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Functional and structural interaction of (−)-lobeline with human α4β2 and α4β4 nicotinic acetylcholine receptor subtypes

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ABSTRACT

To determine the pharmacologic activity of (−)-lobeline between human (h)α4β2 and hα4β4 nicotinic acetylcholine receptors (AChRs), functional and structural experiments were performed. The Ca^{2+} influx results established that (−)-lobeline neither actives nor enhances the function of the studied AChR subtypes, but competitively inhibits hα4β4 AChRs with potency ~10-fold higher than that for hα4β2 AChRs. This difference is due to a higher binding affinity for the [^{3}H]cytisine sites at hα4β4 compared to hα4β2 AChRs, which, in turn, can be explained by our molecular dynamics (MD) results: (1) higher stability of (−)-lobeline and its hydrogen bonds within the α4β4 pocket compared to the α4β2 pocket, (2) (−)-lobeline promotes Loop C to cap the binding site at the α4β4 pocket, but forces Loop C to get apart from the α4β2 pocket, precluding the gating process elicited by agonists, and (3) the orientation of (−)-lobeline within the α4β4, but not the α4β2, subpocket, promoted by the *t*− (or *t*+) rotameric state of α4-Tyr98, remains unchanged during the whole MD simulation. This study gives a detailed view of the molecular and dynamics events evoked by (−)-lobeline supporting the differential binding affinity and subsequent inhibitory potency between hα4β2 and hα4β4 AChRs, and supports the possibility that the latter subtype is also involved in its activity.

1. Introduction

(−)-Lobeline [(−)-2S, 6R, 8S-lobeline, or α-lobeline] is a non-pyridino alkaloid obtained from several *Lobelia* plant species. Pre-clinical and clinical studies have demonstrated that this natural product possess pro-cognitive activity in animals (Decker et al., 1993) and in patients with attention deficit hyperactivity disorder (Martin et al., 2015), as well as anxiolytic (Brioni et al., 1993), and anti-addictive (Harrod et al., 2001; Polston et al., 2006) properties. Although (−)-lobeline was used as a smoking cessation agent, several side effects made this drug to be banned by the FDA (reviewed in Sewester et al., 1997).

Although (−)-lobeline was originally classified as a partial agonist of nicotinic acetylcholine receptors (AChRs), mounting evidence suggests that this compound behaves more like a competitive antagonist. Although (−)-lobeline binds to AChRs with high affinity (K_i ~4 nM) (Flammia et al., 1999), the functional results indicate that it does not activate (Damaj et al., 1997) or slightly activates (Kaniaková et al., 2011) AChRs, but instead it acts as an antagonist at different AChRs (Dwoskin and Crooks, 2002). It has been hypothesized that the previously described pharmacologic activities of (−)-lobeline are mediated by its interaction with α4β2 AChRs. However, the evidence indicating that (−)-lobeline-induced [^{3}H]dopamine overflow from rat striatal slices is not inhibited by mecamylamine (Dwoskin and Crooks, 2002), a nonspecific noncompetitive antagonist of AChRs, and that the spinal analgesic activity of (−)-lobeline does not correlate with its affinity at the α4β2 AChR (Flammia et al., 1999), suggests additional mechanisms of action (Dwoskin and Crooks, 2002; Kaniaková et al., 2011) or the existence of other targets. For example, it has been suggested that (−)-lobeline may interact with a locus different from the

Abbreviations: AChR, nicotinic acetylcholine receptor; (−)-lobeline (α-lobeline) (−), -2S, 6R, 8S-lobeline, Ct-AChBP, acetylcholine binding protein from *Capitella teleta*; Ac-AChBP, acetylcholine binding protein from *Aplysia californica*; CCh, carbamylcholine; RT, room temperature; BS, binding saline; EXD, extracellular domain, ORT, orthosteric; K_i, inhibition constant; K_d, dissociation constant; IC_{50}, ligand concentration that produces 50% inhibition (of binding or of agonist activation); n_H, Hill coefficient; EC_{50}, agonist concentration that produces 50% AChR activation; MD, molecular dynamics; FBS, fetal bovine serum.

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orthosteric sites enhancing agonist-activated α4β2 AChRs (Kaniaková et al., 2011), a pharmacologic property resembling that for positive allosteric modulators (reviewed in Arias, 2011).

There is a large amount of experimental evidence supporting an important role of α4β2 AChRs in the mechanism of nicotine addiction (reviewed in Ortells and Arias, 2010). For example, knockout animal results indicate that the β2 subunit is necessary for nicotine-induced dopamine release (Grady et al., 2001), and for the discriminative (Shoaib et al., 2002) and reinforcing (Picciotto et al., 1998) properties of nicotine. Although α4β4 AChRs are expressed in less proportion compared to α4β2 AChRs, they have been found in several brain regions, including basal ganglia, cerebellum, midbrain, ventral tegmental area, hippocampus, and cortex, where some of these areas are implicated in drug addiction (Azam et al., 2002; Quik et al., 2000).

Studies using the acetylcholine binding proteins (AChBPs) from *Capitella teleta* (Ct-AChBP; Billen et al., 2012), and *Aplysia californica* (Ac-AChBP; Hansen et al., 2005) show that (−)-lobeline induces a strong capping of Loop C (at the principal component) as other antagonists do, as well as exposes an additional subpocket to accommodate the α-hydroxyphenetyl moiety by changing the rotameric state of a Tyr residue from Loop A, the so-called g-to-t or Tyr-flip conformation (reviewed in Arias, 2012). Since (−)-lobeline presents interesting pharmacologic properties and could be clinically important, the binding affinity, agonistic and antagonistic activities of (−)-lobeline, as well as the structural features of its binding site were compared between the human (h) α4β2 and hα4β4 AChRs. In this regard, Ca^{2+} influx and [^{3}H]cytisine competition binding assays, as well as molecular docking and molecular dynamics (MD) studies were applied.

2. Materials and methods

2.1. Materials

[^{3}H]Cytisine (40 Ci/mmol) was obtained from PerkinElmer Life Sciences Products, Inc. (Boston, MA, USA), and stored at −20 °C. Carbamylcholine dihydrochloride (CCh), (−)-lobeline hydrochloride and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas probenecid was obtained from Sigma Chemical Co. (Buchs, Switzerland). (±)-Epibatidine hydrochloride was obtained from Tocris Bioscience (Ellisville, MO, USA). Fluo-4 was purchased from Molecular Probes (Eugene, OR, USA). Fetal bovine serum (FBS) and trypsin/EDTA were purchased form Gibco BRL (Paisley, UK). Salts were of analytical grade.

2.2. Ca^{2+} influx measurements in HEK293-hα4β2 and CHO-hα4β4 cells

Ca^{2+} influx assays were performed as previously described (Pérez et al., 2013; Arias et al., 2013, 2015). Briefly, HEK293-hα4β2 and CHO-hα4β4 cells were seeded 72 h prior to the experiment on black 96-well plates (Costar, New York, USA) at a density of 5×10^4 per well and incubated at 37 °C in a humidified atmosphere (5% CO_2/95% air). 16–24 h before the experiment, the medium was changed to 1% FBS in HEPES-buffered salt solution (HBSS) (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM glucose, 20 mM HEPES, pH 7.4). On the day of the experiment, the medium was removed by flicking the plates and replaced with 100 μL HBSS/1% FBS containing 2 μM Fluo-4 in the presence of 2.5 mM probenecid. The cells were then incubated at 37 °C in a humidified atmosphere (5% CO_2/95% air) for 1 h. Plates were flicked to remove excess of Fluo-4, washed twice with HBSS/1% FBS, and finally refilled with 100 μL of HBSS containing different concentrations of (−)-lobeline and pre-incubated for 5 min. Plates were then placed in the cell plate stage of the fluorimetric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA). (±)-Epibatidine (0.1 μM) was then added from the agonist plate to the cell plate using the 96-tip pipettor simultaneously to fluorescence recordings for a total length of 3 min. A baseline consisting of 5 measurements of 0.4 s each was recorded. To determine the agonistic activity, AChRs were stimulated with increasing concentrations of (−)-lobeline. In parallel experiments, a fixed concentration of (±)-epibatidine (i.e., 1, 3, 10, 30, or 100 nM) was co-injected with increasing concentrations of (−)-lobeline. The excitation and emission wavelengths are 488 and 510 nm, at 1 W, and a CCD camera opening of 0.4 s.

2.3. [^{3}H]Cytisine competition binding experiments

To determine the binding affinity of (−)-lobeline for the hα4β2 and hα4β4 AChRs, [^{3}H]cytisine competition binding experiments were performed as previously published (Pérez et al., 2013; Arias et al., 2015). In this regard, AChR membranes (1.0 mg/mL), first prepared from HEK293-hα4β2 and CHO-hα4β4 cells (Pérez et al., 2013; Arias et al., 2015), were suspended in BS buffer with 10 nM [^{3}H]cytisine, and preincubated for ~30 min at RT. Nonspecific binding was determined in the presence of 1 mM CCh. The total volume was divided into aliquots, and increasing concentrations of (−)-lobeline were added to each tube and incubated for 2 h at RT. AChR-bound [^{3}H]cytisine was then separated from free radioligand by a filtration assay using a 48-sample harvester system with GF/B Whatman filters (Brandel Inc., Gaithersburg, MD, USA), previously soaked with 0.5% polyethylenimine for 30 min. The membrane-containing filters were transferred to scintillation vials with 3 mL of Bio-Safe II (Research Product International Corp, Mount Prospect, IL, USA), and the radioactivity was determined using a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

The concentration–response data were curve-fitted by nonlinear least squares analysis using the Prism software (GraphPad Software, San Diego, CA). The observed IC_{50} values were transformed into inhibition constant (K_i) values using the Cheng–Prusoff relationship (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + ([[^3H]\text{cytisine}]/K_d^{\text{cytisine}})} \quad (1)$$

where [[^{3}H]cytisine] is the initial concentration of [^{3}H]cytisine, and K_d^{cytisine} is the dissociation constant for [^{3}H]cytisine at the hα4β4 (0.1 nM; Slater et al., 2003) and hα4β2 AChRs (0.3 nM; Zhang and Steinbach, 2003), respectively. The calculated K_i values were summarized in Table 1.

2.4. Homology models of the α4β4 and α4β2 subunit pairs

Structural models of the α4β2 and α4β4 subunit pairs were based on the crystal structure of the Ct-AChBP complexed with (−)-lobeline (PDB 4AFH; Billen et al., 2012) as a template for

Table 1
Inhibitory potency (IC_{50}) and binding affinity (K_i) of (−)-lobeline for the hα4β2 and hα4β4 AChRs.

AChR subtype	Ca^{2+} influx		[^{3}H]Cytisine competition binding	
	IC_{50} [a] (μM)	n_H [a]	K_i [b] (nM)	n_H [b]
hα4β4	0.25 ± 0.04	1.67 ± 0.17	1.82 ± 0.07	0.87 ± 0.03
hα4β2	2.58 ± 0.50	1.06 ± 0.11	4.90 ± 0.30	0.79 ± 0.04

n_H, Hill coefficient.
[a] These values were obtained from Fig. 1A and C, respectively.
[b] These values were obtained from experiments as shown in Fig. 2.