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$GABA_A$ receptor open-state conformation determines non-competitive antagonist binding

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ABSTRACT

The γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) is one of the most important targets for insecticide action. The human recombinant β3 homomer is the best available model for this binding site and 4-*n*-[^{3}H]propyl-4′-ethynylbicycloorthobenzoate ([^{3}H]EBOB) is the preferred non-competitive antagonist (NCA) radioligand. The uniquely high sensitivity of the β3 homomer relative to the much-less-active but structurally very-similar β1 homomer provides an ideal comparison to elucidate structural and functional features important for NCA binding. The β1 and β3 subunits were compared using chimeragenesis and mutagenesis and various combinations with the α1 subunit and modulators. Chimera β3/β1 with the β3 subunit extracellular domain and the β1 subunit transmembrane helices retained the high [^{3}H]EBOB binding level of the β3 homomer while chimera β1/β3 with the β1 subunit extracellular domain and the β3 subunit transmembrane helices had low binding activity similar to the β1 homomer. GABA at 3 μM stimulated heteromers α1β1 and α1β3 binding levels more than 2-fold by increasing the open probability of the channel. Addition of the α1 subunit rescued the inactive β1/β3 chimera close to wildtype α1β1 activity. EBOB binding was significantly altered by mutations β1S15′N and β3N15′S compared with wildtype β1 and β3, respectively. However, the binding activity of α1β1S15′N was insensitive to GABA and α1β3N15′S was stimulated much less than wildtype α1β3 by GABA. The inhibitory effect of etomidate on NCA binding was reduced more than 5-fold by the mutation β3N15′S. Therefore, the NCA binding site is tightly regulated by the open-state conformation that largely determines $GABA_A$ receptor sensitivity.

Introduction

The γ-aminobutyric acid (GABA) type A receptor ($GABA_AR$) is a major insecticide target along with the voltage-dependent sodium channel, the nicotinic receptor and acetylcholinesterase (Bloomquist, 1996; Casida and Quistad, 1998). Important insecticides acting at the $GABA_AR$ are lindane, α-endosulfan and fipronil. They bind at the picrotoxinin or non-competitive antagonist (NCA) site to block GABA-induced chloride flux. The safe and effective use of GABAergic insecticides requires detailed knowledge about the structural and functional basis of $GABA_AR$-NCA interactions. The NCA site is readily assayed with 4-*n*-[^{3}H]propyl-4′-ethynylbicycloorthobenzoate ([^{3}H]EBOB) as the radioligand (Casida, 1993; Ratra et al., 2001). Ten years ago in this journal we reported a significant step in establishing the toxicity mechanisms of these insecticides and EBOB by defining that they all bind with very high affinity to the NCA site of human recombinant $GABA_AR$ β3 homomer with a specificity approximating that of the similarly sensitive insect receptor (Ratra et al., 2001). These studies then localized the binding site of the insecticides and EBOB to A2′, T6′ and L9′ of the chloride channel (Chen et al., 2006a). The present investigation uses the human $GABA_AR$ β3 subunit as a homomer, heteromers and chimeras to define the unique structural and functional features of NCA action and interactions.

The $GABA_AR$ consists of 5 subunits arranged around a central ion-conducting pore and each of the subunits has a long extracellular domain and four transmembrane (TM) helices. There are 19 known human $GABA_AR$ subunits (α1–6, β1–4, γ1–3, δ, ε, π, ρ1–3) with sequence identity of about 30% between subunits and 70% between subunit subtypes (Olsen and Sieghart, 2008). Although the $GABA_AR$ is expressed in neurons as a heteromeric pentamer containing two or more different subunits, studies of homomeric receptors can reveal important structural determinants for assembly and ligand selectivity. When somatic cells are transfected with α_1, β_1, β_2, β_3, or γ_2 subunits, only β_3 and occasionally β_1 subunits are detected on the cell surface

Abbreviations: EV, empty vector; $GABA_AR$, γ-aminobutyric acid (GABA) type A receptor; GFP, green fluorescent protein; [^{3}H]EBOB, 4-*n*-[^{3}H]propyl-4′-ethynylbicycloorthobenzoate; NCA, non-competitive antagonist; TM, transmembrane; WT, wildtype.

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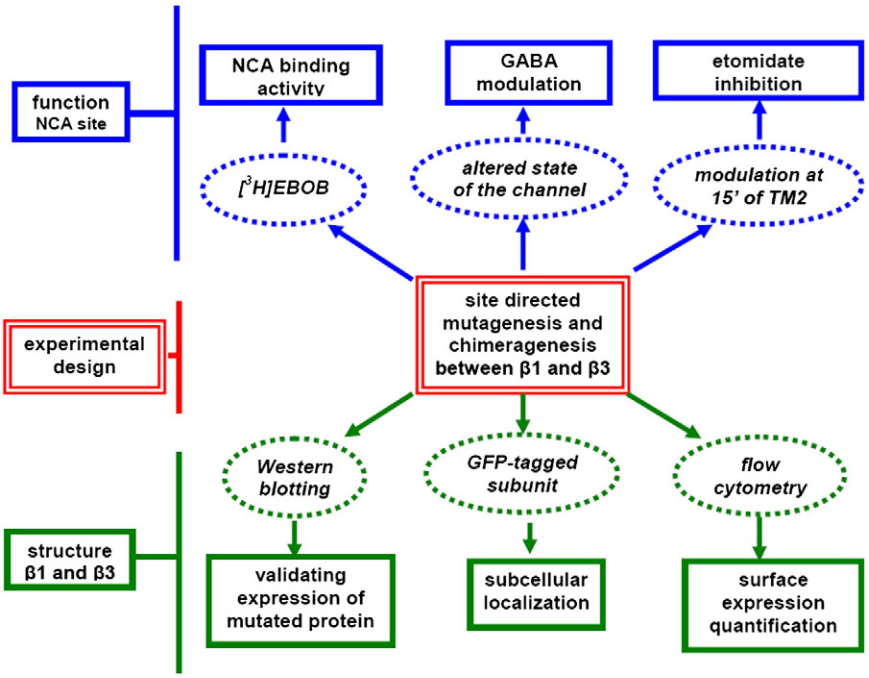

Fig. 1. Strategy and experimental design to define the mechanisms for differential surface expression ability and NCA binding activity between β1 and β3 subunits.

with high spontaneous holding current (Bracamontes and Steinbach, 2008; Connolly et al., 1996a,b; Serafini et al., 2000; Taylor et al., 1999). HEK cells transfected with β_3 subunits not only form dimers or tetramers but also significant amounts of homopentamers (Barnard et al., 1998). Critical residues in the extracellular domain of β3 are important for surface expression of homomers (Bracamontes and Steinbach, 2008; Sarto-Jackson and Sieghart, 2008; Taylor et al., 1999). However, the results often vary with the expression systems and species from which the subunit is obtained. The present study uses insect Sf9 cells as the expression system for human recombinant receptors (Chen et al., 2006a; Ratra et al., 2001).

The human $GABA_AR$ subunits have very different sensitivities to NCA binding. The β subunit is essential for NCA sensitivity in the recombinant multiple-subunit receptors (Ratra et al., 2001). The β_3 is the only single subunit highly sensitive to NCA binding at current knowledge (Chen et al., 2006a,b; Ratra et al., 2001). It is surprising that β1 and β2 are insensitive to NCAs (Ratra and Casida, 2001; Ratra et al., 2001). The β1 homomer has little or no binding activity unless expressed with other subunits (Ratra and Casida, 2001; Ratra et al., 2001). The β3 subunit assembles to form homomeric surface receptors in somatic cells, but human β1 subunits do not (Taylor et al., 1999). NCA binding studies have focused on TM2 of the $GABA_AR$ (Buhr et al., 2001; Chen et al., 2006a,b; Dibas et al., 2002; Jursky et al., 2000; Perret et al., 1999). There are high homologies between β subunits, particularly in TM2. The β3 and β2 subunits contain N15′, while the β1 contains S15′. This residue faces away from the ion channel pore and into a water-filled cavity that appears capable of accommodating drugs (Chen et al., 2006b; Miller and Smart, 2010). Mutation at 15′ of the β subunit is known to affect anesthetics and alcohol action to potentiate $GABA_A$ receptor-mediated electrical responses (Belelli et al., 1997; Hemmings et al., 2005; Jurd et al., 2002).

In this investigation, we employed the β3 homomer as a model to study the differential sensitivity conferred by β3 and β1 subunits using chimeragenesis and site-directed mutagenesis (Fig. 1). Two chimeras (β3/β1 and β1/β3) (Fig. 2A) were constructed to localize the important domains in the β1 and β3 subunits for surface expression of the $GABA_A$ receptor and formation of the NCA site. These chimeras allowed us to study whether lack of surface expression plays a role in the low binding activity of β1. Sequence alignment of the important NCA binding domain of TM2 revealed that the $GABA_AR$ across the subunits is highly conserved in this region (Fig. 2B). The only difference between β3 and β1 in TM2 is at positions N15′ and S15′, respectively. We therefore chose this position to mutate for exploring the binding activity and anesthetic modulation difference between β3 and β1. The agonist GABA regulates the $GABA_AR$ activity or the state of the ion channel which in turn potentially affects the receptor's NCA binding sensitivity. Finally, by co-expression with the α subunit in order to introduce the agonist binding site, we also consider the effect of GABA (modulating channel

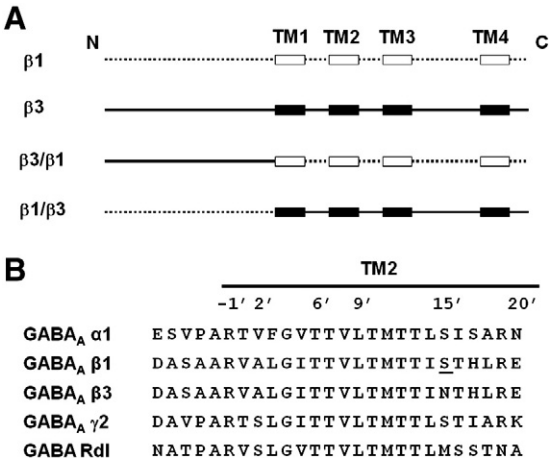

Fig. 2. (A) Design of chimera β3/β1 and chimera β1/β3 based on β1 and β3 predicted extracellular domain and TM helices. The extracellular domain and the loops between the TMs of the β1 subunit are drawn as a dotted line (▪▪▪▪) and the TM helices as open boxes (▭). The β3 subunit's extracellular domain and the loops between the TMs are drawn as a continuous black line (▬) and the TM helices as black boxes (■). (B) TM2 alignment of human GABA receptor subunits α1, β1, β3 and γ2 and *Drosophila* GABA receptor Rdl mutant (ffrench-Constant et al., 1993). The amino acid numbering system is based on Horenstein et al. (2001). The single amino acid difference in β1 from β3 at position 15′ is underlined and was the site for mutagenesis.