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A chimeric prokaryotic-eukaryotic pentameric ligand gated ion channel reveals interactions between the extracellular and transmembrane domains shape neurosteroid modulation

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ABSTRACT

Pentameric ligand-gated ion channels (pLGICs) are the targets of several clinical and endogenous allosteric modulators including anesthetics and neurosteroids. Molecular mechanisms underlying allosteric drug modulation are poorly understood. Here, we constructed a chimeric pLGIC by fusing the extracellular domain (ECD) of the proton-activated, cation-selective bacterial channel GLIC to the transmembrane domain (TMD) of the human ρ1 chloride-selective $GABA_AR$, and tested the hypothesis that drug actions are regulated locally in the domain that houses its binding site. The chimeric channels were proton-gated and chloride-selective demonstrating the GLIC ECD was functionally coupled to the GABAρ TMD. Channels were blocked by picrotoxin and inhibited by pentobarbital, etomidate and propofol. The point mutation, ρ TMD W328M, conferred positive modulation and direct gating by pentobarbital. The data suggest that the structural machinery mediating general anesthetic modulation resides in the TMD. Proton-activation and neurosteroid modulation of the GLIC-ρ chimeric channels, however, did not simply mimic their respective actions on GLIC and GABAρ revealing that across domain interactions between the ECD and TMD play important roles in determining their actions. Proton-induced current responses were biphasic suggesting that the chimeric channels contain an additional proton sensor. Neurosteroid modulation of the GLIC-ρ chimeric channels by the stereoisomers, 5α-THDOC and 5β-THDOC, were swapped compared to their actions on GABAρ indicating that positive versus negative neurosteroid modulation is not encoded solely in the TMD nor by neurosteroid isomer structure but is dependent on specific interdomain connections between the ECD and TMD. Our data reveal a new mechanism for shaping neurosteroid modulation.

1. Introduction

Many clinically important drugs such as anesthetics, barbiturates and neurosteroids exert their CNS effects by binding to gamma-aminobutyric acid type-A receptors ($GABA_ARs$). These drugs bind to distinct sites located far from the orthosteric GABA binding sites, and allosterically modulate $GABA_AR$ function (Forman and Miller, 2011; Miller and Smart, 2010). The structural mechanisms by which these different classes of drugs either enhance or inhibit GABA-activated currents remain poorly understood and represent a major challenge in developing novel therapeutics that target $GABA_ARs$.

$GABA_ARs$ are members of the pentameric ligand gated ion channel superfamily, which include nicotinic acetylcholine receptors (nAChR), serotonin type 3 receptors ($5HT_3R$) and glycine receptors (GlyR). Pentameric ligand-gated ion channels (pLGICs) mediate fast synaptic neurotransmission, and signaling in the brain depends on their activity. For these receptors, neurotransmitter binding promotes opening of an integral membrane-spanning ion channel, which allows ions to flow across the membrane and change the cell's activity (Miller and Smart, 2010). In the last

Abbreviations: pLGIC, pentameric ligand-gated ion channel; $GABA_AR$, gamma amino butyric acid type A receptor; GLIC, *Gloeobacter* ligand-gated ion channel; ECD, extracellular domain; TMD, transmembrane domain; PB, pentobarbital; THDOC, Tetrahydrodeoxycorticosterone.

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decade, prokaryotic pLGIC homologs GLIC (*Gloeobacter* ligand-gated ion channel) (Bocquet et al., 2007, 2009; Hilf and Dutzler, 2009) and ELIC (*Erwinia* ligand-gated ion channel) (Hilf and Dutzler, 2008) have been identified.

With the goal of dissecting molecular mechanisms underlying how different classes of allosteric drugs modulate $GABA_AR$ function, we constructed a chimeric pLGIC by fusing the extracellular domain (ECD) of the prokaryotic proton-gated ion channel GLIC with the transmembrane domain (TMD) of $GABA_AR$ ρ subunit. We examined how anesthetics, barbiturates and neurosteroids modulate chimeric channel function and tested the hypothesis that drug actions are regulated locally in the domain that houses its binding site.

Based on high-resolution structures of prokaryotic (Bocquet et al., 2009; Cecchini and Changeux, 2015; Hilf and Dutzler, 2008; Sauguet et al., 2014) and eukaryotic pLGICs (Althoff et al., 2014; Du et al., 2015; Hassaine et al., 2014; Hibbs and Gouaux, 2011; Miller and Aricescu, 2014), pLGICs have a modular architecture. The N-terminal extracellular domain (ECD) consists mostly of beta sheets and houses the neurotransmitter binding site (Brejc et al., 2001; Miller and Smart, 2010). The transmembrane domain (TMD) consists of alpha helices that span the lipid bilayer, and contains the ion-conducting channel as well as the binding sites for various drugs including anesthetics, barbiturates and neurosteroids (Baenziger and Corringer, 2011; Cecchini and Changeux, 2015; Du et al., 2015; Fourati et al., 2017; Hibbs and Gouaux, 2011; Nemecz et al., 2016; Nury et al., 2011; Spurny et al., 2013). At the ECD-TMD interface, connections between flexible loops in the extracellular binding domain (loops 2, 7, 9) with the transmembrane channel domain (M2-M3 loop) structurally link the two domains and are essential for coupling ligand binding to channel gating (Miller and Smart, 2010). Agonist-mediated closed to open channel gating transitions are accompanied by substantial rearrangements of this interface (Bertozzi et al., 2016; Dellisanti et al., 2013; Gupta et al., 2017; Lee and Sine, 2005; Velisetty et al., 2014; Xiu et al., 2005). In chimeric channels assembled by combining the ECD and TMD of two distinct pLGICs, substantial loop substitutions are required to maintain complementarity and ensure normal channel function (Bouzat et al., 2004, 2008; Eisele et al., 1993).

In this study, we were interested in determining whether allosteric drug modulators, especially those that bind to the TMD, rely on the ECD-TMD interface for coupling their binding to modulation of channel activity. Previous studies using chimeric pLGICs, constructed from different eukaryotic ECDs and TMDs (Eisele et al., 1993; Mihic et al., 1997; Serafini et al., 2000) as well as prokaryotic-eukaryotic (Duret et al., 2011; Moraga-Cid et al., 2015) and prokaryotic-prokaryotic (Alqazzaz et al., 2017) domains, have shown that the pharmacological and functional properties of each domain are retained suggesting that a drug's actions on channel activity are regulated locally in the domain that houses its binding site. However, in an ELIC(ECD)-nAChR(TMD) chimera, only when the ECD-TMD interfacial loops were identical to those of nAChR did nAChR-specific drugs modulate chimeric currents (Tillman et al., 2014), suggesting that across-domain interactions may play important roles in mediating the actions of some drugs.

Crystal structures of prokaryotic pLGICs homologs, GLIC (*Gloeobacter* ligand-gated ion channel) and ELIC (*Erwinia* ligand-gated ion channel), in different conformational states and in the presence of various therapeutic drugs (Bocquet et al., 2009; Fourati et al., 2017; Hilf and Dutzler, 2008, 2009; Nury et al., 2011; Pan et al., 2012a, 2012b; Spurny et al., 2012) have been solved making them attractive models to study pLGIC structure and function (Sauguet et al., 2015). However, common $GABA_AR$ ligands bind with low affinity and have modest effects on GLIC and ELIC (Alqazzaz et al., 2011; Chen et al., 2010; Thompson et al., 2012; Weng et al., 2010). Recently, Moraga-Cid et al. showed that the chimera approach can be used to great advantage to study the structural properties of the glycine receptor, and understand its physiological role in hyperexplexia (Moraga-Cid et al., 2015). Here, we report the construction and characterization of a chimeric pLGIC consisting of the ECD from the proton-activated bacterial channel GLIC and the TMD from the chloride-selective $GABA_AR$ ρ subunit. As expected, this chimeric subunit formed functional Cl^- conducting, proton-gated channels demonstrating that the ECD of GLIC was functionally coupled to the $GABA_AR$ TMD. However, proton-activation and neurosteroid modulation of the chimeric GLIC-ρ receptor did not simply mimic their respective actions on GLIC and $GABA_AR$ ρ revealing that across domain interactions between the ECD and TMD play important roles in determining a ligand's actions.

2. Methods

2.1. Generation of chimeric receptors

GLIC-GABAρI chimeric subunits were constructed by fusing the ECD of GLIC ending at pre-M1 R191 with the human $GABA_A$ receptor ρ subunit TMD beginning at H259 (Fig. 1). GLIC was previously cloned into the pUNIV expression vector (Ghosh et al., 2013; Laha et al., 2013; Venkatachalan et al., 2007). To remove the TMD of GLIC, a unique enzyme restriction site *Xma*I was introduced after Arg191 in pUNIV GLIC by site directed mutagenesis (Quickchange, Strategene). A *Mlu*I restriction site was already present at the 3′ end of GLIC. GABAρ TMD cDNA was PCR amplified between Arg 258 and the C-terminus of the human GABAρ1 subunit using primers with overhanging ends containing *Xma*I and *Mlu*I restriction sites at the 5′ and 3'end, respectively. pUNIV GLIC vector and the amplified GABAρ TMD were digested with the *Xma*I and *Mlu*I enzymes and then ligated overnight using T4 DNA Ligase (Promega). After transformation into *E. coli,* positive colonies containing GLIC-ρ were identified by colony PCR. The resulting GLIC-ρ chimeric construct was then mutated to remove the introduced restriction site in GLIC ECD and restore wild-type GLIC coding sequence upstream of the chimeric junction. This construct was called GLIC-ρ1 (Fig. 1 A).

To remove the 78 residue GABAρ M3-M4 loop from GLIC-ρI and replace it with the tri-peptide (SQP) sequence of GLIC M3-M4 loop, *Age*I and *Sac*II restriction sites were introduced at the C-terminal end of M3 and N-terminal end of M4 respectively in pUNIV-GLIC-ρI. A double-stranded oligonucleotide, encoding the tri-peptide M3-M4 loop of GLIC, was custom synthesized with *Age*I and *Sac*II restriction sites on its 5′ and 3' end, respectively. The oligonucleotide insert and the pUNIV-GLIC-ρI vector with the introduced restriction sites were double digested with *Age*I and *Sac*II restriction enzymes. The insert was ligated to the vector overnight to obtain a chimeric construct with the tri-peptide M3-M4 loop. This construct was called GLIC-ρII (Fig. 1 B). The chimeric construct, GLIC-ρIII, was made by introducing a point mutation, W328M (ρ numbering) in the M3 helix of GLIC-ρII using site-directed mutagenesis (Fig. 1 C). All of the constructs were verified by double stranded DNA sequencing.

2.2. GLIC-ρ electrophysiology

GLIC, GABAρ, GLIC-ρI, II and III chimeric ion channels were expressed in *Xenopus laevis* oocytes and functionally characterized using two-electrode voltage clamp electrophysiology. Heterologous expression of channel proteins in *Xenopus laevis* oocytes is a well-established and widely used approach for measuring drugs effects on ion channel function. The large size of the oocytes, their ability to express large numbers of channel proteins and the relative absence of endogenous channels that might complicate