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Functional impact of serial deletions at the C-terminus of the human GABAρ1 receptor

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ABSTRACT

GABAρ1 receptors are formed by homopentameric assemblies that gate a chloride ion-channel upon activation by the neurotransmitter. Very little is known about the structural and functional roles played by the different domains that form each subunit; but one of them, the fourth transmembrane segment (TM4), is known to form a hydrophobic bundle together with three other TM segments that are necessary to stabilize the structure of the receptor. In this study we progressively removed amino acid residues from the C-terminus of the human GABAρ1 and studied the functional properties of the receptor mutants expressed in *X. laevis* oocytes. We found that deletions of up to the last four residues gave rise to receptors that were still functional, generating currents of 3.92 μA for the wt, 5.75 μA for S479X, 1.82 μA for F478X, 0.52 μA for I477X and 0.27 μA for S476X when exposed to 5 μM GABA; surprisingly, the mutant with one residue removed resulted more sensitive to the agonists. Further deletions, up to residue W475, resulted in receptors that did not gate an ion-channel. In addition, deleting the signal sequence, from R2-A15, in the N-terminus produced non-functional receptors. This study reveals that GABAρ1 can tolerate removal of several residues that form the fourth transmembrane segment up to a critical point, signaled by W475, beyond which the mutant protein is translated but does not form functional receptors. A comparative study is presented of some electrophysiological and pharmacological properties of the deletion mutants that were able to generate GABA currents.

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1. Introduction

Ligand-gated ion channels (LGIC) play a fundamental role in neuronal communication and γ-aminobutyric acid (GABA) receptors are the major inhibitory LGIC in the adult mammalian brain and retina. Accordingly, GABA receptors are one of the most important targets for a variety of clinically prescribed therapeutic compounds [1–3]. Detailed knowledge of the receptor's structure and function would provide important information for understanding fundamental aspects of synaptic transmission and neurological disorders, and would help also to design new therapeutic compounds that target specifically the GABA receptor.

The ionotropic GABAρ receptors permeate chloride ions and modulate presynaptic inhibition at synaptic terminals of the bipolar neurons of the retina [4]. The three known subunits that form the receptors (GABAρ1–ρ3) assemble as functional homopentameric complexes when expressed in heterologous systems such as the *Xenopus laevis* oocytes. GABAρ receptors are insensitive to bicuculline and desensitize very little, in sharp contrast to classic GABA-A receptors [5,6].

Basic structural characteristics of ionotropic GABA receptors are shared by other LGIC, such as the nicotinic acetylcholine (nACh), glycine and serotonin type 3 receptors which form a phylogenetically related group of proteins with diverse functional properties, and which share basic structural characteristics [7]. They all form pentameric assemblies in the membrane, and their individual subunits have a conserved structure consisting of four transmembrane segments (TM1–TM4, Fig. 1). The TM2s of each receptor are aligned in such a way that they form a central ion-permeant channel. In addition, a large extracellular amino-terminal domain contains the agonist binding site, and the intracellular loop between TM3 and TM4 is important for proper targeting of the receptor to the plasma membrane [9].

A combination of chimeric receptors, site-directed mutagenesis and molecular dynamics has permitted to define several distinctive amino acids important for the function of ionotropic GABAρ receptors, for example, the agonist binding site of the receptor, the structural gate of the ion-channel as well as other important modulatory sites, such as the zinc binding site [10–15]. Nevertheless, very little is known about the functional and structural roles of the residues in the

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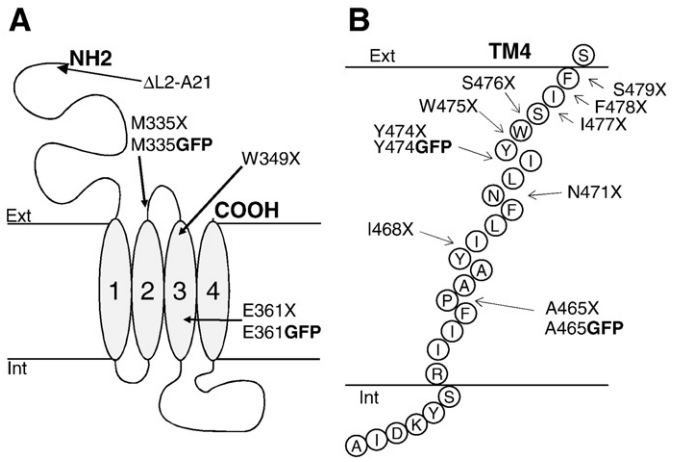

Fig. 1. Schematic representation of one subunit of hGABA ρ1 and its TM4. A) Four transmembrane regions (1–4) span the plasma membrane. Positions of the deletion mutants and sites in which the GFP was fused are indicated by arrows. B) Amino acid sequence of TM4, threading as predicted by the HMMTOP transmembrane topology prediction server [8]. Arrows indicate the position of the mutants generated.

short extracellular C-terminus of the receptor, as well as those that form the TM4.

In this study, we produced deletion mutants of the C-terminus of the human GABAρ1 receptor and assessed the impact of the deletions on the electrophysiological properties of the receptors expressed in *Xenopus* oocytes. In addition, a deletion mutant of the signal peptide of the receptor confirmed the essential role of this domain in addressing the position of the protein in the plasma membrane.

2. Materials and methods

2.1. Deletion mutants

hGABAρ1 was cloned from a human retina cDNA library and introduced into pcDNA3 (Invitrogen, Carlsbad, CA) [16]. The deletions were generated with the high fidelity DNA polymerase *Deep vent* (New England BioLabs, Ipswich, MA) and consisted of: 1) deletions at the C-terminus that introduced a translational stop codon (X in Fig. 1 and Table 1) at: a) the beginning of the short extracellular loop, after S334, b) the end of the short extracellular loop, after L348, c) the end of TM3 after L360; 2) serial amino acid deletions from the C-terminus S479 to N471, that is located in the middle of TM4. Then, stop codons were introduced after Y467 and P464, also embedded in TM4. 3) Deletion of the signal sequence from the second residue L2 to A21, conserving the ATG corresponding to the first M. Some of the receptors that did not generate GABA currents were tagged with GFP towards the C-terminus to assess their ability to reach the plasma membrane (Fig. 1).

cRNAs were synthesized from each deletion mutant using the mMessage mMachine kit (Ambion, Austin, TX). At least three independent cRNA preparations from each construct were tested.

2.2. Expression and electrophysiology in frog oocytes

X. laevis frogs were anesthetized with 0.17% 3-aminobenzoic acid methylester (MS-222) for 20–30 min. The follicles were manually removed, enzymatically defolliculated (with 0.3 μg/μl collagenase type I at room temperature for 45 min) and then kept at 16 °C in Barth's medium: 88 mM NaCl; 1 mM KCl; 0.33 mM $Ca(NO)_3$; 0.41 mM $CaCl_2$; 0.82 mM $MgSO_4$; 2.4 mM $NaHCO_3$; 5 mM HEPES; pH 7.4, containing 0.1 mg/ml gentamicin sulfate. The next day, 50 nl of cRNA of wtGABAρ1 or deletion mutants (1 μg/μl) were injected and electrophysiological recordings were obtained 3–5 days after injection.

Membrane currents elicited by the agonists were recorded using the two-microelectrode voltage-clamp technique [17]. The oocytes were placed in a 1000 μl chamber, impaled with two microelectrodes filled with 3 M KCl (0.5–1.5 MΩ) and clamped usually at −60 and sometimes at −80 mV when the currents were very small or absent. To obtain the equilibrium membrane potential of transmitter action, current–voltage relations were constructed by stepping the oocyte's membrane potential from −60 to −120 and to +40 (in 20 mV steps) in the absence or presence of GABA, β-alanine (β-ala), taurine (Tau), or glycine (Gly). All recordings were made at room temperature (20–23 °C) in a chamber continually perfused (5–10 ml/min) with Ringers solution (115 mM NaCl; 2 mM KCl; 1.8 mM $CaCl_2$; 5 mM HEPES; pH 7.4).

2.3. Drugs and statistics

All drugs were purchased from Sigma (St. Louis, MO) except MS-222 which was purchased from Argent Chemical Laboratories

Table 1
Summary of mutants constructed.

Construct	Description	5 mM GABA current (μA)	EC_{50} (GABA)	Hill no. (GABA)	Fluorescence (GFP)	Detected by external Ab
wt	Wild type	**3.92**	3.4 ± 1	0.5		+
S479X	ΔSer^{479}	**5.75**	2.1 ± 1[a]	0.4		+
F478X	ΔPhe^{478}-Ser^{479}	**1.82**	5.4 ± 3	1.2		+
I477X	ΔIle^{477}-Ser^{479}	**0.52**	6.4 ± 2	1.2		+
S476X	ΔSer^{476}-Ser^{479}	**0.27**	3000 ± 2	1.5		+
W475X	ΔTrp^{475}-Ser^{479}	NC				+
Y474X	ΔTyr^{474}-Ser^{479}	NC				+
Y474**GFP**	ΔTyr^{474}-Ser^{479}**GFP**	NC			+	+
I473X	ΔIle^{473}-Ser^{479}	NC				NT
L472X	ΔLeu^{472}-Ser^{479}	NC				NT
N471X	ΔAsn^{471}-Ser^{479}	NC				NT
I468X	ΔIle^{468}-Ser^{479}	NC				NT
A465X	ΔAla^{465}-Ser^{479}	NC				NT
A465**GFP**	ΔAla^{465}-Ser^{479}**GFP**	NC			+	NT
E361X	ΔGlu^{361}-Ser^{479}	NC				NT
E361X**GFP**	ΔGlu^{361}-Ser^{479}**GFP**	NC			+	NT
W349X	ΔTrp^{349}-Ser^{479}	NC				NT
M335X	ΔMet^{335}-Ser^{479}	NC				+
M335X**GFP**	ΔTyr^{335}-Ser^{479}**GFP**	NC			+	NT
ΔL2-A21	ΔLeu^{2}-Ala^{21}	NC				NT

NC, no current; NT, not tested; Ab, antibody.
[a] $P<0.05$ vs. wt (Student's *t*-test).