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Point-to-point ligand–receptor interactions across the subunit interface modulate the induction and stabilization of conformational states of alpha7 nAChR by benzylidene anabaseines☆

Matthew D. Isaacson [a,1], Nicole A. Horenstein [b], Clare Stokes [a], William R. Kem [a], Roger L. Papke [a,*]

[a] Department of Pharmacology and Therapeutics, University of Florida College of Medicine, P.O. Box 100267, Gainesville, FL 32610, United States
[b] Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, FL 32611, United States

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ABSTRACT

The homomeric α7 nicotinic acetylcholine receptor is a well-studied therapeutic target, though its characteristically rapid desensitization complicates the development of drugs with specific agonist effects. Moreover, some experimental compounds such as GTS-21 (2,4diMeOBA), a derivative of the α7-selective partial agonist benzylidene anabaseine (BA), produce a prolonged residual desensitization (RD) in which the receptor remains non-activatable long after the drug has been removed from extracellular solution. In contrast, the desensitization caused by GTS-21's dihydroxy metabolite (2,4diOHBA) is relatively short-lived. RD is hypothetically due to stable binding of the ligand to the receptor in its desensitized state. We can attribute the reduction in RD to a single BA hydroxyl group on the 4′ benzylidene position. Computational prediction derived from homology modeling showed the serine36 (S36) residue of α7 as a reasonable candidate for point-to-point interaction between BA compounds and the receptor. Through evaluating the activity of BA and simple derivatives on wild-type and mutant α7 receptors, it was observed that the drug–receptor pairs which were capable of hydrogen bonding at residue 36 exhibited significantly less stable desensitization. Further experiments involving the type II positive allosteric modulator (PAM) PNU-120596 showed that the various BA compounds' preference to induce either a PAM-sensitive (D_s) or PAM-insensitive (D_i) desensitized state is concentration dependent and suggested that both states are destabilized by S36 H-bonding. These results indicate that the fine-tuning of agonists for specific interaction with S36 can facilitate the development of therapeutics with targeted effects on ion channel desensitization properties and conformational state stability.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are transmembrane cation-selective ligand-gated ion channels, belonging to the large cysteine-loop superfamily of receptors, which include GABA, glycine, and serotonin-gated channels [1]. All nAChRs are pentameric in structure, comprised of protein subunits which form a ring around a central, water-filled pore. Seventeen unique subunits (α1–10, β1–4, γ, δ, and ε) have been identified, and most nAChRs are heteromeric, comprising both α and non-α subunits [2]. However, one of the most prevalent subtypes of neuronal nAChRs is homomeric, composed of five putatively identical α7 subunits. In the brain, such α7 receptors are found most abundantly in the hippocampus, thalamus, hypothalamus, and neocortex [3,4]. The α7 receptor is distinguished by high calcium permeability, low probability of opening, and rapid desensitization following ligand binding. The α7 receptor has been implicated as influential in neuroprotection [5–8], attentional and cognitive enhancement [9], and the regulation of inflammatory signaling [10–12], demonstrating its widespread influence and significance as a therapeutic target.

The α7 nAChR contains five potential ligand-binding domains (LBD) at the subunit interfaces, and at least a few structurally distinct allosteric sites [13]. In the absence of agonist, the channel exists in a resting "closed" state, preventing the flow of ions

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Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; BA, benzylidene anabaseine; RD, residual desensitization; Ds, PAM-sensitive desensitization; Di, PAM-insensitive desensitization; PAM, positive allosteric modulator; LBD, ligand-binding domain; CRC, concentration–response curve; 2,4diMeOBA (GTS-21), 3-(2,4-dimethoxybenzylidene)anabaseine; 2,4diOHBA, 3-(2,4-dihydroxybenzylidene)anabaseine; PNU-120596, N-(5-Chloro-2,4-dimethoxyphenyl)-N′-(5-methyl-3-isoxazolyl)-urea; 4OHBA, (E)-3-(4-hydroxybenzylidene)anabaseine; 4MeBA, 3-(4-methylbenzylidene)anabaseine.

* Corresponding author. Tel.: +1 352 392 4712; fax: +1 352 392 9696.
E-mail addresses: misaacson01@gmail.com (M.D. Isaacson), horen@chem.ufl.edu (N.A. Horenstein), clear@ufl.edu (C. Stokes), wrkem@ufl.edu (W.R. Kem), rlpapke@ufl.edu (R.L. Papke).
[1] Present address: 49 Convent dr. bldg. 49 rm. 1a16, Bethesda, MD 20814, United States.

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through its pore. Binding of agonist can temporarily promote conversion of receptors to a very short-lived, cation-permeable "open" conformational state or, more likely, to ligand-bound, closed states. These "desensitized" states are relatively stable as long as agonist is bound, preventing the receptor from any further activation. Upon complete drug washout from a population of desensitized receptors, some agonists such as acetylcholine will rapidly dissociate from the receptors, allowing them to return to the resting activatable state. Other agonists, such as nicotine or GTS-21, are capable of producing a form of desensitization from which the receptor takes significantly longer to recover [14]. This form of desensitization was first termed "residual inhibition or desensitization", since it was unclear whether the unresponsive state was due to the induction of ligand-bound nonconducting (desensitized) conformations or due to channel block. It was subsequently shown that although GTS-21 and nicotine could block the channel, such channel block was readily reversible, while the desensitization induced by these ligands was relatively stable [14]; therefore, we more accurately refer to the phenomenon simply as residual desensitization (RD).

Two distinct classes of desensitized conformational states have been identified for α7, referred to as the D_s and D_i states, distinguished by being sensitive or insensitive to conversion to open states by a type II [15] positive allosteric modulator (PAM), respectively [16]. RD may be associated with both D_s and D_i states, depending on the relative stabilities of these states, as determined by the chemical structure of the agonist and its interaction with the receptor binding pocket [17], as well as by the levels of PAM and agonist binding site occupancy [16]. The preferential induction of either D_s or D_i states may be an important consideration in the development of nAChR therapeutics, since the relative induction of these states would have significantly different effects when therapeutically combined with a type II PAM, and they also may be involved differently in intracellular signaling [16].

Benzylidene anabaseines (BAs) are one of several classes of α7-selective agonists [18], many of whose derivatives have been studied and observed to vary widely in potency and efficacy for activation [19]. Some BAs have therapeutic potential, such as 3-(2,4-dimethoxybenzylidene)anabaseine (alternatively called GTS-21, DMXBA, or 2,4diMeOBA), which has been shown to be an effective anti-inflammatory agent [11,20] and has been proposed as a potential treatment for Alzheimer's disease [21] and schizophrenia [22]. This particular compound was shown to cause significant amounts of RD, while a dihydroxy analog (a GTS-21 metabolite in vivo), 3-(2,4-dihydroxybenzylidene)-anabaseine (2,4diOHBA), produces practically none [14]. Such significant differences in RD production resulting from minor differences in the agonist molecule may give clues as to the fundamental mechanism of desensitization. The purpose of this project was to investigate the mechanism of RD production or elimination, so that its modulation may be harnessed for the design of novel therapeutic compounds.

2. Materials and methods

2.1. Reagents and synthetic chemistry

Solvents and reagents were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), Fisher scientific (Pittsburgh, PA) and TCI America (Portland, OR). PNU-120596 was synthesized following the method previously described [16,23]. Benzylidene anabaseine compounds were synthesized by the reaction of anabaseine dihydrochloride with the appropriate benzaldehyde, as previously reported [24]. The purity of each BA dihydrochloride salt was verified by silica gel HPLC, NMR, mass spectral, and elemental analyses. NMR analysis indicated that the vinyl group in each compound was in the [E]configuration, as was found for other BAs [25]. The basicity, polarity, and rat nAChR binding properties of these compounds have been reported [26].

2.2. Molecular modeling

A human α7 analog was created using the *Aplysia californica* AChBP structure (PDB ID 2WN9) as the template. The α7 sequence was modeled with SwissModel (http://swissmodel.expasy.org/). The resulting monomeric model was superimposed five times on each chain of the AChBP pentameric crystal structure in order to generate a pentameric model. The model was then examined for clashes, which were subsequently resolved by variation of side chain rotomers or in combination with an initial constrained minimization using the GROMOS force field resident in the SwissPDB viewer 4.0, followed by Amber 10 [27] molecular mechanics refinement with the bound 2WN9 ligand included. The model quality was assessed with the Molprobity server [28]. Docking was performed with Dock 6.5 [29].

2.3. cDNA clones and RNA

The human α7 nAChR receptor clone was obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA), and the RIC-3 clone from Dr. Millet Treinin (Hebrew University, Jerusalem, Israel) for the purpose of co-injection with α7 to improve the level and speed of receptor expression [30]. Mutations at position 36 were introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. Mutations were confirmed with automated fluorescent sequencing. After linearization and purification of cloned cDNA's, RNA transcripts were prepared using the appropriate mMessage mMachine kit from Ambion (Austin, TX).

2.4. Expression in X. laevis oocytes

Oocytes were obtained from mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson, WI). Frogs were anesthetized in 0.7 g/L solution of ethyl 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate, followed by surgical removal of oocytes through an abdominal incision. Harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES, 12 mg/L tetracycline, pH 7.6) for 3–4 h to remove the follicular layer. Stage-5 oocytes were isolated and injected with 50 nl (5–20 ng) of wild type (WT) or mutant α7 and RIC-3 cRNA. Suitable levels of receptor expression were typically achieved 2–6 days after injection of cRNA. For experiments involving the PAM PNU-120596, where standard levels of expression result in ion currents too large to be recorded in voltage clamp, experiments were typically conducted 1–3 days after RNA injection.

2.5. Electrophysiology

Two-electrode voltage clamp experiments were conducted using OpusXpress6000A (Molecular Devices, Sunnyvale, CA), an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the current and voltage electrodes were filled with 3 M KCl and oocytes were clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 1.8 mM $CaCl_2$, pH 7.3), and agonist solutions were delivered from a 96-well drug plate using disposable tips. Flow rates were set at 2 ml/min, with each drug or control application delivered in 12 s