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Functional sites involved in modulation of the $GABA_A$ receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital

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ABSTRACT

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the brain and are the target for many clinically important drugs. Among the many modulatory compounds are also the intravenous anesthetics propofol and etomidate, and barbiturates. The mechanism of receptor modulation by these compounds is of mayor relevance. The site of action of these compounds has been located to subunit interfaces in the intra-membrane region of the receptor. In $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors there are five such interfaces, two $\beta+/\alpha-$ and one each of $\alpha+/\beta-$, $\alpha+/\gamma-$ and $\gamma+/\beta-$ subunit interfaces. We have used reporter mutations located in the second trans-membrane region in different subunits to probe the effects of changes at these subunit interfaces on modulation by propofol, etomidate and pentobarbital. We provide evidence for the fact that each of these compounds either modulates through a different set of subunit interfaces or through the same set of subunit interfaces to a different degree. As a $GABA_A$ receptor pentamer harbors two $\beta+/\alpha-$ subunit interfaces, we used concatenated receptors to dissect the contribution of individual interfaces and show that only one of these interfaces is important for receptor modulation by etomidate.

1. Introduction

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian central nervous system. Numerous subunits have been cloned (for review see: Barnard et al., 1998; Macdonald and Olsen, 1994; Olsen and Sieghart, 2008; Rabow et al., 1995; Sigel and Steinmann, 2012), indicating that numerous receptor isoforms exist (Olsen and Sieghart, 2008). The subunits show homology to subunits of other Cys-loop receptors (Betz, 1990; Miller and Smart, 2010). Many $GABA_A$ receptors are heteromeric protein complexes consisting of five subunits, which are arranged around a central Cl^--selective channel (Macdonald and Olsen, 1994). The major receptor isoform of the $GABA_A$ receptor in the brain consists of α_1, β_2 and γ_2 subunits (Barnard et al., 1998; Macdonald and Olsen, 1994; Olsen and Sieghart, 2008; Rabow et al., 1995; Sigel and Steinmann, 2012). Different approaches have indicated a $2\alpha:2\beta:1\gamma$ subunit stoichiometry for this receptor (Baumann et al., 2001; Chang et al., 1996; Farrar et al., 1999; Tretter et al., 1997) with a subunit arrangement $\gamma\beta\alpha\beta\alpha$ anti-clockwise as seen from the synaptic cleft (Baumann et al., 2001, 2002; Baur et al., 2006). The pharmacological properties of a receptor depend on subunit composition (Sigel et al., 1990) as well as their arrangement (Minier and Sigel, 2004). $GABA_A$ receptors do not exclusively locate to synapses. Some receptor subtypes, among them the ones containing the δ subunit, have been found in extra-synaptic regions where they mediate tonic inhibition (Farrant and Nusser, 2005).

The $GABA_A$ receptor is the target of many clinically used and experimental drugs (for review see Sieghart, 1995; Sieghart and Ernst, 2005; Middendorp et al., 2014), including benzodiazepines, barbiturates, volatile anesthetics, and the intravenous anesthetics propofol and etomidate. There is good evidence that the intravenous anesthetics act near the extracellular end of the membrane-spanning domain (M) of various subunits. Amino acid residues located in the non-channel lining face of the βM2, and in αM1, βM1, βM3 and γM3 have been proposed to form part of the binding site(s) (Bali and Akabas, 2004; Chiara et al., 2013; Jayakar et al., 2014; Richardson et al., 2007; Yip et al., 2013).

Co-crystallization of homomeric *Caenorhabditis elegans* GluCl receptors with the allosteric modulator ivermectin revealed that the latter binds at each of the five interfaces in the transmembrane

Abbreviations: GABA, gamma-aminobutyric acid; $GABA_A$ receptor, gamma-aminobutyric acid type A receptor.

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domain of the receptor. S260 (M2-15') located within M2 forms a hydrogen bond with ivermectin (Hibbs and Gouaux, 2011). The homologous position in β subunits (β_2N265) of $GABA_A$ receptors is also important for modulation by anesthetics (Belelli et al., 1997; Jurd et al., 2003; Stewart et al., 2014). A docking analysis performed with etomidate in the $GABA_A$ receptor placed this compound in a binding pocket homologous to the ivermectin-pocket in the GluCl receptor (Chiara et al., 2012). In $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors there exist in principle 5 different interfaces that harbor the corresponding pocket.

β_2N265 (βM2-15') was first identified as a determinant of the modulatory action of loreclezole (Wingrove et al., 1994). Later work has shown that this residue, located in the β+/α− interface is crucial for modulation by etomidate and propofol. Many point mutations of this residue abolished or reduced sensitivity to etomidate, among them β_3N265M, β_2N265S, β_2N265C, β_2N265M (Belelli et al., 1997; Fernandez et al., 2012; Siegwart et al., 2002; Stewart et al., 2014; Wingrove et al., 1994). In β_3N265M mutant mice its anesthetic action is impaired (Jurd et al., 2003). However, direct photo-labeling of this residue has never been observed. Moreover, propofol and etomidate failed to protect β_2N265C from covalent modification from sulfhydryl-reactive reagents (Bali and Akabas, 2004; Li et al., 2009; Stewart et al., 2014). Thus, it is not clear if the β_2N265 residue has a direct role in binding, or in allosteric effects (Stewart et al., 2014). Modulation by the barbiturate pentobarbital is not affected by the β_2N265C mutation (McCracken et al., 2010), although in a β_3N265M knock-in mouse model some of the anesthetic responses towards this agent were reduced (Zeller et al., 2007). The influence of this residue in barbiturate action remains uncertain (McCracken et al., 2010).

In an attempt to further characterize the site of action of these compounds, photo-affinity labeling has been used. Photo-reactive etomidate analogs identified two equivalent anesthetic binding sites in the trans-membrane domain in the two β+/α− interfaces, which also contain the GABA binding sites in the extracellular domain. A second class of anesthetic binding site has been labeled using a photo-reactive derivative of barbiturates, at the α+/β− and γ+/β− subunit interfaces. In $\alpha_1\beta_3\gamma_2$ and $\alpha_1\beta_3$ receptor types, propofol produced a concentration-dependent inhibition of photolabeling by etomidate and barbiturate analogs >90%. Propofol bound with similar affinities to β+/α− sites and α+/β−/γ+/β− sites, with an IC_{50} of ~40 μM in $\alpha_1\beta_3\gamma_2$ receptors. Since this concentration is nearly 10-fold higher than the concentrations of propofol necessary to potentiate the receptor, the authors suggested that propofol may not only bind to the aforementioned sites, but additionally to other unidentified sites (Chiara et al., 2013). Barbiturates failed to inhibit with high affinity etomidate binding, and etomidate does not inhibit the labeling by the barbiturate derivative (Chiara et al., 2013; Jayakar et al., 2014). Thus, the authors propose that occupation of any of these sites is enough for anesthetic action. Given the structural diversity of intravenous anesthetics it is also unlikely that all anesthetics bind to a common class of pockets (Chiara et al., 2012).

Use of photo-affinity labeling is a direct method for the identification of amino acid residues located in or close to drug binding sites through an irreversible covalent reaction, although the lack of labeling does not provide evidence that a certain amino acid residue is not involved in drug interaction (Yip et al., 2013). Successful photo-affinity labeling alone also can not provide information on whether the corresponding site mediates weak or strong positive allosteric properties or antagonist properties. In any case functional data using low concentrations of the compounds are required.

Here we performed a detailed study of the functional consequences of the β_2N265I point mutation, and homologous mutations in other subunits (α_1S269I, γ_2S280I). All these mutations are located at subunit interfaces. Earlier work has shown that combined mutation of the three residues eliminated the low affinity potentiation by diazepam in mutated $\alpha_1\beta_2\gamma_2$ receptors (Walters et al., 2000). Our results indicate multiple sites of action all located at subunit interfaces for low concentrations of etomidate, propofol and pentobarbital. These sites mediate channel potentation to a different degree. Using subunit concatenation, we demonstrate that the two β+/α− subunit interfaces differentially affect modulation by etomidate.

2. Methods

2.1. Construction of mutated receptor subunits

The point mutations α_1S269I, β_2N265I and γ_2S280I were prepared using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

2.2. Construction of concatenated subunits

Construction of tandem and triple subunit cDNAs. The tandem construct γ_2-β_2, and triple construct α_1-β_2-α_1 has been described previously (Boulineau et al., 2005). Site-directed mutagenesis of β_2N265 to I was done in the tandem construct and the triple construct using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

2.3. Expression of $GABA_A$ receptors in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C.

Xenopus laevis oocytes were prepared, injected and defolliculated as described previously (Sigel, 1987; Sigel and Minier, 2005; Animal research permit by the Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE98/12)). They were injected with 50 nL of the cRNA solution containing wild type or mutated rat α_1, β_2 and γ_2 subunits of the $GABA_A$ receptors at a concentration of 10 nM:10 nM:50 nM (Boileau et al., 2002). For concatenated tandem and triple constructs, cRNA combinations ratios of 25: 25 nM were used. Injected oocytes were incubated in modified Barth's solution at 18 °C for at least 24 h before the measurements.

2.4. Functional characterization of the $GABA_A$ receptors

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. The holding potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4). Concentration response curves for GABA were fitted with the equation $I(c) = I_{max}/$