexed littermates. DNA was extracted from tail clippings obtained from 40-day old mice and genotypes were determined as described previously (Salminen et al., 2004). Mice were allowed free access to food (Rodent Chow, Harlan-Teklad, Madison, WI) and water. The animal vivarium was maintained at a temperature of 23 ± 2 °C with a 12 h light/12 h dark cycle (lights on 7 AM–7 PM). All procedures used in this study were reviewed and approved by the Animal Care and Utilization Committee of the University of Colorado.

3.3. Temperature measurements

Body temperature measurement was chosen for these experiments because the hypothermic effects of nAChR ligands have been reported before for the two different mice strains used in this study (Marks et al., 1983a; Tritto et al., 2004). DBA/2J, C57/BL6 and wild type and α7-, β2- and β4-null mutant mice were tested under similar conditions for determination of body temperature. All drugs were administered intraperitoneally (IP) (10 μl/g of body weight in every injection). All antagonists were injected 10 min before the 3-IC injections. Temperature was measured with a Thermalert rectal probe lubricated with peanut oil and inserted 1.5 cm into the mouse's rectum.

3.4. Chronic 3-IC treatment

DBA/2J mice were anesthetized with pentobarbital (45 mg/kg)-chloral hydrate (63 mg/kg). A cannula was implanted in the right jugular vein (Barr et al., 1979) and after surgery the animals were placed in individual cages (15 × 15 × 25 cm). Following 2 days of continuous saline infusion (35 μl/h), treatment with 3-IC was begun. Fresh drug and saline solution was supplied every day. Mice were randomly assigned to two groups: saline infused (control) or 3-IC 0.2 mg/kg/h. Stock 3-IC solutions (10 mg/ml) were prepared in sterile saline, neutralized with HCl and stored in the dark at 4 °C. Dilutions of this stock solution were prepared to give the appropriate hourly dose. Mice were subsequently infused with 3-IC for 7 days. Following the 7-day treatment, infusion was terminated. Two hours after ending treatment mice were injected IP with 0.2 mg/kg 3-IC and body temperature was measured at 5-min intervals for the first 20 min and at 10 min intervals thereafter.

3.5. Membrane preparation

Two hours following completion of the tolerance test (5 h after withdrawal from drug infusion), mice that had been treated chronically with saline or 0.2 mg/kg/h 3-IC were killed by cervical dislocation. Each brain was placed on an ice-cold platform and dissected into the following brain regions: cerebral cortex (Cx), striatum (St), hippocampus (Hp), thalamus (Th), superior colliculus (SC), inferior colliculus (IC) and midbrain (MB). Each brain region was homogenized in ice-cold hypotonic buffer (NaCl, 14.4 mM; KCl, 0.2 mM; $CaCl_2$, 0.2 mM; $MgSO_4$, 0.1 mM; HEPES 2 mM; pH = 7.5) using a Teflon-glass tissue grinder. The particulate fractions were obtained by centrifugation at 20,000 × *g* (10 min, 4 °C; Sorvall RC-2B centrifuge). The pellets were resuspended in fresh homogenization buffer and washed four times by resuspension/centrifugation, and stored (in pellet form under homogenization buffer) at −70 °C until used.

3.6. [^{125}I]Epibatidine binding

[^{125}I]Epibatidine binding was measured as described previously (Whiteaker et al., 2000). Frozen, washed pellets were resuspended in the overlying buffer and centrifuged at 20,000 × *g* for 20 min. The supernatant was discarded and the pellet was resuspended in ice-cold water. Resuspension volume varied among brain regions, and was adjusted such that less than 10% of the [^{125}I]epibatidine was bound to the protein at the highest ligand concentration. Samples (10–50 μg protein) were incubated in 96-well polystyrene plates for 3 h at room temperature in a binding buffer of the following composition: NaCl, 144 mM; KCl, 2.2 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; HEPES hemisodium, 25 mM; pH = 7.5. Final incubation volume was 30 μl. At the completion of the incubation, samples were diluted with 200 μl of ice-cold binding buffer and filtered under vacuum (0.2 atm.) onto glass fiber filters that had been treated with 0.5% PEI (top filter, MFS Type B; bottom filter, Gelman A/E). An Inotech Cell Harvester (Inotech Biosystems International, Rockville, MD) was used to collect the samples, which were subsequently washed five times with ice-cold buffer. Filters containing the washed samples were transferred to glass culture tubes and radioactivity counted at 80% efficiency using a Packard Cobra Auto-Gamma Counter (Packard Instruments, Downers Grove, IL). For all the experiments, non-specific binding was measured by including 100 μM cytisine in the incubation medium. All brain regions from 3-IC- and saline-treated groups were assayed for [^{125}I]epibatidine binding using 200 pM ligand. Since [^{125}I]epibatidine binds with high affinity to several different nAChR subtypes, differential inhibition by the agonists cytisine (50 and 150 nM) and A85380 (10 and 50 nM) was used to distinguish four binding sites: cytisine-sensitive (primarily α4β2*), cytisine-resistant (mixed population), A85380-sensitive (β2*-nAChR), and A85380-resistant (β4*-nAChR) (Whiteaker et al., 2000).

Inhibition of [^{125}I]epibatidine binding by nicotine, cytisine and 3-IC was measured using a ligand concentration of 200 pM and an incubation volume of 30 μl. Eleven concentrations of drugs (0.01 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM and 10 μM) were used. Blanks were established by measuring binding in the presence of 100 μM cytisine.

3.7. [^{125}I]α-Bungarotoxin binding

Binding of [^{125}I]α-bungarotoxin to membrane preparations was performed as described (Whiteaker et al., 2008). Membrane samples (25–200 mg of membrane preparations) were incubated with 1 nM [^{125}I]α-bungarotoxin in binding buffer