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RESEARCH PAPER

An intracellular redox sensor for reactive oxygen species at the M3-M4 linker of $GABA_A\rho1$ receptors

Andrea N Beltrán González, Javier Gasulla and Daniel J Calvo

Ingebi Conicet UBA, Buenos Aires, Argentina

Correspondence
Dr Daniel J Calvo, Laboratorio de Neurobiología Celular y Molecular, Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI) 'Dr. Héctor N. Torres', Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Vuelta de Obligado 2490, Ciudad Autónoma de Buenos Aires, CP 1428, Argentina. E-mail: danieljcalvo@gmail.com

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BACKGROUND AND PURPOSE
Reactive oxygen species (ROS) are normally involved in cell oxidative stress but also play a role as cellular messengers in redox signalling; for example, modulating the activity of neurotransmitter receptors and ion channels. However, the direct actions of ROS on $GABA_A$ receptors were not previously demonstrated. In the present work, we studied the effects of ROS on $GABA_A\rho1$ receptor function.

EXPERIMENTAL APPROACH
$GABA_A\rho1$ receptors were expressed in oocytes and GABA-evoked responses electrophysiologically recorded in the presence or absence of ROS. Chemical protection of cysteines by selective sulfhydryl reagents and site-directed mutagenesis studies were used to identify protein residues involved in ROS actions.

KEY RESULTS
$GABA_A\rho1$ receptor-mediated responses were significantly enhanced in a concentration-dependent and reversible manner by H_2O_2. Potentiating effects were attenuated by a free radical scavenger, lipoic acid or an inhibitor of the Fenton reaction, deferoxamine. Each ρ1 subunit contains only three cysteine residues, two extracellular at the Cys-loop (C^{177} and C^{191}) and one intracellular (C^{364}) at the M3-M4 linker. Mutant $GABA_A\rho1$ receptors in which C^{364} was exchanged by alanine were completely insensitive to modulation, implying that this site, rather than a cysteine in the Cys-loop, is essential for ROS modulation.

CONCLUSION AND IMPLICATIONS
Our results show that the function of $GABA_A\rho1$ receptors is enhanced by ROS and that the intracellular C^{364} is the sensor for ROS actions.

Abbreviations
DFX, deferoxamine; LA, lipoic acid; NEM, N-ethyl-maleimide; ROS, reactive oxygen species

Introduction
Reactive oxygen species (ROS) such as superoxide ($O_2^{-}\cdot$), hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2), are highly active molecules, inducing oxidative stress in cells (Adam-Vizi, 2005; Rhee, 2006; Halliwell, 2011). ROS are involved in brain processes underlying normal aging and the development of neurodegenerative disorders (Parkinson's

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and Alzheimer's diseases, schizophrenia, amyotrophic lateral sclerosis and ischaemia-reperfusion injury) (Annunziato *et al.*, 2002; Brennan and Kantorow, 2009; Do *et al.*, 2009). Beside their role in pathological processes, some ROS act as cellular messengers in redox signalling (Bao *et al.*, 2009; Toledano *et al.*, 2010; Rice, 2011). ROS are primarily produced as by-products of the mitochondrial oxidative metabolism and act as local diffusible messengers that regulate neuron-glia and interneuronal communication (Giniatullin *et al.*, 2005; Safiulina *et al.*, 2006; Kishida and Klann, 2007). In the CNS, ROS can be also generated secondary to the activation of neuronal NMDA and AMPA receptors (Kishida and Klann, 2007). It was also suggested that ROS levels modulate neurotransmission and eventually cause changes in neuronal activity and induce synaptic plasticity (Colton *et al.*, 1989; Bernard *et al.*, 1997; Frantseva *et al.*, 1998; Knapp and Klann, 2002; Garcia *et al.*, 2011). Considering the production of ROS and their effects on many ligand- and voltage-gated ion channels (Aizenman *et al.*, 1990; Vega-Saenz De Miera and Rudy, 1992; Li *et al.*, 1998; Annunziato *et al.*, 2002; Dirksen, 2002; Campanucci *et al.*, 2008; Coddou *et al.*, 2009; Rice, 2011), we hypothesized that ionotropic GABA receptors might also be targets for ROS actions. Previous work showed that diverse redox agents modulate the activity of native and cloned ionotropic GABA receptors (Amato *et al.*, 1999; Pan *et al.*, 2000; Calero and Calvo, 2008; Calero *et al.*, 2011; Gasulla *et al.*, 2012), but direct effects of ROS on GABA receptors had not been examined before. A number of studies have indicated that GABAergic neurotransmission is sensitive to ROS (Sah and Schwartz-Bloom, 1999; Sah *et al.*, 2002; Takahashi *et al.*, 2007; Saransaari and Oja, 2008; Tarasenko *et al.*, 2012) and high levels of these agents are normally generated in the retina (Brennan and Kantorow, 2009). However, the molecular targets for ROS actions on the synaptic GABAergic machinery remained so far elusive.

$GABA_A$ receptors are GABA-gated pentameric chloride channels, members of the Cys-loop receptor superfamily, that mediate most of the inhibitory neurotransmission in the CNS (Moss and Smart, 2001; Farrant and Nusser, 2005). $GABA_A$ receptors are made up by combination of diverse functionally distinct subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ, ρ_{1-3}) that commonly form heterooligomeric complexes (e.g. $GABA_A\alpha_1\beta_2\gamma_2$ receptors) (Farrant and Nusser, 2005; receptor nomenclature follows Alexander *et al.*, 2013). Most of these heteromeric $GABA_A$ receptors are antagonized by bicuculline or picrotoxin and modulated by benzodiazepines and barbiturates (Moss and Smart, 2001). In contrast, $GABA_A\rho$ receptors appear to be exclusively composed of ρ subunits which are widely distributed in the CNS, but are highly expressed only in the retina, densely and selectively concentrated in bipolar cells (Enz *et al.*, 1995; Boué-Grabot *et al.*, 1998; Wegelius *et al.*, 1998). $GABA_A\rho$ receptor-mediated responses are blocked by picrotoxin but are typically insensitive to bicuculline (Zhang *et al.*, 2001). $GABA_A\rho1$ receptors display both high affinity for GABA and poor desensitization (Farrant and Nusser, 2005), and these distinctive properties allow them to mediate several modes of inhibitory signalling in the retina (Matthews, 1994; Zhang and Slaughter, 1995; Dong and Werblin, 1998; Hartveit, 1999; McCall *et al.*, 2002; Lukasiewicz *et al.*, 2004; Hull *et al.*, 2006; Chávez *et al.*, 2010).

In the present work, $GABA_A\rho1$ receptor activity was measured before, during and after ROS generation using an *in vitro* cell model. $GABA_A\rho1$ receptors were heterologously expressed in *Xenopus laevis* oocytes and $GABA_A\rho1$ receptor-mediated Cl^- currents electrophysiologically recorded. Our results showed that responses mediated by $GABA_A\rho1$ receptors were potentiated by ROS. Additionally, experiments involving the chemical modification of sulfhydryl groups and site-directed mutagenesis indicated that Cys^{364}, located at the intracellular M3-M4 linker of the ρ_1 subunits, was essential for modulation by ROS.

Methods

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the CONICET-University of Buenos Aires Animal Care and Use Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of N animals were used in the experiments described here.

RNA preparation, oocyte isolation and cell injection

Human cDNA encoding the ρ_1 $GABA_A$ receptor subunit, cloned in the *in vitro* transcription-suitable vector pGEM, was used as a template to synthesize cRNAs *in vitro*. Site-directed mutagenesis was achieved by the PCR overlap extension method using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). cRNA solutions (0.3–1 ng nL^{-1}) were prepared in Rnase-free H_2O and stored at −70°C. *Xenopus laevis* (Nasco, Modesto, CA, USA) oocytes at stages V and VI were used for expression of exogenous cRNAs. Isolation and maintenance of cells were carried out as previously described (Miledi and Woodward, 1989). Briefly, frogs were anaesthetized with 3-aminobenzoic-acid ethylester (~1 mg mL^{-1}) and ovaries surgically removed. Ovaries were incubated with 400 U mL^{-1} collagenase for 4 h at 23–24°C and isolated oocytes maintained in an incubator at 18°C in Barth's medium (in mM: 88 NaCl; 0.33 $Ca(NO_3)_2$; 0.41 $CaCl_2$; 1 KCl; 0.82 $MgSO_4$; 2.4 $NaHCO_3$; 10 HEPES and 0.1 mg mL^{-1} gentamicin; pH adjusted to 7.4 with NaOH). After 1 day, each oocyte was manually microinjected (microinjector Drummond Sci. Co., Broomall, PA, USA) with 50 nL of a solution containing 5–50 ng of cRNA.

Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed 3–7 days after oocyte injection, with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA, USA). Standard glass recording electrodes were made in a Narishige PB-7 puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and filled with 3 M KCl. Pipette resistance values were approximately 1 MΩ. The holding potential was set to −70 mV and current traces acquired by a PC through a Labmaster TL-1 DMA interface (Scientific solutions Inc., Solon, OH, USA) using AXOTAPE software (Axon Instruments). Cells

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were placed in a chamber (volume 100 µL) continuously superfused (12 mL min^{-1}) with frog Ringer's solution (in mM: 115 NaCl; 2 KCl; 1.8 $CaCl_2$; 5 HEPES; pH 7.0). GABA and other drugs were applied through the perfusion system (Goutman *et al.*, 2005). N-ethyl maleimide (NEM) was freshly prepared prior to each experiment in normal Ringer's. A stock solution of H_2O_2 (1 M) was stored at –20°C and its concentration was confirmed spectrophotometrically at 240 nm. pH was adjusted to 7.0 with NaOH (1 M) or HCl (1 M). All the experiments were carried out at room temperature (23–24°C) and were replicated in at least five different oocytes isolated from at least two different frogs.

Data analysis

Data are expressed as means ± SEM and were analysed with Prism v. 5.0 (Graphpad Software, Inc., San Diego, CA, USA). Concentration-response curves for GABA and concentration-effect curves for H_2O_2 were fit with a logistic equation of the following form: $I_{GABA}/B = [A^n/(A^n + EC_{50}{}^n)] \times 100$ where A is the agonist concentration, B the maximal response, EC_{50} the concentration of agonist that elicits half-maximal responses and n the Hill coefficient. Percentage of potentiation was calculated as $[(I_{GABA\rho1H2O2} \times 100/I_{GABA\rho1control}) - 100]$, where $I_{GABA\rho1H2O2}$ indicates the current amplitude evoked at each particular GABA concentration in the presence of H_2O_2 and $I_{GABA\rho1control}$ the corresponding responses in the absence of modulator. Student's *t*-tests (two-tailed) were employed to evaluate significant differences between parameters.

Materials

The transcription kit mMessage mMachine was purchased from Ambion (Austin, TX, USA), QuickChange Site-Directed Mutagenesis Kit was from Stratagene and type I or type II collagenase from Worthington (Freehold, NJ, USA). The agonist and all the drug and salts, HEPES, 3-aminobenzoic-acid ethylester and Rnase-free H_2O were purchased from Sigma-Aldrich (St Louis, MO, USA).

Results

Functional modulation of GABA$_A$ρ1 receptors by H_2O_2

Application of GABA to oocytes expressing homomeric GABA$_A$ρ1 receptors induced large inward Cl^- currents displaying all features of the bicuculline resistant component of the retinal GABA receptor-mediated responses (Zhang *et al.*, 2001; Hull *et al.*, 2006). For example, in addition to their bicuculline insensitivity, they are antagonized by 1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) and picrotoxin, non-desensitizing blockers and display the same pharmacological profile for agonists. Figure 1 illustrates representative responses elicited by 0.3 µM GABA in the absence or presence of H_2O_2, recorded at –70 mV. Significant potentiation of the GABA-evoked responses was produced by H_2O_2 (500 µM). In order to characterize H_2O_2 effects, we mainly used two different procedures with equivalent results namely, H_2O_2 applied *during* the plateau of the GABA responses (Figure 1A), or *co-applied* with GABA (Figure 1B). Pre-incubation with H_2O_2 (up to 10 min) was also tested and gave

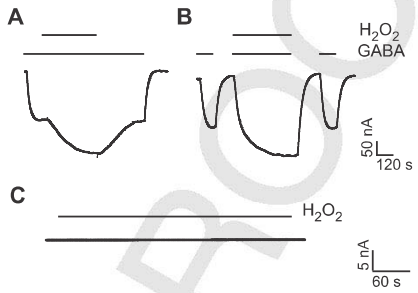


Figure 1

Potentiating effects of H_2O_2 on responses mediated by GABA$_A$ρ1 receptors expressed in *Xenopus laevis* oocytes. Representative traces of GABA$_A$ρ1 receptors mediated Cl^- currents elicited by GABA (0.3 µM) applications (indicated as bars) in the absence (control) or presence of H_2O_2 (500 µM). H_2O_2 was either applied during the GABA-evoked responses (A) or co-applied with GABA (B), flanked by control responses to GABA. (C) Lack of effect of H_2O_2 on a representative baseline current recorded from a non-transfected oocyte. For this and the subsequent figures, the oocytes were voltage-clamped at –70 mV. Scale bars indicate current amplitude and time.

the same results. H_2O_2 effects were reversible (Figure 1A) and a second application (not shown) produced similar results. Our previous work showed that several redox modulators of the GABA$_A$ρ1 receptors, such as ascorbic acid and glutathione, act rapidly and extracellularly (Calero and Calvo, 2008; Calero *et al.*, 2011). In contrast, potentiation of the GABA$_A$ρ1 receptor activity by H_2O_2 showed a relatively slow onset (Figure 1A). Because H_2O_2 is membrane-permeable (Desagher *et al.*, 1997), this slow time course of action might indicate an intracellular mechanism. No appreciable effects on oocyte properties, such as membrane potential, membrane resistance or current baseline under voltage-clamp, were observed during H_2O_2 applications (500 µM, up to 10 min) (Figure 1C).

Concentration-response curves for GABA were also performed in the absence (control) or presence of H_2O_2 (Figure 2A). H_2O_2 (500 µM) produced a leftward shift in $GABA_{EC50}$ without significantly affecting the maximal responses to GABA and nH ($EC_{50\ GABA} = 0.76 \pm 0.03$ µM, nH = 2.4 ± 0.3, $n = 6$; $EC_{50\ GABA+H2O2} = 0.64 \pm 0.03$ µM, nH = 2.5 ± 0.4, $n = 6$; $P < 0.005$). In order to determine the concentration range for effective H_2O_2 modulation, we tested increasing concentrations of H_2O_2. A concentration-effect curve (Figure 2B) was fitted to a sigmoid equation (see Methods). No saturation was observed at the maximal H_2O_2 concentration tested (2 mM), but higher concentrations of H_2O_2 significantly increased leak currents and were toxic to the oocyte membrane. Similar to effects displayed by other GABA$_A$ρ1 receptor redox modulators previously studied (Calero and Calvo, 2008; Calero *et al.*, 2011), the degree of potentiation exerted by H_2O_2 on GABA$_A$ρ1 receptor responses decreased as GABA concentration increased (Figure 2C). For example, the amplitude of currents evoked by 0.3 µM GABA was enhanced by 74 ± 3% ($n = 10$), whereas potentiation of currents evoked by 30 µM GABA was only 1.3 ± 0.7% ($n = 5$). For GABA concentrations lower than 3 µM, potentiation induced by H_2O_2 was always significant ($P < 0.05$). Current-voltage

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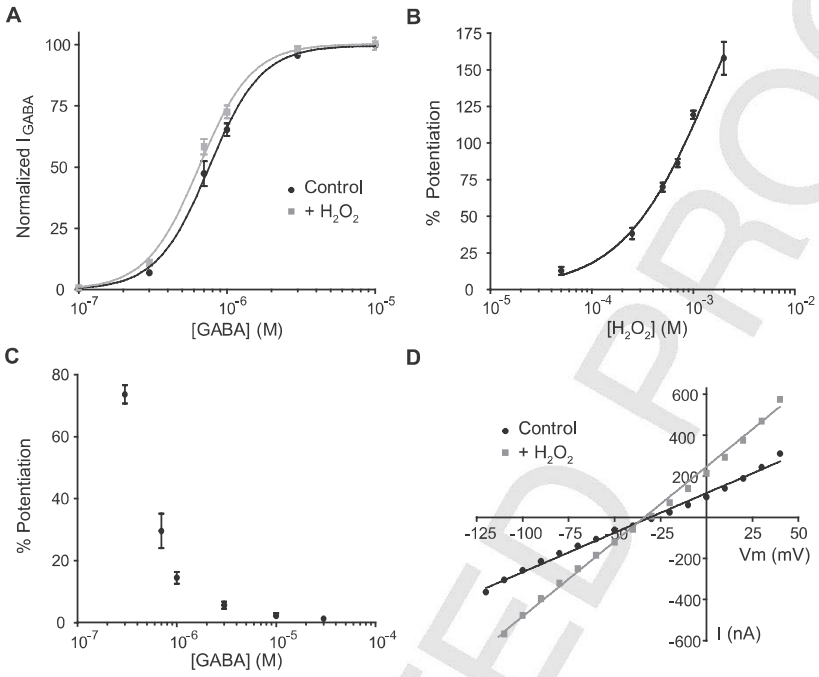


Figure 2

Analysis of H_2O_2 effects on $GABA_A\rho1$ receptors. (A) C-R curves for GABA in the absence (control) or presence of H_2O_2 (500 μM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response). (B) Potentiation of $GABA_A\rho1$ receptors responses (0.3 μM GABA) by increasing concentrations of H_2O_2. (C) GABA concentration dependence of the induced H_2O_2 (500 μM) potentiation of $GABA_A\rho1$ receptors responses. (D) I-V relationship for $GABA_A\rho1$ receptors responses evoked by GABA (0.3 μM) in the absence or presence of H_2O_2 (500 μM).

relationships (I-V curves) were carried out, in the presence or absence of H_2O_2. In the presence of H_2O_2 (500 μM), a significant change in slope without alteration in linearity of the I-V relationship or reversal potential (between –120 and +40 mV) was observed (Figure 2D). Therefore, H_2O_2 effects were voltage-independent and not due to variations in intracellular Cl^- levels.

Potentiation of $GABA_A\rho1$ receptors by H_2O_2 is mediated by an intracellular cysteine residue

Many ionic channels sensitive to redox modulation can be chemically modified through oxidation of cysteine residues. We have previously shown that reducing and oxidizing thiol agents are also effective modulators of the $GABA_A\rho1$ receptor function (Calero and Calvo, 2008). The reversible effect of H_2O_2 on $GABA_A\rho1$ receptors is consistent with a direct modulatory action and thiol groups located at the ρ_1 subunits are the most reactive candidates to be oxidized by this agent. In order to elucidate if cysteines are involved in this modulation, we examined the effect of DTT, a membrane permeable reagent which reversibly reduces the thiol groups (Lauriault and O'Brien, 1991). Pre-incubation with DTT (2 mM for 120 s) enhanced the potentiating effects of H_2O_2 on $GABA_A\rho1$ receptor responses (Figure 3A). We further examined if H_2O_2 actions on $GABA_A\rho1$ receptor-mediated responses were affected by the presence of a reagent that irreversibly modifies

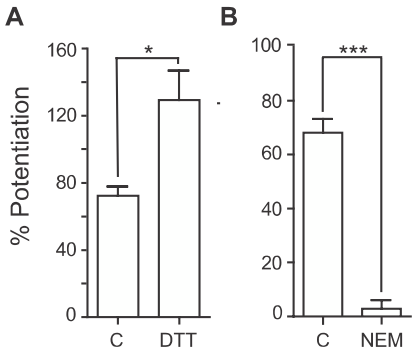


Figure 3

Cysteine thiols are involved in H_2O_2 modulation of responses mediated by $GABA_A\rho1$ receptors. Potentiation of $GABA_A\rho1$ receptors responses (0.3 μM GABA) by H_2O_2 (500 μM) was (A) enhanced when cysteine thiols were reduced with DTT (2 mM); $^*P < 0.03$, n = 6 and (B) prevented when sulfhydryl residues were alkylated by NEM (30 μM); $^{***}P < 0.0001$, n = 8.

cysteine thiol groups. NEM is a membrane-permeable, irreversible alkylating reagent which selectively forms covalent bonds with free sulfhydryl groups, preventing any further chemical reaction at these sites (at pH = 7) (Means and

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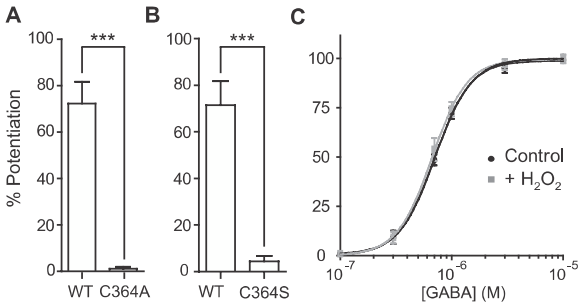


Figure 4

Intracellular cysteine C^{364} mediates H_2O_2 modulation of responses mediated by GABA$_A$ρ1 receptors. Mutation of the C^{364} affects the potentiating actions of H_2O_2. (A) H_2O_2 (500 μM) failed to potentiate GABA$_A$ρ$_{1C364A}$; ***P < 0.0001, n = 5 and (B) GABA$_A$ρ$_{1C364S}$ receptor responses (0.3 μM GABA); ***P < 0.0001, n = 8. (C) Concentration-response curves for GABA$_A$ρ$_{1C364S}$ receptors in the absence (control) or presence of H_2O_2 (500 μM). Response amplitudes were expressed as fraction of 30 μM GABA-evoked currents (maximal response).

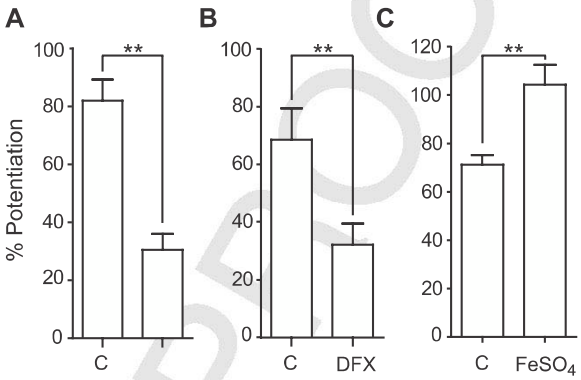


Figure 5

Hydroxyl radicals are involved in H_2O_2 potentiation of GABA$_A$ρ1 receptors responses. Potentiation of GABA$_A$ρ1 receptor responses by H_2O_2 (500 μM) was decreased in the presence of (A) the free radical scavenger LA (200 μM); **P < 0.001, n = 6 or (B) the iron chelator deferoxamine (100 μM); **P < 0.002, n = 6 and was enhanced (C) after pre-incubation with $FeSO_4$ (100 μM); **P < 0.005, n = 6.

Feeney, 1971). The concentration of NEM was kept as low as possible and incubation periods as short as possible to prevent non-specific effects (Calero *et al.*, 2011). Pre-incubation with NEM (30 μM for 120 s) completely prevented the potentiating effects of H_2O_2 on GABA$_A$ρ1 receptor responses (Figure 3B). These results suggest that H_2O_2 modulates GABA$_{Aρ1}$ receptor function by interacting with one or more cysteines.

Each ρ1 subunit contains only three cysteine residues, two extracellular located in the N-terminal domain that form the characteristic Cys-loop (C^{177} and C^{191}) of the GABA$_{Aρ1}$ receptors and one intracellular (C^{364}), located at the M3-M4 linker (Zhang *et al.*, 2001). Mutations of the cysteines at the Cys-loop are known to render receptors non-functional (Amin *et al.*, 1994). To determine whether C^{364} was involved in the modulation of GABA$_A$ρ1 receptors by H_2O_2, we performed site-directed mutagenesis, replacing this amino acid residue by alanine, a small neutral amino acid insensitive to redox modulation. Mutant GABA$_{Aρ1C364A}$ receptors expressed in oocytes responded to GABA ($EC_{50\ GABAAρ1C364A}$ = 0.52 ± 0.03 μM, n = 7; $EC_{50\ GABAAρ1wt}$ = 0.70 ± 0.04 μM, n = 6) and showed a similar pharmacological profile. Interestingly, in sharp contrast with wild-type receptors, the mutant GABA$_{Aρ1C364A}$ receptors were largely insensitive to H_2O_2 applications and there was no potentiation of 0.3 μM GABA responses by 500 μM H_2O_2 (Figure 4A). Because H_2O_2 potentiation observed in wild-type receptors could be due to the polar environment of C^{364},and because GABA$_{Aρ1C364A}$ receptors showed changes in GABA affinity, compared with wild-type receptors, we performed a more conservative mutation, replacing this cysteine by serine, an amino acid of similar polarity and size. Mutant GABA$_{Aρ1C364S}$ receptors expressed in oocytes showed typical responses to GABA and EC_{50} values for GABA were not significantly different from the EC_{50} values obtained for wild-type receptors ($EC_{50\ GABAAρ1C364S}$ = 0.69 ± 0.04 μM, n = 14; $EC_{50\ GABAAρ1wt}$ = 0.71 ± 0.04 μM, n = 7; *n.s.*). However, H_2O_2 (500 μM) failed to potentiate GABA responses elicited from this mutant C364S GABA$_{Aρ1}$ receptor (Figure 4B). To further analyse if the insensitivity of GABA$_{Aρ1C364S}$ receptor to H_2O_2 depended on GABA concentration, we carried out concentration-response curves for GABA either in the absence (control) or the presence of H_2O_2 (Figure 4C). H_2O_2 (500 μM) did not affect GABA EC_{50} in the mutant receptors ($EC_{50\ GABAAρ1C364S}$ = 0.71 ± 0.02 μM, nH = 2.6 ± 0.2; $EC_{50\ GABAAρ1C364S+H2O2}$ = 0.67 ± 0.02 μM, nH = 2.7 ± 0.3; n = 5; *n.s*).

In summary, these results indicated that the intracellular cysteine C^{364}, located at the intracellular M3-M4 linker of the ρ$_1$ subunits, acted as a specific target for the action of ROS and that its chemical modification by ROS potentiated the function of GABA$_{Aρ1}$ receptors.

Hydroxyl radicals are involved in the potentiation of GABA$_A$ρ1 receptor function by H_2O_2

In the presence of low concentrations of Fe^{2+}, H_2O_2 generates hydroxyl radicals (OH·) via Fenton reaction (Sah *et al.*, 2002). In order to determine if H_2O_2 can act indirectly through the production of hydroxyl radicals, we studied the effect of H_2O_2 on GABA responses in the presence of lipoic acid (LA; a free radical scavenger), deferoxamine (DFX), an iron chelator that inhibits the Fenton reaction, and iron (II) sulfate, a source of Fe^{2+} to enhance the Fenton reaction. The potentiation of GABA$_A$ρ1 receptor current responses elicited by 500 μM H_2O_2 *during* applications of 0.3 μM GABA were decreased in the presence of 200 μM LA (Figure 5A) or 100 μM DFX (Figure 5B) and increased after pre-incubation with 100 μM $FeSO_4$ for 2 min (Figure 5C). Potentiation of the GABA$_A$ρ1 receptor-mediated current was reversible in the presence of iron as it was in the presence of H_2O_2 alone (not shown). None of these agents produced significant changes in the current baseline or modulated GABA$_A$ρ1 receptor responses, when applied

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alone. These results suggest that hydroxyl radicals contributed to the potentiation of $GABA_A\rho1$ receptor function by H_2O_2.

Discussion and conclusions

The present findings are the first to demonstrate the existence of a putative intracellular redox sensor at an ionotropic GABA receptor. We showed here the potentiation of the homomeric $GABA_A\rho1$ receptor function by ROS and identified the intracellular C^{364} residue, located at the M3-M4 cytoplasmic linker of the ρ1 subunits, as the target for ROS actions.

$GABA_A\rho1$ receptors can be considered a simple and suitable model for studying the sensitivity of ionotropic GABA receptors to ROS for several reasons. $GABA_A\rho1$ receptors are key players in synaptic inhibition in the retina, a tissue that produces high levels of ROS. In addition, homomeric ρ receptors present less structural complexity compared with classic heteromeric $GABA_A\alpha\beta\gamma$ receptors. In fact, ρ1 subunits contain only two molecular sites contributing potential reactive cysteine residues (the extracellular Cys-loop and the single intracellular C^{364}), whereas other $GABA_A$ receptor subunit subtypes contain many intracellular cysteine residues as potential targets for ROS modulation (Sedelnikova *et al.*, 2005; Lo *et al.*, 2008).

Mechanisms underlying the potentiation of $GABA_A\rho1$ receptors by ROS

The sensitivity of $GABA_A\rho1$ receptors to ROS was unknown, but earlier work indicated that ROS were capable of modulating GABAergic neurotransmission, presumably via both presynaptic and postsynaptic mechanisms (Colton *et al.*, 1986; Sah *et al.*, 2002; Safiulina *et al.*, 2006; Takahashi *et al.*, 2007; Saransaari and Oja, 2008; Yowtak *et al.*, 2011; Tarasenko *et al.*, 2012). Sah *et al.* showed that exposure of hippocampal slices to H_2O_2 concomitantly altered $GABA_A$ receptor binding characteristics and increased $GABA_A$ receptor-mediated Cl^- influx in CA1 pyramidal cells (Sah and Schwartz-Bloom, 1999; Sah *et al.*, 2002). These results raised the question of how the postsynaptic ROS effects were exerted at the GABAergic synapses. Were they direct, acting through redox-sensitive sites on the GABA receptors or indirect, acting by peroxidation of membrane lipids located near to the Cl^- channel (Sah *et al.*, 2002)? We have demonstrated here that ROS were capable of inducing functional changes on $GABA_A\rho1$ receptors, a $GABA_A$ receptor subtype highly expressed in retinal bipolar cells. These changes were similar to that reported for classic $GABA_A$ receptors from different brain regions (Sah *et al.*, 2002). However, we also provided experimental evidence indicating that $GABA_A\rho1$ receptors are directly modulated by ROS. Potentiation of $GABA_A\rho1$ receptors by H_2O_2 was reversible, concentration-dependent, voltage-independent and strongly dependent on the GABA concentration. H_2O_2 effects were partially prevented in the presence of the free radical scavenger LA or by DFX, an inhibitor of the Fenton reaction. In contrast, potentiation of $GABA_A\rho1$ receptors by H_2O_2 was enhanced if the Fenton reaction was amplified by using iron (II) sulfate. These data suggested that H_2O_2 acted as a precursor for the generation of hydroxyl radicals that eventually exerted their effects on the GABA receptors. H_2O_2-induced potentiation of $GABA_A\rho1$ receptors persisted in the presence of DFX, thus H_2O_2 might also be acting directly on the receptor to produce these modulatory effects, without involving products of the Fenton reaction.

Due to their reactivity, the cysteine residues in the receptor protein were good candidates for sensing ROS. Chemical protection studies, using the selective membrane-permeable sulfhydryl reagent NEM, and site-directed mutagenesis experiments, where C^{364} was replaced by alanine or serine, indicated that this particular intracellular residue was essential for ROS effects. The slow onset of potentiation (illustrated in Figure 1) was also consistent with an intracellular mechanism of action. External H_2O_2 concentrations below the range of those normally used in previous studies (Vega-Saenz De Miera and Rudy, 1992; Rice, 2011) had significant effects on $GABA_A\rho1$ receptors. The effective intracellular concentrations sensed by the $GABA_A\rho1$ receptors are expected to be lower than bath concentrations, due to the high reducing power of the oocyte cytoplasm. The intracellular antioxidant network that maintains redox balance of amphibian oocytes is composed of many enzymic activities and metabolites, including superoxide dismutase, catalase, ascorbic acid and glutathione (Ferrari *et al.*, 2008). We found that the ROS effects on $GABA_A\rho1$ receptors were completely washed out in the absence of supplementary reducing agents, probably because the intracellular environment caused potentiation to cease after H_2O_2 applications were stopped. This also suggests that C^{364} may undergo a reversible chemical modification producing a transient conformational change in the receptor that, in the absence of ROS, rapidly relaxed to a lower energy state. One possible interpretation is that oxidation of the thiol group of C^{364} by ROS induced protein structural rearrangements that affected GABA binding. The leftward shift produced in concentration-response curves for GABA in the presence of H_2O_2 is compatible with this hypothesis. In addition, because H_2O_2 treatment did not change the reversal potential of the I-V curves is unlikely that, in our experiments, ROS actions were due to a change in the intracellular Cl^- levels.

Potential physiological relevance of the modulation of ionotropic GABA receptors by ROS

ROS production in neurons can affect many targets, including several neurotransmitter receptors (Rice, 2011). The modulation of nicotinic cholinergic and purinergic receptors by ROS was exerted through specific intracellular cysteines (Campanucci *et al.*, 2008; Coddou *et al.*, 2009) and such actions could be involved in neuropathological events (Campanucci *et al.*, 2008; 2010). Concerning $GABA_A$ receptors, is quite remarkable that all ρ subunits display a conserved single intracellular cysteine residue at the M3-M4 linker (C^{364} in ρ_2 and C^{379} in ρ_3). Moreover, most of the $GABA_A$ receptor subunits contain also one or more cysteine residues at their intracellular loops. Particularly, the M3-M4 intracellular loop is known to interact directly with several cellular regulatory proteins which can be involved in $GABA_A$ receptor oligomerization, assembly, forward trafficking and clustering (Boué-Grabot *et al.*, 2004; Lo *et al.*, 2008). Thus, the importance that these intracellular cysteines might have for $GABA_A$

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receptor function during endogenous ROS generation in neurons deserves to be further studied. Given that H_2O_2 is generated normally during cell activity, whereas hydroxyl radicals are typically generated under pathological conditions, it will be important to establish whether redox modulation of GABA receptors is physiological or pathophysiological. It will be also interesting to examine whether oxidation of these intracellular cysteines by ROS might represent a common mechanism for regulating the activity of diverse GABA$_A$ receptor subtypes and other members of the Cys-loop receptor superfamily.

As GABA$_A$ρ1 receptors provide significant inhibitory drive to the synaptic terminals of retinal bipolar cells, including tonic, reciprocal and lateral inhibition (Zhang and Slaughter, 1995; Lukasiewicz *et al.*, 2004; Hull and von Gersdorff, 2004; Chávez *et al.*, 2010), modulation of the GABA$_A$ρ1 receptor activity by ROS could eventually shape ganglion cell responses via control of glutamate release at these terminals. Nevertheless, whether or not ROS modulation of ionotropic GABA receptors represents a physiologically relevant mechanism for controlling the activity of retinal neuronal circuits, will need to be assessed by using both retinal slices and *in vivo* models.

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Conflict of interest

None.

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