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Intra-subunit flexibility underlies activation and allosteric modulation of neuronal nicotinic acetylcholine receptors

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ABSTRACT

Allosteric modulation is a general feature of nicotinic acetylcholine receptors, yet the structural components and movements important for conversions among functional states are not well understood. In this study, we examine the communication between the binding sites for agonist and the modulator morantel (Mor) of neuronal α3β2 receptors, measuring evoked currents of receptors expressed in *Xenopus* oocytes with the two-electrode voltage-clamp method. We hypothesized that movement along an interface of β sheets connecting the agonist and modulator sites is necessary for allosteric modulation. To address this, we created pairs of substituted cysteines that span the cleft formed where the outer β sheet meets the β sheet constituting the (−)-face of the α3 subunit; the three pairs were L158C-A179C, L158C-G181C and L158C-K183C. Employing a disulfide trapping approach in which bonds are formed between neighboring cysteines under oxidation conditions, we found that oxidation treatments decreased the amplitude of currents evoked by either the agonist (ACh) or co-applied agonist and modulator (ACh + Mor), by as much as 51%, consistent with the introduced bond decreasing channel efficacy. Reduction treatment increased evoked currents up to 89%. The magnitude of the oxidation effects depended on whether agonists were present during oxidation and on the cysteine pair. Additionally, the cysteine mutations themselves decreased Mor potentiation, implicating these residues in modulation. Our findings suggest that these β sheets in the α3 subunit move with respect to each other during activation and modulation, and the residues studied highlight the contribution of this intramolecular allosteric pathway to receptor function.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are widely distributed throughout the central and peripheral nervous systems and are implicated in a range of normal and pathological functions (Albuquerque et al., 2009; Hurst et al., 2013). Neuronal nAChRs are implicated in memory loss and diminished cognitive ability associated with Alzheimer's disease and other dementias (Haydar and Dunlop, 2010), and mediate nicotine addiction (Ortells and Arias, 2010). Although the molecular and physiological mechanisms of these disorders are not completely understood (Parri et al., 2011; Picciotto and Kenny, 2013), the known nicotinic ligands rivastigmine (e.g., Grossberg et al., 2010) and varenicline (e.g., Mills et al., 2012), for example, are currently in clinical use. Recently, interest has grown in allosteric modulators of nAChRs as possible therapeutic targets (e.g., Maelicke and Albuquerque, 2000; Williams et al., 2011).

nAChRs are pentameric, membrane-bound ligand-gated ion channels that are part of the Cys-loop superfamily (Hurst et al., 2013). Binding sites for agonists and competitive antagonists are located at subunit interfaces. Due to the radial asymmetry of subunits (Brejc et al., 2001), these sites have sidedness, with α subunits contributing (+)-face residues to the canonical site, and the (−)-face residues coming from a diverse set of neighboring subunits, depending on whether the receptor is homo- or heteromeric. Based on several crystal structures determined for the muscle-type nAChR (Unwin, 2005), bacterial homologs (Bocquet et al., 2009; Hilf and Dutzler, 2008) and the extracellular domain and homologs thereof (e.g., Brejc et al., 2001; Dellisanti et al., 2007; Hansen et al., 2005; Li et al., 2011), the general structure of nAChRs and related Cys-loop proteins is well-known. However, the diversity among ion channel subunit genes, receptor stoichiometries and subunit arrangements means that homology models are of limited use at the

Abbreviations: nAChR, nicotinic acetylcholine receptor; Mor, morantel; MTS, methanesthiosulfonate; TCEP, tris(2-carboxyethyl)phosphine; $GABA_A$, γ-aminobutyric acid A (receptor); OR2, oocyte Ringer's.

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atomic scale for specific subtype residues (Hurst et al., 2013). Understanding how individual residues contribute to ligand binding and receptor movement at this scale is critical for rational drug design.

In a similar way, homology-based structural models give little information about the conformational changes that are the hallmark of receptor function. Unwin (2005) compared closed and putatively open forms of the muscle-type nAChR to reveal the major structural changes in the full receptor. These changes have been substantiated and refined by the comparison of the ELIC (Hilf and Dutzler, 2008) and GLIC structures (Bocquet et al., 2009; Hilf and Dutzler, 2009), bacterial homologs of the Cys-loop receptors, which are thought to correspond to closed and open forms, respectively. Many studies, using a wide range of techniques, have indicated that the C loop, a component of the canonical binding site, moves to "cap" the agonist (e.g., Hansen et al., 2005; Mukhtasimova et al., 2009; Wang et al., 2009). However, most of this work has employed the acetylcholine binding protein, a homolog of just the nAChR extracellular domain, meaning that the picture of movement for this region of the full receptor is incomplete. Disulfide trapping in receptors with substituted cysteines has been useful to deduce regional motions in both $GABA_A$ receptors (e.g., Horenstein et al., 2001; Venkatachalan and Czajkowski, 2008) and nAChRs (e.g., Mukhtamisova and Sine, 2007). On the whole, a major focus in this body of work has been to understand the allosteric communication between agonist binding sites and the channel pore (e.g., Lee and Sine, 2005; Purohit and Auerbach, 2007). Thus, while progress has been made elucidating the nature of movement in Cys-loop receptors and their intra-molecular pathways of communication, much remains to be discovered.

A related interest is the communication of allosteric modulator sites with agonist sites and the channel gate. We have demonstrated that the anthelmintics morantel (Mor) and oxantel potentiate α3β2 nAChRs from a site in the β2(+)/α3(−) interface, which is structurally homologous to the canonical agonist site (Cesa et al., 2012; Seo et al., 2009). Several small-molecule modulators, both positive and negative, specific for the α4β2 subtype have been discovered (reviewed in Pandya and Yakel, 2011), but for only a few of these have the binding sites been identified. For certain ligands of α7 nAChRs such as galanthamine and anthelmintics, evaluating the interactions of modulator and agonist sites is more complicated because each occurs at the same type of α7(+)/α7(−) interface (Bartos et al., 2006; Ludwig et al., 2010; Rayes et al., 2009). In addition, two groups have recently determined that an α4(+)/α4(−) site in the α4β2 receptors binds agonist, dictating the overall receptor sensitivity (Harpsøe et al., 2011; Mazzaferro et al., 2011). The emerging picture from these studies is the generality of the nAChR interfaces as potential ligand binding sites, which might have been predicted from the symmetry of the system and allostery theory (Wyman and Gill, 1990).

Given the spatial relationship of the Mor and ACh sites at the (−) and (+) interfaces, respectively, of the α3 subunit (Fig. 1), we hypothesize that a subset of α3 residues mediates communication between these two sites. The β sandwich structure of Cys-loop receptor extracellular domains suggests intra-subunit movement might occur where the β sheets comprised of the β9-10-7 strands and β1-2-6-5/5′ strands meet. Using cysteines substituted into this region for a disulfide trapping approach, we investigated the role of these residues in channel activation, aiming to find evidence of movement in this region by discriminating among resting and active states of the system. We demonstrate that disulfide bonds formed here perturb both ACh activation and Mor modulation. Thus, the residues we studied appear to be involved in α3 intra-subunit movement that may functionally connect agonist and modulator sites.

2. Materials and methods

2.1. Reagents

All chemicals used, unless otherwise noted, were reagent grade and obtained from Sigma (St. Louis, MO). Morantel (Mor) is 1,4,5,6-tetrahydro-1-methyl-2-(2-[3-methyl-2-thienyl]ethenyl)pyrimidine, tartrate salt. MTS-dansyl (dansylamidoethyl methanesthiosulfonate) was obtained from Toronto Research Chemicals (Toronto, ON, Canada); some experiments also employed MTSET (2-(trimethylammonium) ethyl methanethiosulfonate) and MTS-biotin, also from TRC.

2.2. Nicotinic receptor clones and mutagenesis

Wild type rat α3 and β2 subunits in pGEMHE-based vectors were a gift from Dr. Charles Luetje (University of Miami); clones were originally isolated in the lab of Dr. Jim Patrick (Baylor University; Boulter et al., 1987). All mutant subunits were custom synthesized by GenScript (Piscataway, NJ), except α3G181C which was prepared in our laboratory using standard thermocycling methods (e.g., Seo et al., 2009). Mutations were verified by sequencing of the entire extracellular domain using capillary electrophoresis of dye-detected, dideoxy-generated fragments. Unless otherwise noted all α3 and β2 residue numbering corresponds to that in the structure a3b2rr.pdb (http://www.ebi.ac.uk/compneur-srv/LGICdb/HTML/a3b2rr.html) (Sallette et al., 2004); these position numbers are smaller by two compared with numbering used elsewhere in the literature, a discrepancy which arises due to homology modeling based on a crystal structure of a protein of different sequence. The cDNA plasmids were linearized with a unique restriction enzyme, and then made RNase-free by phenol-chloroform extraction. cRNAs were transcribed in vitro from these using the T7 kit from Ambion (Life Technologies; Carlsbad, CA), and were diluted to 0.5 μg/μL with RNase-free water and stored at −20 °C.

2.3. Oocyte preparation and injection

Xenopus laevis oocytes were harvested from oocyte-positive frogs (obtained from Nasco, Ft. Atkinson, WI), using procedures approved by the Grinnell College Institutional Animal Care and Use Committee and in accord with National Institutes of Health guidelines; all efforts to minimize the number of animals used and minimize suffering were made. On some occasions oocytes were prepared from whole ovary tissue obtained directly from Nasco. In both cases stage V and VI oocytes were prepared by collagenase treatment and manual selection, following published procedures (Bertrand et al., 1991). Additionally, several experiments used pre-sorted stage VI oocytes obtained from Ecocyte Bioscience (Austin, TX). Oocytes were maintained at 16 °C in Barth's medium: 88 mM NaCl, 1.0 mM KCl, 2.5 mM $NaHCO_3$, 0.30 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 2.5 mM sodium pyruvate, pH 7.6, supplemented with 100 U/mL penicillin/streptomycin and 50 μg/mL gentamicin (Life Technologies; Carlsbad, CA). A Nanoject microinjector (Drummond; Broomall, PA) was used to inject each oocyte with 46 nL of a 1:1 (v:v) combination of the desired α and β subunits, prepared from the respective 0.5 μg/μL stock solutions. Following 2–3 days for protein expression, current responses could be recorded for up to 7 subsequent days. During this time the Barth's solution was changed daily and any dead cells removed.

2.4. Voltage-Clamp recordings

Macroscopic currents were recorded using a Geneclamp 500B amplifier and a Digidata 1322A data acquisition system (Molecular Devices; Sunnyvale, CA) using the two-electrode voltage-clamp method, as previously described (Cesa et al., 2012; Seo et al., 2009; Wu et al., 2008). Voltage was clamped at −60 mV, and leak currents were generally 0–200 nA, although in some cases higher leak currents were tolerated if the baseline was stable. Recording electrodes were filled with 3.0 M KCl and selected for resistances between 0.5 and 4.0 MΩ. Perfusion and drug administration were controlled via VC-6 solenoid valve systems (Warner Instruments; Hamden, CT). Cells were perfused with oocyte Ringer's medium (OR2; 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3) until the baseline stabilized prior to recording, typically 90–120 s. Drug challenges lasted 5 s unless otherwise noted, and the oocytes were typically washed with OR2 for 100 s between challenges, sufficient time for the current to return to baseline.

In experiments involving oxidation or reduction (throughout referred to as *treatments*) of the putative disulfide bonds, oxidation was typically accomplished via perfusion for 5 min with a solution that was 4.4 mM H_2O_2, 100 μM ACh and 10 μM Mor. The solution was prepared fresh before every second or third cell from a refrigerated 30% (w/v) H_2O_2 stock. Reduction was typically accomplished via perfusion for 5 min with a 40 μM DTT solution, which was prepared fresh every second or third cell from frozen stock. Following treatment with either H_2O_2 or DTT, oocytes were washed for 100 s with OR2 before any challenges (5-s application of ACh or ACh + Mor) were made. As an emphasis of this study, we altered the nature of the treatments, the conditions of which are given in the figure legends and text. Typically two challenges were administered before and after a given treatment, with deviations from these regular conditions noted where appropriate. We refer to the responses prior to the treatment as $I_{control}$, and those after H_2O_2 (which may contain ACh and/or Mor) and DTT treatments as $I_{oxidation}$ and $I_{reduction}$, respectively. For