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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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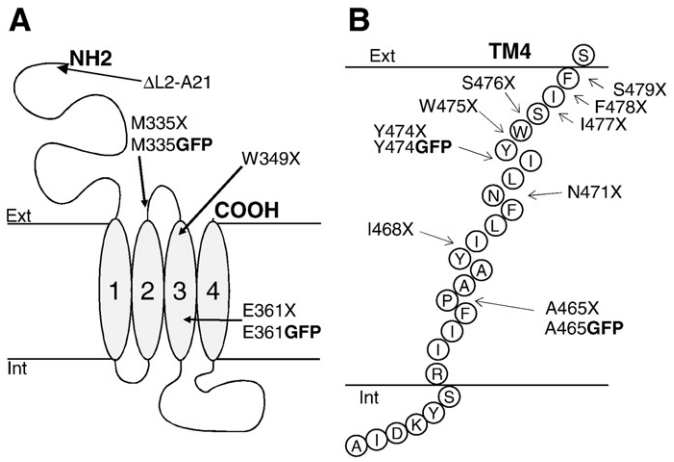

Fig. 1. Schematic representation of one subunit of hGABA ρ1 and its TM4. A) Four transmembrane regions (1–4) span the plasma membrane. Positions of the deletion mutants and sites in which the GFP was fused are indicated by arrows. B) Amino acid sequence of TM4, threading as predicted by the HMMTOP transmembrane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

(Redmond, WA). The GABA, β-ala, Tau and Gly stock solutions (0.5–1 M) were stored frozen. All the perfused solutions were at pH 7.0. Membrane currents are expressed as percentages of the control responses obtained with GABA alone. Control GABA responses were obtained before and after each test drug application to account for possible shifts in the control current amplitude. The data are expressed as means ± SEM. Dose–response relationships were fitted with the Hill equation: $R/R_{max} = [C]^{\eta}/\{[C]^{\eta} + (EC_{50})^{\eta}\}$, where R is the current elicited by the drug at concentration $[C]$; R_{max} = maximum response at saturation; η = Hill coefficient; and EC_{50} (or IC_{50} in the case of an inhibitory effect), is the concentration at which half-maximum response is obtained. Data from each experiment were collected from at least 12 oocytes, and the kinetics of GABA, β-ala, Gly or Tau activation (τ_o) or deactivation (τ_c) responses, were fit using Origin 6.0 software (Northampton, MA) to a second-order exponential decay function of the form: $I_t = Ae^{-t/\tau 1} + Be^{-t/\tau 2}$, where I_t is the current recorded at time t, A and B are the amplitudes of each decay component at $t = 0$, and τ_1 and τ_2 are the time constants of each phase of the current decline towards its asymptotic value. Experimental data are shown as mean ± SEM. Differences between two groups were statistically analyzed with the Student's t-test.

2.4. Immunolocalization and fluorescence microscopy

Two to three days after RNA injection, oocytes were scored for expression of the receptor(s) tagged with GFP. A Carl Zeiss LSM Meta 510 was used with the filter BP 505–530, wave length 488 (for GFP) and LP 560, wave length 543 (for Alexa Fluor 568). For immunofluorescence, oocytes were labeled with anti-GABAρ1 antibodies to assess the location and relative abundance of the receptors. Briefly, oocytes were incubated in Barth's media with albumin 5% and FBS 10%, for 30 min at room temperature, to block unspecific binding. After that, the oocytes were incubated overnight (16 °C) in Barth's with anti-GABA ρ1 (0.8 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA). After washout, the oocytes were incubated in Alexa Fluor 568 goat anti-mouse antibody (2 μg/ml, Invitrogen) for 2 h at room temperature. Non-injected oocytes were used as specificity control for the antibodies. The oocytes were then immediately observed in the confocal microscope using a Plan-Neofluar 10×/0.3 objective. Finally, to study the actual translation of the wt receptor and mutants, Western blots were performed on whole membrane preparations of injected oocytes, as previously described [18].

3. Results

3.1. General properties of hGABA ρ1 and mutations

Deletion mutants whose signal peptide, TM3 or the whole TM4, or a significant part of it, were removed failed to produce evident GABA-gated ion currents (Table 1); although the deletions tagged with GFP emitted fluorescence diffusely near the plasma membrane. In Fig. 2A the fluorescence emitted by an oocyte injected with ρ1GFP is contrasted with that exhibited by 2 deletion mutants that did not induce GABA-currents (ρ1Y474GFP, Fig. 2B and ρ1M335GFP, Fig. 2C). The images did not show obvious differences in fluorescence distribution between oocytes that gave rise to functional GABA receptors and those that did not. In contrast, the deletion mutants, where we removed progressively residues of TM4 from the last

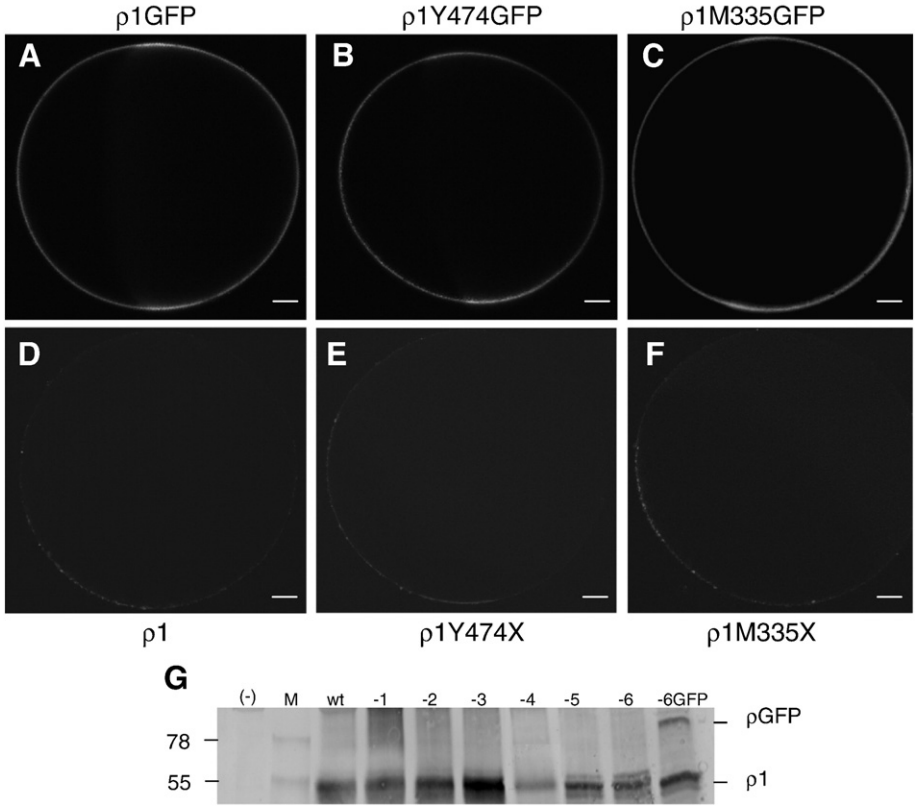

Fig. 2. Expression of receptors in oocytes. A–C) No major differences in GFP fluorescence were detected between wild type and mutant receptors. D) wt ρ1, E) ρ1Y474X and F) ρ1M335X were immunolabeled with antibody coupled to Alexa Fluor 568, also showing similar distribution. Images show a single optical section of the oocyte and in all of them the animal pole is positioned to the left. Non-injected oocytes exposed to the antibodies showed no signal. Bar = 100 μm. G). Western blot of membranes isolated from oocytes injected with cRNA coding for the indicated receptor, number indicates the amino acids removed, (−) non-injected oocytes. M is for marker (SeeBlue Pre-Stained Standard, Invitrogen). Each lane was loaded with membranes from 6 oocytes.

residue in the C-terminus (S479) to S476, formed receptors that elicited GABA-currents of various magnitudes and, therefore, we proceeded to study the electrophysiological properties of those receptors.

3.2. Deletion mutants reach the plasma membrane

To determine if the deletion mutants that did not generate functional receptors were able to reach the plasma membrane, we did immunofluorescence studies using an antibody directed to the extracellular amino-terminal domain of the receptor. Confocal laser microscopy revealed that the oocytes injected with different deletions and exposed to the Alexa Fluor 568 antibody, showed fluorescence along the plasma membrane, towards the animal pole. For example, Fig. 2D shows the label in an oocyte injected with the wt GABAρ1 and Fig. 2E and F that of oocytes injected with ρ1Y474X and ρ1M335X respectively, which did not generate GABA-currents. Non-injected oocytes exposed to the antibodies did not show fluorescence. Those results suggested that the mutant receptors were inserted in the plasma membrane, but were unable to assemble properly and gate the ion-channel. Western blots of whole oocyte membranes confirmed the presence of the receptors in all the samples of oocyte plasma membrane that were tested (Fig. 2G).

3.3. Functional properties of the deletion mutants

All the mutants having serial deletions of amino acids spanning the last four residues generated GABA-currents, and responded also to other agonists with a sequential potency of GABA>β-ala>Gly>Tau (Tables 1 and 2, Figs. 3 and 4). Removal of the last residue (S479) resulted in receptors that were more sensitive to GABA than the wild type receptors, and generated the largest currents: about 6 μA vs. 3 μA for the wild type with 5 μM GABA, a concentration close to the EC_{50} (Table 1 and Figs. 3 and 4). In contrast the receptors whose four last amino acids were deleted gave small GABA-currents (about 250 nA with 5 μM GABA) (Table 1 and Fig. 4). When the last four residues (S476X-S479X) were removed, the opening and closing kinetics of the channels changed notably (Fig. 4), from $\tau_o = 4 \pm 1$ s to $\tau_o = 7.5 \pm 1$ s and a $\tau_c = 45.6 \pm 2$ s to a $\tau_c = 29.43 \pm 1$ s for the wt and S476X respectively ($n = 12$, each). Moreover, and in sharp contrast to the wt GABAρ1 and all other GABAρ receptors, the GABA-currents generated by this mutant desensitized in the presence of the agonist (Fig. 4E).

None of the mutants changed the ion selectivity of the channel as judged by the equilibrium potential, which in all cases ranged from −24 to −30 mV, suggesting that Cl^- is the main ion flowing through the channel gated by the agonists. Moreover, in all cases the current/voltage relations were fairly linear, within the potential range explored (−120 to +40 mV) (data not shown). As is the case for the wt GABAρ receptors, La^{3+} modulated positively the currents of all the mutants; with EC_{50}s for GABA between 111 μM and 4.0 mM. The peculiar characteristics of the S476X receptors were reflected also in their sensitivity to La^{3+}, since they were affected less by La^{3+} (Table 3). On the other hand, the negative modulator Zn^{2+} [29] reversibly blocked the currents generated by GABA, β-Ala, Gly and Tau from all the tested mutants. The IC_{50}s to this metal ranged from 10 μM to 2.8 mM, being S479X the most sensitive (Table 4). We also examined the effect of the TPMPA, a selective GABAρ antagonist [19]. This compound potently and reversibly blocked the currents induced by the agonists in all mutant receptors. The S479X showed major sensitivity to the TPMPA, than the other receptors, even the wt. In the case of S476X, the TPMPA concentration required for half-maximum inhibition (IC_{50}) was more than 100 μM (data not shown).

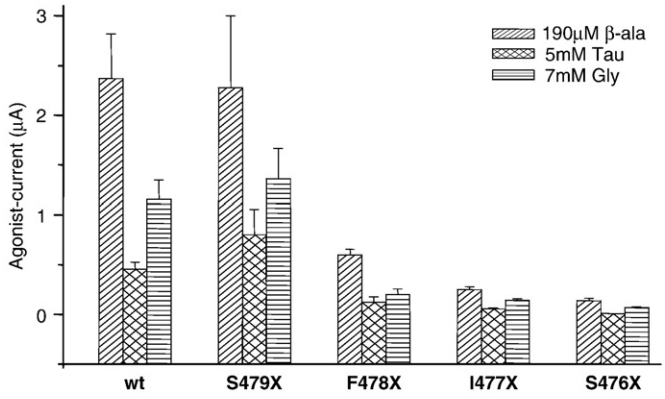

Fig. 3. Membrane currents elicited by the agonists indicated in oocytes expressing hGABAρ1 or its deletion mutants. Notice that S479X generated the largest currents while the subsequent mutations elicited progressively smaller currents.

Table 2
Agonists half-maximal responses (EC_{50} in μM) and Hill number of wt and mutant receptors. Observe the shift in the neurotransmitter EC50 of the mutants; the largest deletions had reduced sensitivity to the agonists, except for S479X, that was more sensitive (wt $n = 12$, each mutant $n = 16$, 4 frogs). Mean ± SEM. *$P < 0.05$, vs. wt (Student's *t*-test).

Agonist (μM)	Receptor				
	wt	S479X	F478X	I477X	S476X
β-alanine	150 ± 1	73.5 ± 3*	740 ± 3	810 ± 3	10,000 ± 2
Hill no.	1.7	1.2	1.2	1.1	1.3
Taurine	4200 ± 2	3600 ± 3*	13,600 ± 3	15,000 ± 2	62,000 ± 2
Hill no.	0.8	2.8	1.6	1.9	1.9
Glycine	5000 ± 3	3800 ± 2*	31,000 ± 2	41,600 ± 1	63,000 ± 3
Hill no.	1.9	1.3	1.6	1.9	1.6

*$P < 0.05$ vs. wt (Student's *t*-test).

4. Discussion

An attempt was made here to determine the functional relevance of the residues that form the C-terminus of the GABAρ1 receptor. Very little was known about the roles played by the fourth transmembrane segment and the short carboxy-end of ionotropic GABAρ-receptors on the function of the receptors. We therefore conducted this study to assist any future and more detailed determination of structure/function relations of this fairly unique receptor.

Few studies have been made on the C-terminus of human ion channels. It has been demonstrated recently that the C-terminus plays an important role in the formation of functional h5-HT_3A receptors [20]. Moreover, the structure of the heteromeric nicotinic Ach receptor suggests that, whether the channel is open or closed, the TM4 segment physically contacts the TM1 and TM3 segments [21] and, considering the well conserved amino acid sequence between the nACh and GABAρ1 receptors, it can be assumed that this will also be true for the homopentameric human receptor used in this study. Thus, deleting the whole or part of the α-helix of TM4 will predictably destabilize the bundle arrangement formed by the TM segments of each subunit. This prediction was partially fulfilled by our results, but unexpectedly we found that the GABAρ1 receptors in which we removed the last amino acid (S479) and up to three more of the residues that form part of the α-helix of TM4 (F478, I477, S476), still generated GABA-currents. Interestingly, the mutant which had only the last amino acid removed (S479X) was the most sensitive to all the agonists tested (cf Tables 1 and 2). The sequential removal of the next amino acids produced a gradual loss of receptor sensitivity; and some channel kinetics were also altered, indicating that the structure was further affected. Introduction of a stop codon after W475, as well as further deletions extending towards the N-end, resulted in non-

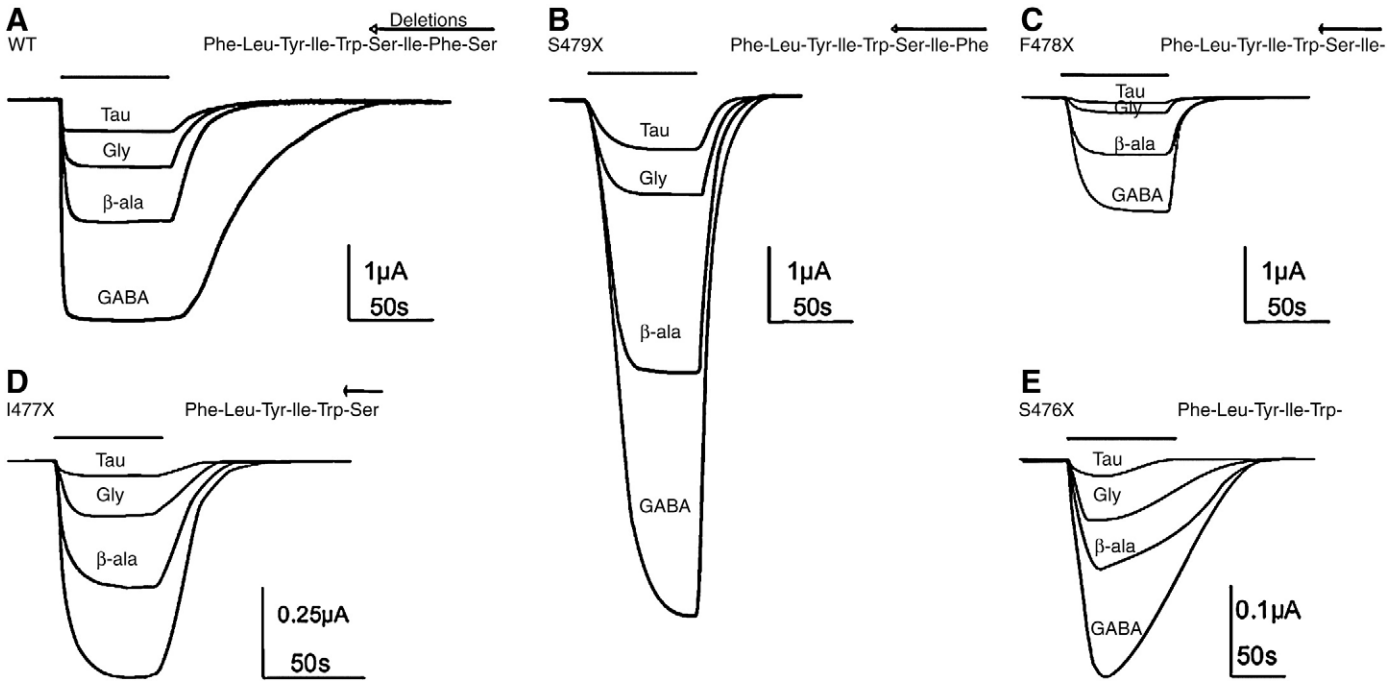

Fig. 4. Neurotransmitter-gated ion currents. Sample traces generated by near EC_{50} concentrations of GABA (5 μM), β-ala (200 μM), glycine (7 mM) and taurine (5 mM) in the oocytes expressing hGABA ρ1 (A) or the indicated deletion mutant (B–E). The horizontal bar indicates the time of application of the agonist. Note the largest currents generated by S479X (B), and the smaller currents of the subsequent deletions (F478X, I477X and S476X.)

functional receptors, although they were still localized in the plasma membrane. Further experiments will be necessary to probe if the ethanol sensitivity of the GABAρ receptor has been modified, such as is the case for the glycine receptor in which several residues of the TM4 are important to form a water-accessible cavity important for alcohol binding [22].

All the functional deletion mutants studied were efficiently activated by β-ala, Gly and Tau; and the currents generated by those agonists were all effectively blocked by TPMPA (not shown) and zinc. This is consistent with the presumed mechanism of action of both molecules, since TPMPA is known to compete for the agonist binding site [19,23,24], and zinc binds to residue H156 present in the extracellular domain of the receptor [10], and both sites were unaffected in the functional deletions. La^{3+} acts on $GABA_A$ receptors at a site different from the sites of action of barbiturates, benzodiazepines, picrotoxin, or zinc [25–28]. It positively modulated ionotropic GABAρ1 receptors [29], and it also potentiated the currents of all the functional deletion mutants, in agreement with a presumed direct lanthanide–receptor interaction at a site capable of allosterically modulating channel properties [30].

All ionotropic GABA receptors, including the GABAρ1, have a predicted signal sequence that spans 16–24 residues of the amino-end of the protein [6]. Since experimental analysis of this region has not been extensible we decided to see if GABAρ1 was properly targeted to the membrane after deleting this hydrophobic tract of residues. Three independent cRNAs were injected and all failed to produce receptors capable of generating GABA-currents, thus confirming the fundamental role of the signal peptide in this family of receptors. However, the precise role of this signal sequence in the biogenesis and proper membrane targeting of the receptor still remains to be determined.

We still have to study how the structural modification of the C-terminus impacts the gating of the ion-channel. The altered activation kinetics of the receptors that lack one or four residues, and the higher sensitivity to GABA of the mutant that lacks only one residue are worth studying at the single channel level to see if their characteristics have been altered. All these will be very important in the future, when the atomic structure of this family of receptors is determined.

Table 3
Effect of lanthanum. Positive modulation of lanthanum on receptors activated by the corresponding EC_{50} for each agonist [GABA (3.4 μM), β-ala (150 μM), Tau (4.2 mM) and Gly (4.2 mM)]. wt $n=12$, each mutant = 12–14, 4 different frogs. Mean ± SEM.

Agonist (μM)	Receptor				
	wt	S479X	F478X	I477X	S476X
GABA	111 ± 3	90 ± 2	174 ± 2	205 ± 3	4000 ± 4
Hill no.	0.7	1.1	1.9	1.9	1.2
β-alanine	130 ± 2	97 ± 4	151 ± 3	217 ± 4	5600 ± 3
Hill no.	1.2	0.7	1.3	1.9	1.6
Taurine	157 ± 3	125 ± 3	195 ± 1	245 ± 4	45,000 ± 2
Hill no.	1.2	0.9	1.73	1.9	1.1
Glycine	198 ± 3	162 ± 1	221 ± 3	300 ± 1	51,000 ± 3
Hill no.	1.0	0.7	1.7	1.7	1.4

Table 4
Effect of zinc. The Zn^{2+} blocking on the receptors activated by the corresponding EC_{50} of GABA (3.4 μM), β-ala (150 μM), Tau (4.2 mM) and Gly (4.2 mM). wt $n=11$, each mutant $n=18$, 4 frogs. Mean ± SEM.

Agonist (mV)	Receptor				
	wt	S479X	F478X	I477X	S476X
GABA	22.6 ± 3	9.9 ± 4	30 ± 6	50 ± 5	623 ± 3
Hill no.	1.9	1.1	1.3	1.2	1.9
β-alanine	26.7 ± 2	13.2 ± 6	43.7 ± 4	196 ± 3	631 ± 5
Hill no.	0.8	1.0	1.7	1.3	2.0
Taurine	30.8 ± 3	17.4 ± 4	92.5 ± 4	152 ± 6	1300 ± 7
Hill no.	1.2	0.7	1.3	1.5	1.7
Glycine	57.4 ± 4	25.7 ± 5	111 ± 6	211 ± 4	2800 ± 5
Hill no.	1.2	0.9	1.7	1.4	1.2

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