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Pharmacological and molecular studies on the interaction of varenicline with different nicotinic acetylcholine receptor subtypes. Potential mechanism underlying partial agonism at human α4β2 and α3β4 subtypes

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ABSTRACT

To determine the structural components underlying differences in affinity, potency, and selectivity of varenicline for several human (h) nicotinic acetylcholine receptors (nAChRs), functional and structural experiments were performed. The Ca^{2+} influx results established that: (a) varenicline activates (μM range) nAChR subtypes with the following rank sequence: hα7 > hα4β4 > hα4β2 > hα3β4 >>> hα1β1γδ; (b) varenicline binds to nAChR subtypes with the following affinity order (nM range): hα4β2 ~ hα4β4 > hα3β4 > hα7 >>> *Torpedo* α1β1γδ. The molecular docking results indicating that more hydrogen bond interactions are apparent for α4-containing nAChRs in comparison to other nAChRs may explain the observed higher affinity; and that (c) varenicline is a full agonist at hα7 (101%) and hα4β4 (93%), and a partial agonist at hα4β2 (20%) and hα3β4 (45%), relative to (±)-epibatidine. The allosteric sites found at the extracellular domain (EXD) of hα3β4 and hα4β2 nAChRs could explain the partial agonistic activity of varenicline on these nAChR subtypes. Molecular dynamics simulations show that the interaction of varenicline to each allosteric site decreases the capping of Loop C at the hα4β2 nAChR, suggesting that these allosteric interactions limit the initial step in the gating process. In conclusion, we propose that in addition to hα4β2 nAChRs, hα4β4 nAChRs can be considered as potential targets for the clinical activity of varenicline, and that the allosteric interactions at the hα3β4- and hα4β2-EXDs are alternative mechanisms underlying partial agonism at these nAChRs.

1. Introduction

The addictive properties of nicotine (the active alkaloid involved in smoking addiction) as well as the activity of varenicline (Chantix®, Champix®) for smoking cessation therapy [1] are primarily mediated by their interactions with nicotinic acetylcholine receptors (nAChRs). The interaction of nicotine with α4β2 nAChRs in the mesocorticolimbic system, the so-called "brain reward system", increases the synaptic levels of dopamine, which in turn produces the pleasurable effects mediated by nicotine [2–4]. There is a large amount of experimental evidence supporting an important role of α4β2 nAChRs in the mechanism of nicotine addiction. For example, animal studies show that agonists specific for α4β2 nAChRs produce similar discriminative stimulus as nicotine [5], and knockout animal results indicates that the β2 subunit is necessary for the reinforcing [6] and discriminative [7] properties

Abbreviations: nAChR, nicotinic acetylcholine receptor; [^{3}H]MLA, [^{3}H]methyllycaconitine; [^{3}H]TCP, piperidyl-3, 4-^{3}H(*N*)]-(*N*-(1-(2 thienyl)cyclohexyl)-3, 4-piperidine; α-BTx, α-bungarotoxin; Varenicline, 7,8,9,10-tetrahydro-6,10-methano-6*H*-azepino[4,5-*g*]quinoxaline; RT, room temperature; BS, binding saline; A, allosteric; EXD, extracellular domain, ORT, orthosteric; A, allosteric; TMD, transmembrane domain, K_i, inhibition constant; K_d, dissociation constant; IC_{50}, ligand concentration that produces 50% inhibition (of binding or of agonist activation); EC_{50}, agonist concentration that produces 50% nAChR activation; n_H, Hill coefficient; MD, molecular dynamics; NVT, constant number of particles, volume, and temperature; NPT, constant number of particles, pressure, and temperature; RMSD, root mean square deviation; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; FLIPR, fluorescent imaging plate reader

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of nicotine and for nicotine-induced dopamine release [8]. Nevertheless, other nAChR subtypes (i.e., α7, α3β4, and α6β2-containing nAChRs) are also involved in the mechanism of nicotine addiction [2,3].

Pharmacologically, varenicline behaves as a partial agonist of α4β2 nAChRs and a full agonist of α7 nAChRs [9–12]. Through its intrinsic partial activation of α4β2 nAChRs, varenicline elicits a moderate and sustained increase of dopamine levels in the brain reward system, which would elevate low dopamine levels observed during smoking cessation attempts [4,11–14]. In addition, varenicline competitively inhibits nicotine binding to α4β2 nAChRs, preventing nicotine-induced dopaminergic activation. This dual effect ultimately decreases craving, withdrawal symptoms, smoking satisfaction and reward. Studies using β2-subunit knockout animals and animals where the β2 subunit has been re-expressed indicate that β2-containing nAChRs are involved in the dopaminergic effects mediated by varenicline [15]. Although there is a good idea of how varenicline acts clinically, we still do not have a complete understanding of the structural and functional aspects underlying its receptor selectivity, specifically regarding the α4β4 nAChR. α4 and β4 subunits, potentially forming α4β4-containing nAChRs, are also expressed in several brain regions implicated in drug addiction, including basal ganglia, cerebellum, midbrain, ventral tegmental area, hippocampus, and cortex [16,17]. To have a more comprehensive idea of the interaction of varenicline with different nAChR subtypes, we decided to determine which structural components are important for the different binding affinities, agonistic and antagonistic potencies, and receptor selectivity of varenicline for several nAChR subtypes including, the human (h) α4β2, hα4β4, hα3β4, hα7, *Torpedo* and hα1β1γδ nAChRs. In this study we applied structural and functional approaches including radioligand binding assays, Ca^{2+} influx-induced fluorescence detections, as well as homology modeling, molecular docking, and molecular dynamics studies.

2. Materials and methods

2.1. Materials

[^{3}H]Epibatidine (45.1 Ci/mmol), [^{3}H]cytisine (34.1 Ci/mmol), [piperidyl-3,4-^{3}H(*N*)]-(*N*-(1-(2 thienyl)cyclohexyl)-3,4-piperidine) ([^{3}H]TCP; 45.0 Ci/mmol), and [^{3}H]imipramine (47.5 Ci/mmol) were obtained from PerkinElmer Life Sciences Products, Inc. (Boston, MA, USA). [^{3}H]Methyllycaconitine (100 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA). The radioligands were stored at −20 °C. Methyllycaconitine citrate, carbamylcholine dihydrochloride, imipramine hydrochloride, and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (±)-Epibatidine hydrochloride was obtained from Tocris Bioscience (Ellisville, Missouri, USA). Fetal bovine serum (FBS) and trypsin/EDTA were purchased form Gibco BRL (Paisley, UK). Ham's F-12 Nutrient Mixture was obtained from Invitrogen (Paisley, UK). Varenicline hydrochloride and phencyclidine hydrochloride (PCP) were obtained through the National Institute on Drug Abuse (NIDA) (NIH, Baltimore, USA). Salts were of analytical grade.

2.2. Ca^{2+} influx measurements in cells containing different nAChR subtype

Ca^{2+} influx measurements were performed in GH3-hα7, HEK293-hα4β2, HEK293-hα3β4, and TE671-hα1β1γδ cells incubated at 37 °C as previously described [17–19]. In the particular case of CHO-hα4β4 cells, a density of 5×10^4 per well was used. Under these conditions, the majority of expressed nAChRs has the $(\alpha x)_3(\beta x)_2$ stoichiometry (see [18] and references therein). To determine the agonistic activity, varenicline or (±)-epibatidine was added to the cell plate using the 96-tip pipettor simultaneously to fluorescence recordings for a total length of 3 min. To determine the antagonistic activity, cells were pretreated (5 min) with different concentrations of varenicline before testing the activity of (±)-epibatidine (0.1 μM for neuronal nAChRs, and 1 μM for hα1β1γδ nAChRs).

2.3. Radioligand competition binding experiments

To determine receptor selectivity, the effect of varenicline on [^{3}H]MLA (4.1 nM) binding to hα7 nAChRs, on [^{3}H]epibatidine (4.6 nM) binding to hα3β4 nAChRs, and on [^{3}H]cytisine (9.1 nM) binding to hα4β2, hα4β4, and *Torpedo* nAChRs, respectively, was studied as previously described [19–22]. To determine whether varenicline interacts with the *Torpedo* and hα4β2 nAChR ion channels, additional studies were conducted using [^{3}H]TCP (20 nM) [21] and [^{3}H]imipramine (13 nM) [23]. The effect of varenicline on [^{3}H]cytisine, in the absence (nAChRs are in the resting but activatable state) and presence of 200 μM proadifen [24], and [^{3}H]TCP binding, in the presence of 1 mM CCh (nAChRs are mainly in the desensitized state), was also determined as previously described [21,23]. Nonspecific binding was determined in the presence of 10 μM MLA ([^{3}H]MLA experiments), 1 mM CCh ([^{3}H]cytisine experiments), 0.2 μM (±)-epibatidine ([^{3}H]epibatidine experiments), 100 μM PCP ([^{3}H]TCP experiments), or 100 μM imipramine ([^{3}H]impramine experiments).

After incubation (2 h), nAChR-bound radioligand was separated from free radioligand by a filtration assay [19–23]. 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 [25]:

$$K_i = IC_{50} \Big/ \left\{1 + \left(\left[\left[{}^3H \right] \text{ligand} \right] \Big/ K_d^{\text{ligand}} \right) \right\} \quad (1)$$

where [[^{3}H]ligand] is the initial concentration of [^{3}H]MLA, [^{3}H]cytisine, or [^{3}H]epibatidine, and K_d^{ligand} is the dissociation constant for [^{3}H]MLA (1.86 nM for the hα7 nAChR [26]), [^{3}H]cytisine (0.1 nM for the hα4β4 nAChR [27], 0.3 nM for the hα4β2 nAChR [28] and 0.45 μM for the desensitized *Torpedo* nAChR [22]), and [^{3}H]epibatidine (89 pM for the hα3β4 AChR [29]). The calculated K_i values were summarized in Table 2.

2.4. Homology models of the hα3β4, hα4β4, hα4β2, and hα7 nAChRs

The crystal structure of the acetylcholine binding protein (AChBP) (PDB 4AFT) [30] was used as a template for the extracellular domain of the human (h)α3β4, hα4β4, hα4β2, and hα7 nAChRs, whereas the *Torpedo* nAChR model (PDB 2BG9) [31] was used as a template for the transmembrane domains (TMD). Water molecules were added to the model according to the AChBP-cytisine structure (PDB 4AFO) [30]. The amino acid sequence of each nAChR subunit (i.e., hα7, hα3, hα4, hβ2, and hβ4) was first aligned with corresponding sequences of the AChBP and *Torpedo* nAChR subunits by using the ClustalW2 server (www.ebi.ac.uk/Tools/msa/clustalw2) [32]. A hundred homology models for each nAChR subtype were generated using Modeller v.9.9 [33], and subsequently assessed by Modeller objective function and Discrete Optimized Protein Energy profiles [34]. The best model of each nAChR subtype was subjected to quality assessments using the Molecular Environment module for Ramachandran plots (http://www.chemcomp.com/software.htm) and the web-based tools of Annolea [35], Verify3D [36], and ProCheck [37].

2.5. Molecular docking

Docking simulations were performed using the same protocol as reported previously [19]. In addition, water molecules were incorporated within the binding pockets. The crystal structure of varenicline, transferred from its crystal model with AChBP, was used for the subsequent step of molecular docking. Molegro Virtual Docker (MVD v 5.0.0,