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Quercetin antagonism of $GABA_{A\rho1}$ receptors is prevented by ascorbic acid through a redox-independent mechanism

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ABSTRACT

Quercetin is a natural flavonoid widely distributed in plants that acts as a neuroprotective agent and modulates the activity of different synaptic receptors and ion channels, including the ionotropic GABA receptors. $GABA_{A\rho1}$ receptors were shown to be antagonized by quercetin, but the mechanisms underlying these antagonistic actions are still unknown. We have analyzed here if the antagonistic action produced by quercetin on $GABA_{A\rho1}$ receptors was related to its redox activity or due to alternative mechanism/s.

Homomeric $GABA_{A\rho1}$ receptors were expressed in frog oocytes and GABA-evoked responses electrophysiologically recorded. Quercetin effects on $GABA_{A\rho1}$ receptors were examined in the absence or presence of ascorbic acid. Chemical protection of cysteines by selective sulfhydryl reagents and site directed mutagenesis experiments were also used to determine ρ_1 subunit residues involved in quercetin actions.

Quercetin antagonized $GABA_{A\rho1}$ receptor responses in a dose-dependent, fast and reversible manner. Quercetin inhibition was prevented in the presence of ascorbic acid, but not by thiol reagents that modify the extracellular Cys-loop of these receptors. H141, an aminoacidic residue located near to the ρ_1 subunit GABA binding site, was involved in the allosteric modulation of $GABA_{A\rho1}$ receptors by several agents including ascorbic acid. Quercetin similarly antagonized GABA-evoked responses mediated by mutant $^{H141D}GABA_{A\rho1}$ and wild-type receptors, but prevention exerted by ascorbic acid on quercetin effects was impaired in mutant receptors. Taken together the present results suggest that quercetin antagonistic actions on $GABA_{A\rho1}$ receptors are mediated through a redox-independent allosteric mechanism.

1. Introduction

Flavonoids are plant-derived compounds showing a wide range of biological activities, including antioxidant properties (Cao et al., 1997; Williams et al., 2004) and neuropharmacological actions such as proconvulsant, anticonvulsant, sedative and anxiolytic effects (Avallone et al., 2000; Griebel et al., 1999; Karim et al., 2011; Kavvadias et al., 2004; Loscalzo et al.; Medina et al., 1997; Nielsen et al., 1988; Ren et al., 2010). Flavonoid's effects on the nervous system can involve multiple targets including synaptic receptors and ion channels (Elliott et al., 1992; Goutman et al., 2003; Ji et al., 1996; Koh et al., 1994; Lee et al., 2008; Mall et al., 2000; Saponara et al., 2002).

The ionotropic γ-aminobutyric acid (GABA) receptors are GABA-gated chloride (Cl^-) channels, members of the Cys-loop receptor superfamily (Farrant and Nusser, 2005). Diverse $GABA_A$ receptor isoforms (e.g: $GABA_{A\alpha1\beta2\gamma2}$) are widely distributed in the mammalian brain; in contrast, $GABA_{A\rho}$ receptors are highly expressed only in the retina and other visual areas (Boue-Grabot et al., 1998; Enz et al., 1995). $GABA_{A\rho}$ receptors exhibit a distinct pharmacological profile; they are insensitive to the competitive $GABA_A$ antagonist bicuculline and show a very low or null affinity for classical $GABA_A$ allosteric modulators such as benzodiazepines, steroids and barbiturates (Abdel-Halim et al., 2008; Johnston et al., 2010; Zhang et al., 2001). $GABA_A$ receptors are pharmacological targets

Abbreviations: GABA, γ-aminobutyric acid; NEM, N-ethylmaleimide

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for diverse natural, synthetic and semi-synthetic flavonoids (Hanrahan et al., 2011). Modulation of $GABA_A$ receptors by flavonoids is exerted through different sites and mechanisms of action. Depending on the flavonoid structure and the particular $GABA_A$ receptor subtype examined, either potentiating or blocking effects were described, both in the presence or absence of GABA (Dekermendjian et al., 1999; Gavande et al., 2011; Goutman et al., 2003; Haberlein et al., 1994; Hanrahan et al., 2003; Hanrahan et al., 2011; Karim et al., 2011; Karim et al., 2012; Marder and Paladini, 2002). Still, many issues concerning the mechanisms underlying flavonoid modulation of ionotropic $GABA_A$ receptors are unknown. For example, flavonoid actions on $GABA_{A\rho}$ receptors were only characterized to some extent (Goutman and Calvo, 2004; Goutman et al., 2003; Hall et al., 2004).

Quercetin is a natural flavonoid with redox properties (Boots et al., 2008a) that showed an antagonistic profile on $GABA_{A\rho1}$ receptors (Goutman and Calvo, 2004). As many other ionic channels, $GABA_{A\rho1}$ receptors can be modulated by several reducing and oxidizing agents (Calero and Calvo, 2008). However, whether quercetin effects on $GABA_{A\rho1}$ receptors are mediated by a redox mechanism or by an allosteric interaction (or both) is not established. We have recently shown that ascorbic acid potentiates the activity of retinal ionotropic GABA receptors (Calero et al., 2011) through two independent and concomitant modulatory events, namely a redox modification and an allosteric interaction both involving amino acidic residues located near to the agonist binding site. Interestingly, due to its structural similarities to the ascorbic acid molecule, quercetin was shown to be capable to inhibit ascorbate transport mediated by the sodium-dependent vitamin C transporters 1 and 2 (SVCT1 and 2) in a redox-independent, non-competitive and reversible manner (Caprile et al., 2009; Song et al., 2002). Based on these evidences, we analyzed if a similar mechanism is involved during quercetin modulation of $GABA_{A\rho1}$ receptors, or its effects are due to its redox activity.

GABA-evoked Cl^- currents were recorded in *Xenopus laevis* oocytes expressing homomeric $GABA_{A\rho1}$ receptors and quercetin effects were tested in the absence or presence of ascorbic acid. Data show that quercetin antagonism of $GABA_{A\rho1}$ receptors can be prevented by ascorbic acid. We also used chemical protection of cysteines by selective sulfhydryl reagents and site-directed mutagenesis of amino acidic residues critical for $GABA_{A\rho1}$ receptor modulation, both located in the N-terminal extracellular domain of the ρ1 subunits, to study the molecular mechanisms implicated in quercetin actions. Taken together, our results suggest that quercetin inhibitory effects on $GABA_{A\rho1}$ receptors are not related to its antioxidant properties and that are more likely due to an allosteric modulation.

2. Materials and methods

2.1. RNA preparation, oocyte isolation and cell injection

A human cDNA encoding the ρ1 GABA receptor subunit cloned in the vector suitable for *in vitro* transcription pGEM was used as a template to synthesize cRNAs *in vitro* (mMessage mMachine kit Ambion; Austin, TX, USA). Site-direct mutagenesis was achieved by the polymerase chain reaction overlap extension method using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). cRNA solutions (0.1–0.3 ng/nl) 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 oocytes were carried out as previously described (Goutman et al., 2003). Briefly, frogs were anaesthetized with 3-aminobenzoic-acid ethylester (~1 mg/ml) and ovaries surgically removed. Ovaries were incubated with 200 U/ml collagenase for 30 min at room temperature (RT), and isolated oocytes were maintained in an incubator at 17 °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 gentamycin; 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.

2.2. 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 puller Narishige PB-7 (Narishige Scientific Instrument Lab., 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 in a PC through Labmaster TL-1 DMA interface (Scientific solutions Inc, Solon, OH, USA) using AXOTAPE software (Axon Instruments). Cells 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). The agonist and other drugs were applied through the perfusion system. Stock solutions were prepared freshly each day as follows: Quercetin (Que) in DMSO; ascorbic acid (Asc) and N-ethylmaleimide (NEM)

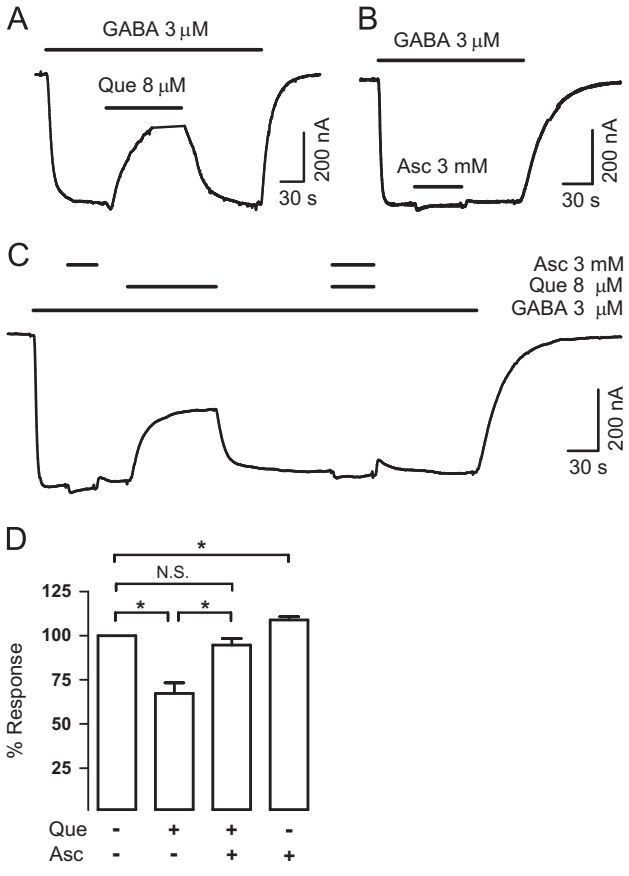

Fig. 1. Ascorbic acid prevents quercetin inhibition of $GABA_{A\rho1}$ receptors expressed in *Xenopus laevis* oocytes. (A, B, C) Representative traces of $GABA_{A\rho1}$ responses (Cl^- currents) elicited by GABA (3 µM). Bars indicate *on-top* applications of (A) quercetin (Que = 8 µM) or (B) ascorbic acid (Asc = 3 mM). In (C) quercetin and ascorbic acid were separately or simultaneously applied. (D) Histogram summarizing experiments illustrated in (C). For this and subsequent figures oocytes were voltage-clamped at −70 mV. Scale bars indicate current amplitude (nA) (*y*-axis) and time (sec) (*x*-axis).

in Ringer's solution and dithiothreitol (DTT) in water. The pH was always adjusted to 7.0 with NaOH (1 M). All the experiments were carried out at RT (23–24 °C). 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).

2.3. Data analysis

Data were analyzed with Prism v. 5.0 (MicroCal, Northampton, MA, USA). Statistical analysis was performed using Dunnett's Multiple Comparison Test, One-way Anova and post-Test Turkey's Multiple Comparison Test. Dose–response curves (D–R) for GABA were fit with the expression of the mentioned logistic equation: $I_{GABA}/B=[A^n/(A^n+EC_{50}{}^n)]\times 100$ where A is the agonist concentration, B is the maximal response, EC_{50} is the concentration of agonist that elicits half-maximal responses, and n is the Hill coefficient. The percentages (%) of inhibition were calculated as a fraction of the inhibition induced by quercetin on the corresponding GABA responses (as indicated in the text and figures) in the absence (Fig. 3) or presence (Fig. 4) of different ascorbic acid concentrations.

3. Results

3.1. Antagonistic actions of quercetin on $GABA_{A\rho1}$ receptors are prevented by ascorbic acid

Human homomeric $GABA_{A\rho1}$ receptors were heterologously expressed in frog oocytes. GABA applications to oocytes expressing $GABA_{A\rho1}$ receptors induced large inward Cl^- currents displaying all of the features of the so-called retinal $GABA_C$ receptor-mediated responses (Hull et al., 2006). For example, they were bicuculline-insensitive, TPMPA and picrotoxin sensitive, non-desensitizing and display the same pharmacological profile for agonists (Kusama et al., 1993; Woodward et al., 1993). Fig. 1A illustrates a representative trace of a $GABA_{A\rho1}$ response elicited by GABA (3 μM). Once the ionic current reached the plateau, quercetin (Que=8 μM) was delivered through the superfusion system (*on-top* application, indicated by the bar) producing its characteristic inhibitory effect (Goutman and Calvo, 2004).

As mentioned above, we have recently reported that $GABA_{A\rho1}$ receptor activity is potentiated by ascorbic acid in a manner strongly dependent on GABA concentration. For example, 3 mM ascorbic acid produced approximately a 200% enhancement in

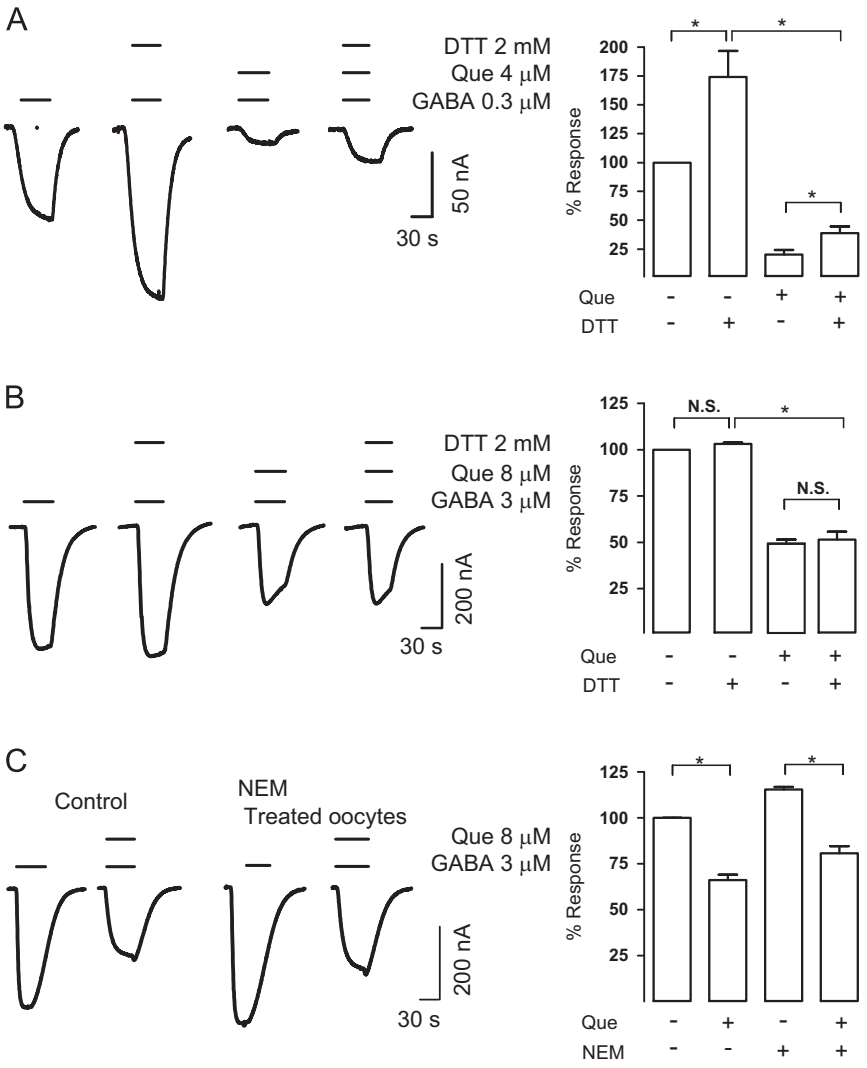

Fig. 2. Thiol reagents do not prevent quercetin inhibition of $GABA_{A\rho1}$ receptor responses. (A) Left: $GABA_{A\rho1}$ responses elicited by consecutive applications of GABA: alone (0.3 μM; control), with DTT (2 mM), with quercetin (4 μM) or with both modulators. Applications were spaced by 2 min washings. Right: bar chart summarizing these results. (B) Left: same as in (A), but GABA 3 μM and quercetin 8 μM. (C) Quercetin effects in control oocytes or in oocytes pretreated (2 min 30 s) with NEM (30 μM).

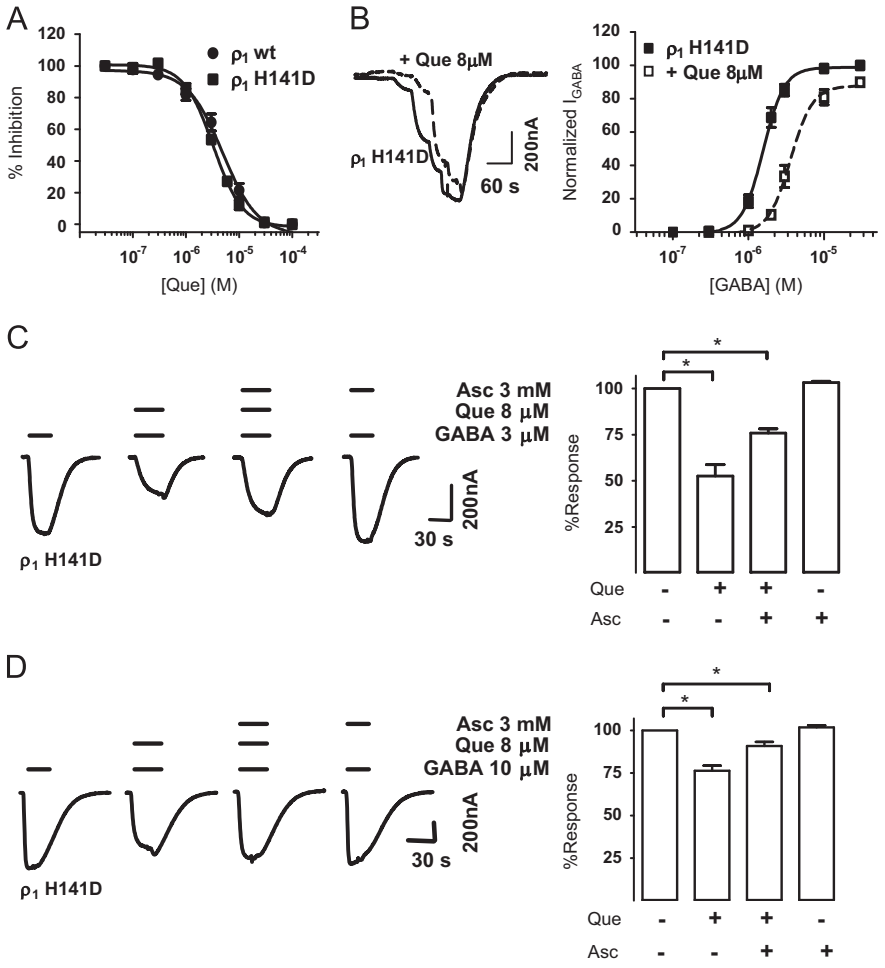

Fig. 3. Extracellular histidine 141 in ρ1 subunits is essential for ascorbic acid prevention of quercetin antagonism of $GABA_{A\rho1}$ receptors. (A) Quercetin inhibition of $GABA_{A\rho1}$ responses in wild-type receptors or mutant $^{H141D}GABA_{A\rho1}$ receptors, expressed as a fraction of control values obtained in the absence of flavonoid. (B) Left: representative $^{H141D}GABA_{A\rho1}$ receptor responses induced by increasing GABA concentrations (in steps), before (filled line) and after (dotted line) quercetin application. Right: D–R curves for GABA in the absence (closed squares), or presence (open squares) of quercetin. Current amplitudes expressed as a fraction of the maximal response evoked by 30 μM GABA. Each point represents the mean and SEM of the responses obtained in 6 oocytes (some error bars are hidden by symbols). (C) Ascorbic acid effects were impaired in $^{H141D}GABA_{A\rho1}$ receptors. Left: representative traces illustrating quercetin inhibition of $^{H141D}GABA_{A\rho1}$ receptor responses (GABA=3 μM) in the presence or absence of ascorbic acid. Right: bar chart summarizing these experiments. (D) Same as (C) but GABA was 10 μM. In the bar charts responses were normalized to control current (3 μM or 10 μM GABA).

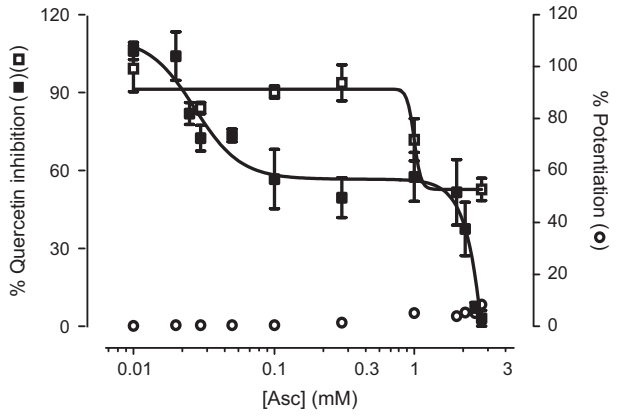

Fig. 4. D–R analysis. Degree of inhibition produced by quercetin on wild-type (closed squares) or mutant $^{H141D}GABA_{A\rho1}$ receptor (open squares) responses (left *y* axis) in the presence of increasing concentrations of ascorbic acid. Inhibition expressed as a fraction of the decrease in the GABA response (3 μM) induced by quercetin alone (8 μM) or in the presence of different ascorbic acid concentrations {[% Inhibition (Que+Asc)/Inhibition (Que)] × 100} (left *y* axis). Abscissa represents a logarithmic scale. Effects of ascorbic acid alone on wild-type $GABA_{A\rho1}$ receptor responses (right *y* axis, open circles, error bars hidden by symbols).

$GABA_{A\rho1}$ receptor response amplitudes (GABA=0.3 μM) (Calero et al., 2011), but this potentiating effect was completely surmounted by saturating concentrations of agonist (over 5 mM). Consistently, in the present study *on-top* applications of 3 mM ascorbic acid only produced a very mild but significant enhancement in the amplitude of $GABA_{A\rho1}$ responses evoked by high but non-saturating ($EC_{75\%}$) GABA concentrations (Fig. 1B) (% potentiation = 8.9 ± 1.9%, $n=6$). To further examine the mechanism involved in quercetin modulation, the effects of quercetin and ascorbic acid on $GABA_{A\rho1}$ receptor responses were independently or simultaneously tested (Fig. 1C). We found that quercetin inhibition of $GABA_{A\rho1}$ responses was largely prevented by bath applications of ascorbic acid and the degree of prevention cannot be explained by the small potentiating effect produced by applications of ascorbic acid when applied alone. In fact, the percentage of potentiation induced by ascorbic acid on $GABA_{A\rho1}$ responses was identical in the absence or presence of quercetin. All these actions were fast, easily reversible and equivalent results were obtained by using co-incubations (similar to that shown in experiments of Fig. 2) instead of *on-top* applications of the modulators (as in Fig. 1D). It is also important to remark that quercetin and ascorbic acid do not react with each other in aqueous solution at the

concentrations and pH used in this study (Boots et al., 2003; Caprile et al., 2009; Song et al., 2002). Thus, a direct interaction between these compounds can be disregarded. Results were summarized in Fig. 1D. Percentages of the responses were normalized as a fraction of the control current values obtained during applications of 3 μM GABA alone and results were as follows: (% Response) Que = 67.2 ± 6.0%, Que+Asc = 94.6 ± 3.7%, Asc = 108.9 ± 1.9% (n = 6, *$p < 0.05$ One-way Anova, post test analysis Dunnett's Multiple Comparison Test).

3.2. Quercetin antagonism of $GABA_{A\rho1}$ receptors is not affected by thiol-selective agents

Previous studies have shown that thiol reducing or methylating reagents potentiate the activity of $GABA_{A\rho1}$ receptors by selectively acting at the ρ1 subunit extracellular Cys-loop (Calero and Calvo, 2008; Calero et al., 2011). Thus, we examined whether the antagonism of $GABA_{A\rho1}$ receptors by quercetin could also involve a redox mechanism. The first two records in Fig. 2A illustrate the significant potentiation produced by the reversible sulfhydryl agent DTT on $GABA_{A\rho1}$ receptor responses. The second two current traces show that although receptor responses were potentiated by DTT inhibition caused by quercetin could be quite well reproduced in the presence of this reducing agent. Similar results were obtained by using glutathione instead of DTT as reducing agent (data not shown). Quercetin concentration for these experiments was kept at 4 μM, because GABA responses evoked by 0.3 GABA would be fully blocked at higher doses of this flavonoid. Results summarized in the bar chart were as follows: (% Response) DTT = 174.1 ± 22.5%, Que = 20.1 ± 3.9%, Que+DTT = 38.8 ± 5.8% (n = 4, *$p < 0.05$ One-way Anova, post-test analysis Dunnett's Multiple Comparison Test). This experimental set was reproduced at a higher concentration of GABA (3 μM), a condition where the DTT-induced redox-mediated potentiation of the $GABA_{A\rho1}$ receptor responses is minimized, and equivalent results were obtained (Fig. 2B). (% Response) Que = 49.3 ± 2.2%, DTT = 103.1 ± 3.8%, Que +DTT = 51.4 ± 4.4% (n = 4, N.S. $p < 0.05$ One-way Anova, post-test analysis Dunnett's Multiple Comparison Test). In addition, the use of NEM, which irreversibly methylates cysteine residues at the Cys-loop, produced identical results (Fig. 3C). Quercetin effects were equivalent in control oocytes or in oocytes pretreated with NEM. (% Response): Que = 66.2 ± 2.8 (n = 3), GABA+NEM = 117.8 ± 2.1%, GABA+NEM+Que = 78.1 ± 3.9 (n = 3, *$p < 0.05$ One-way Anova post-test analysis Turkey's Multiple Comparison Test).

These data show that redox status of the ρ1 subunit Cys-loop was not relevant for quercetin antagonism.

3.3. Histidine 141 is essential for ascorbic acid prevention of quercetin antagonism of $GABA_{A\rho1}$ receptors

Given the fact that quercetin and ascorbic acid show structural similarities, the simplest way to explain the preventive effects of ascorbic acid on quercetin antagonism of $GABA_{A\rho1}$ receptors would be to assume a competitive mechanism as proposed for the ascorbate transporter (Song et al., 2002). A histidine residue (H141) located at the extracellular N-terminal domain of the ρ1 subunits was shown to be critical for allosteric potentiation of $GABA_{A\rho1}$ responses by ascorbic acid, but not required for the redox modulatory component which involves opening of the disulfide bridge at the Cys-loop (Calero et al., 2011). Thus, if quercetin and ascorbic acid shared one or more binding sites at these receptors, the substitution of H141 by D must impact on quercetin effects. Inhibition curves for quercetin were performed at their corresponding GABA $EC_{50's}$ ($GABA_{A\rho1}$ receptors = 1 μM, n = 4; $^{H141D}GABA_{A\rho1}$ receptors = 2 μM, n = 5) (Fig. 3A). Interestingly, we found that wild type and mutant $^{H141D}GABA_{A\rho1}$ receptors are similarly antagonized by quercetin. In addition, Fig. 3B shows that quercetin effects on D–R curves for GABA in oocytes expressing $^{H141D}GABA_{A\rho1}$ receptors did not differ from those effects previously reported for quercetin acting on wild-type $GABA_{A\rho1}$ receptors (Goutman and Calvo, 2004). In contrast, the preventive effects of ascorbic acid on quercetin inhibition of $GABA_{A\rho1}$ receptor responses were impaired in mutant receptors. The bar chart in Fig. 3C summarizes these results (% Response): Que = 52.6 ± 6.3%, Que+Asc = 75.9 ± 2.3% and Asc = 103.2 ± 0.7% (n = 5, *$p < 0.01$ One-way Anova, post test analysis Dunnett's Multiple Comparison Test). As shown, while quercetin antagonism of wild-type $GABA_{A\rho1}$ receptors ([GABA] = 3 μM, Que 8 μM, Vm = −70 mV) in the presence of ascorbic acid (3 mM) was negligible, inhibition of mutant $^{H141D}GABA_{A\rho1}$ receptor current responses by quercetin reached a 24% under equivalent experimental conditions (compare bar charts in Fig. 1D and C). Since the apparent affinity for GABA of mutant $^{H141D}GABA_{A\rho1}$ receptors (GABA EC_{50} = 1 μM) is slightly higher than that showed by wild-type $GABA_{A\rho1}$ receptors (GABA EC_{50} = 2 μM) (Calero et al., 2011), as a control we repeated the experimental scheme shown in Fig. 3C by only raising GABA concentration (10 μM). Fig. 3D shows that under these conditions ascorbic acid still was unable to prevent quercetin inhibition of the $GABA_{A\rho1}$ receptor responses. (% Response): Que = 76.3 ± 3.0%, Que +Asc = 90.9 ± 2.4% and Asc = 101.8 ± 1.2%, (n = 3, *$p < 0.01$ One-way Anova, post-test analysis Dunnett's Multiple Comparison Test). Thus, the lack of ascorbic acid effect in mutant receptors appears not to be related to the lower affinity of these receptors for GABA, but related to the fact that ascorbic acid might be less effective to exert an allosteric-induced structural transition on these receptors that impact on quercetin binding. The present results also support the idea that a competitive interaction between quercetin and ascorbic acid is improbable, and that modulatory effects of quercetin and ascorbic acid at the $GABA_{A\rho1}$ receptors would operate differently.

3.4. Dose response analysis

We had previously reported a more systematic concentration response analysis of the effects of quercetin on $GABA_{A\rho1}$ receptors (Goutman and Calvo, 2004; Goutman et al., 2003). Now we show that the degree of inhibition produced by quercetin on responses mediated by $GABA_{A\rho1}$ receptors could be gradually blocked by increasing concentrations of ascorbic acid (Fig. 4, closed squares, left axis). Dose-inhibition curve fits with a double sigmoid function which may well be consistent with the actions of two allosteric modulators acting together (Bardsley and Childs, 1975). The effect of ascorbic acid becomes significant beyond 20 μM, reaching a first plateau between 100 μM and 1 mM. At ascorbic acid concentrations above 1 mM, quercetin inhibition of $GABA_{A\rho1}$ receptor responses is fully blocked by increasing concentrations of ascorbic acid. At higher levels of ascorbic acid and high but not saturating GABA concentrations potentiating effects on $GABA_{A\rho1}$ receptors are small (Fig. 4, open circles, right axis) and may not contribute significantly to the preventive effect (see also Fig. 1C).

The effect of increasing concentrations of ascorbic acid on quercetin antagonistic actions on $GABA_{A\rho1}$ receptors was also evaluated for responses mediated by $^{H141D}GABA_{A\rho1}$ receptors. Even though the dose-inhibition curve followed a similar trend (Fig. 4, open squares), the substitution of H141 notably modified the degree of prevention induced by ascorbic acid all over the range of concentrations tested. Ascorbic acid was considerably less effective in preventing the inhibitory actions of quercetin on $^{H141D}GABA_{A\rho1}$ receptors even at the mM range, confirming observations described in Fig. 3B and C.

4. Discussion

The present study provides key evidence about the mechanisms of action of quercetin on $GABA_{A\rho1}$ receptors. Quercetin actions were analyzed through two different approaches, by using modulators whose mechanisms of action on $GABA_{A\rho1}$ receptors were previously defined (e.g: ascorbic acid and sulfhydryl reagents) and by mutational analysis to identify critical aminoacidic residues. Our findings suggest that antagonism exerted by quercetin on $GABA_{A\rho1}$ receptors is mediated through a redox-independent allosteric mechanism, a hypothesis based on different facts discussed below.

Thiol reagents with the ability to reversibly reduce disulfide bridges to sulfydryl groups (e.g.: DTT), as well as the irreversible thiol alkylating agent NEM that forms covalent bonds with free sulfhydryl groups preventing any further chemical reaction at these sites, were both unable to block quercetin effects. Therefore, the two extracellular cysteine residues forming the characteristic disulfide bridge (C177 and C191) would not be direct targets of quercetin actions. These data also confirmed that quercetin was not oxidized in the course of our assays, because if this was the case quercetin quinone derivatives should react with extracellular cysteines at the $GABA_{A\rho1}$ receptor subunits producing NEM-sensitive changes in channel activity (Boots et al., 2003). To our knowledge there are no other amino acidic residues in the ρ_1 subunits susceptible to undergo a redox modification by quercetin, so the involvement of an alternative redox reaction during quercetin antagonism of $GABA_{A\rho1}$ receptors is unlikely. Additionally, ascorbic acid was capable of preventing quercetin antagonism of $GABA_{A\rho1}$ responses even at concentrations below the range of those producing significant potentiation of the $GABA_{A\rho1}$ receptor activity. The preventive effects of ascorbic acid were dose dependent, at high ascorbic acid concentrations quercetin inhibition was completely surmounted, but a mild and yet significant potentiation of the $GABA_{A\rho1}$ receptors responses was observed. Interestingly, even though 1 mM ascorbic acid was unable to induce any changes in $GABA_{A\rho1}$ receptor responses elicited by 3 μM GABA, this concentration was enough to prevent up to 50% of the quercetin (8 μM) inhibition (Fig. 3). A plausible interpretation for these data is that ascorbic acid allosterically produces a conformational change in quercetin binding sites and results obtained using mutant receptors were in agreement with this hypothesis. Ascorbic acid failed to completely prevent quercetin antagonism of $^{H141D}GABA_{A\rho1}$ receptors at any of the concentrations used. These data suggest that the extracellular H141 is directly or indirectly involved in the mechanism of ascorbic acid action. Previous studies demonstrated that quercetin and ascorbic acid can be recognized at a same protein domain (Caprile et al., 2009; Song et al., 2002). In those studies, the reversible and non-competitive inhibition produced by quercetin on ascorbic acid transport could not be explained by ascorbic acid oxidation, because quercetin did not decrease ascorbic acid stability. Based on these evidences it was suggested that similarities between the chemical structure of certain flavonoids and ascorbic acid are crucial for this inhibition to take place. In particular, the presence of a double bond at C2–C3, a ketone at C4 and the hydrogen at C8 is critical (Song et al., 2002). In contrast, our present data suggest that chemical interactions of quercetin and ascorbic acid at the $GABA_{A\rho1}$ receptors might be rather different, because the H141 mutation did not affect quercetin inhibition curves but significantly changed the way ascorbic acid prevents quercetin antagonism of $GABA_{A\rho1}$ receptors, indicating that a pure competitive interaction is unlikely. Presumably ascorbic acid could turn $GABA_{A\rho1}$ receptors into a conformation less sensitive to quercetin and/or hide target sites for flavonoid action.

$GABA_{A\rho}$ receptors are much less abundant than other $GABA_A$ receptor subtypes in the CNS and display high levels of expression only in the retina (Enz et al., 1995; Farrant and Nusser, 2005). $GABA_{A\rho}$ receptors are mainly extrasynaptic in retinal bipolar cells and regulate the overall level of excitability of postsynaptic ganglionic neurons by controlling glutamate release at the synaptic terminal via tonic inhibition (Hull et al., 2006). Thus, $GABA_{A\rho}$ receptor modulators may have an important role in reducing or increasing tonic inhibition (Chebib et al., 2009; Johnston et al., 2003). The present data help to recognize structural determinants and the mechanisms underlying quercetin modulation of one particular class of ionotropic GABA receptor. Thus, we hope that our work will eventually contribute to the development of selective agents for GABA receptor subtypes.

On the other hand, quercetin can be used as an *in vivo* antioxidant agent in human subjects (Boots et al., 2008b). These effects were reported to be more evident when the basal level of oxidative stress is high, indicating that quercetin administration might be a helpful therapeutical approach for the treatment of related pathological processes (Boots et al., 2008a). Quercetin is frequently combined with ascorbic acid as herbal supplement for synergistic actions. Interestingly, we found that ascorbic acid seems to be capable to prevent redox-independent actions of quercetin on retinal ionotropic GABA receptors. Thus, the co-administration of ascorbic acid could also prevent possible undesirable secondary effects of quercetin on retinal inhibitory neurotransmission. However, considering the diverse functional effects of flavonoids and ascorbic acid in the retina and/or the visual system (Chiou and Xu, 2004; Calero et al., 2011), further studies will be necessary to elucidate the therapeutic significance of our observations.

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References

Abdel-Halim, H., Hanrahan, J.R., Hibbs, D.E., Johnston, G.A., Chebib, M., 2008. A molecular basis for agonist and antagonist actions at GABA(C) receptors. Chemical Biology and Drug Design 71, 306–327.

Avallone, R., Zanoli, P., Puia, G., Kleinschnitz, M., Schreier, P., Baraldi, M., 2000. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. Biochemical Pharmacology 59, 1387–1394.

Bardsley, W.G., Childs, R.E., 1975. Sigmoid curves, non-linear double-reciprocal plots and allosterism. Biochemical Journal 149, 313–328.

Boots, A.W., Haenen, G.R., Bast, A., 2008a. Health effects of quercetin: from antioxidant to nutraceutical. European Journal of Pharmacology 585, 325–337.

Boots, A.W., Wilms, L.C., Swennen, E.L.R., Kleinjans, J.C.S., Bast, A., Haenen, G.R., 2008b. In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. Nutrition 24, 703–710.

Boots, A.W., Kubben, N., Haenen, G.R., Bast, A., 2003. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochemical and Biophysical Research Communications 308, 560–565.

Boue-Grabot, E., Roudbaraki, M., Bascles, L., Tramu, G., Bloch, B., Garret, M., 1998. Expression of GABA receptor rho subunits in rat brain. Journal of Neurochemistry 70, 899–907.

Calero, C.I., Calvo, D.J., 2008. Redox modulation of homomeric rho1 GABA receptors. Journal of Neurochemistry 105, 2367–2374.

Calero, C.I., Vickers, E., Moraga Cid, G., Aguayo, L.G., von Gersdorff, H., Calvo, D.J., 2011. Allosteric modulation of retinal GABA receptors by ascorbic acid. Journal of Neuroscience 31, 9672–9682.

Cao, G., Sofic, E., Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radical Biology and Medicine 22, 749–760.

Caprile, T., Salazar, K., Astuya, A., Cisternas, P., Silva-Alvarez, C., Montecinos, H., Millan, C., de Los Angeles Garcia, M., Nualart, F., 2009. The Na^+-dependent L-ascorbic acid transporter SVCT2 expressed in brainstem cells, neurons, and neuroblastoma cells is inhibited by flavonoids. Journal of Neurochemistry 108, 563–577.

Chebib, M., Hinton, T., Schmid, K.L., Brinkworth, D., Qian, H., Matos, S., Kim, H.L., Abdel-Halim, H., Kumar, R.J., Johnston, G.A., Hanrahan, J.R., 2009. Novel, potent, and selective GABAC antagonists inhibit myopia development and facilitate learning and memory. Journal of Pharmacology and Experimental Therapeutics 328, 448–457.

Chiou, G.C.Y., Xu, X.-R., 2004. Effects of some natural flavonoids on retinal function recovery after ischemic insult in the rat. Journal of Ocular Pharmacology and Therapeutics 20, 107–113.
Dekermendjian, K., Kahnberg, P., Witt, M.R., Sterner, O., Nielsen, M., Liljefors, T., 1999. Structure–activity relationships and molecular modeling analysis of flavonoids binding to the benzodiazepine site of the rat brain GABA (A) receptor complex. Journal of Medicinal Chemistry 42, 4343–4350.
Elliott, A.J., Scheiber, S.A., Thomas, C., Pardini, R.S., 1992. Inhibition of glutathione reductase by flavonoids. A structure-activity study. Biochemical Pharmacology 44, 1603–1608.
Enz, R., Brandstatter, J.H., Hartveit, E., Wassle, H., Bormann, J., 1995. Expression of GABA receptor rho 1 and rho 2 subunits in the retina and brain of the rat. European Journal of Neuroscience 7, 1495–1501.
Farrant, M., Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nature Reviews Neuroscience 6, 215–229.
Gavande, N., Karim, N., Johnston, G.A., Hanrahan, J.R., Chebib, M., 2011. Identification of benzopyran-4-one derivatives (isoflavones) as positive modulators of GABA(A) receptors. ChemMedChem 6 (1340–1346), 1317.
Goutman, J.D., Calvo, D.J., 2004. Studies on the mechanisms of action of picrotoxin, quercetin and pregnanolone at the GABA rho 1 receptor. British Journal of Pharmacology 141, 717–727.
Goutman, J.D., Waxemberg, M.D., Donate-Oliver, F., Pomata, P.E., Calvo, D.J., 2003. Flavonoid modulation of ionic currents mediated by GABA(A) and GABA (C) receptors. European Journal of Pharmacology 461, 79–87.
Griebel, G., Perrault, G., Tan, S., Schoemaker, H., Sanger, D.J., 1999. Pharmacological studies on synthetic flavonoids: comparison with diazepam. Neuropharmacology 38, 965–977.
Haberlein, H., Tschiersch, K.P., Schafer, H.L., 1994. Flavonoids from *Leptospermum scoparium* with affinity to the benzodiazepine receptor characterized by structure activity relationships and in vivo studies of a plant extract. Pharmazie 49, 912–922.
Hall, B.J., Chebib, M., Hanrahan, J.R., Johnston, G.A., 2004. Flumazenil-independent positive modulation of gamma-aminobutyric acid action by 6-methylflavone at human recombinant alpha1beta2gamma2L and alpha1beta2 GABAA receptors. European Journal of Pharmacology 491, 1–8.
Hanrahan, J.R., Chebib, M., Davucheron, N.L., Hall, B.J., Johnston, G.A., 2003. Semisynthetic preparation of amentoflavone: a negative modulator at GABA (A) receptors. Bioorganic and Medicinal Chemistry Letters 13, 2281–2284.
Hanrahan, J.R., Chebib, M., Johnston, G.A., 2011. Flavonoid modulation of GABA (A) receptors. British Journal of Pharmacology 163, 234–245.
Hull, C., Li, G.L., von Gersdorff, H., 2006. GABA transporters regulate a standing GABAC receptor-mediated current at a retinal presynaptic terminal. Journal of Neuroscience 26, 6979–6984.
Ji, X.D., Melman, N., Jacobson, K.A., 1996. Interactions of flavonoids and other phytochemicals with adenosine receptors. Journal of Medicinal Chemistry 39, 781–788.
Johnston, G.A., Chebib, M., Hanrahan, J.R., Mewett, K.N., 2003. GABA(C) receptors as drug targets. Current Drug Targets CNS and Neurological Disorders 2, 260–268.
Johnston, G.A., Chebib, M., Hanrahan, J.R., Mewett, K.N., 2010. Neurochemicals for the investigation of GABA(C) receptors. Neurochemical Research 35, 1970–1977.
Karim, N., Curmi, J., Gavande, N., Johnston, G.A., Hanrahan, J.R., Tierney, M.L., Chebib, M.. 2′-Methoxy-6-methylflavone: a novel anxiolytic and sedative with subtype selective activating and modulating actions at GABA(A) receptors. British Journal of Pharmacology 165, 2012, pp. 880–896.
Karim, N., Gavande, N., Wellendorph, P., Johnston, G.A., Hanrahan, J.R., Chebib, M., 2011. 3-Hydroxy-2′-methoxy-6-methylflavone: a potent anxiolytic with a unique selectivity profile at GABA(A) receptor subtypes. Biochemical Pharmacology 82, 1971–1983.
Kavvadias, D., Sand, P., Youdim, K.A., Qaiser, M.Z., Rice-Evans, C., Baur, R., Sigel, E., Rausch, W.D., Riederer, P., Schreier, P., 2004. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsive effects. British Journal of Pharmacology 142, 811–820.
Koh, D.S., Reid, G., Vogel, W., 1994. Activating effect of the flavonoid phloretin on $Ca^{(2+)}$-activated K^+ channels in myelinated nerve fibers of *Xenopus laevis* (corrected). Neuroscience Letters 165, 167–170.
Kusama, T., Spivak, C.E., Whiting, P., Dawson, V.L., Schaeffer, J.C., Uhl, G.R., 1993. Pharmacology of GABA rho 1 and GABA alpha/beta receptors expressed in *Xenopus* oocytes and COS cells. British Journal of Pharmacology 109, 200–206.
Lee, B.H., Choi, S.H., Shin, T.J., Pyo, M.K., Hwang, S.H., Lee, S.M., Paik, H.D., Kim, H.C., Nah, S.Y., 2008. Effects of quercetin on alpha9alpha10 nicotinic acetylcholine receptor-mediated ion currents. European Journal of Pharmacology 650, 79–85.
Loscalzo, L.M., Yow, T.T., Wasowski, C., Chebib, M., Marder, M. Hesperidin induces antinociceptive effect in mice and its aglycone, hesperetin, binds to mu-opioid receptor and inhibits GIRK1/2 currents. Pharmacology Biochemistry and Behavior 99, 2011, pp. 333–341.
Mall, M., Wissner, A., Seydewitz, H.H., Hubner, M., Kuehr, J., Brandis, M., Greger, R., Kunzelmann, K., 2000. Effect of genistein on native epithelial tissue from normal individuals and CF patients and on ion channels expressed in *Xenopus* oocytes. British Journal of Pharmacology 130, 1884–1892.
Marder, M., Paladini, A.C., 2002. GABA(A)-receptor ligands of flavonoid structure. Current Topics in Medicinal Chemistry 2, 853–867.
Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1997. Overview – flavonoids: a new family of benzodiazepine receptor ligands. Neurochemical Research 22, 419–425.
Nielsen, M., Frokjaer, S., Braestrup, C., 1988. High affinity of the naturally-occurring biflavonoid, amentoflavon, to brain benzodiazepine receptors in vitro. Biochemical Pharmacology 37, 3285–3287.
Ren, L., Wang, F., Xu, Z., Chan, W.M., Zhao, C., Xue, H., 2010. GABA(A) receptor subtype selectivity underlying anxiolytic effect of 6-hydroxyflavone. Biochemical Pharmacology 79, 1337–1344.
Saponara, S., Sgaragli, G., Fusi, F., 2002. Quercetin as a novel activator of L-type $Ca^{(2+)}$ channels in rat tail artery smooth muscle cells. British Journal of Pharmacology 135, 1819–1827.
Song, J., Kwon, O., Chen, S., Daruwala, R., Eck, P., Park, J.B., Levine, M., 2002. Flavonoid inhibition of sodium-dependent vitamin C transporter 1 (SVCT1) and glucose transporter isoform 2 (GLUT2), intestinal transporters for vitamin C and glucose. Journal of Biological Chemistry 277, 15252–15260.
Williams, R.J., Spencer, J.P., Rice-Evans, C., 2004. Flavonoids: antioxidants or signalling molecules? Free Radical Biology and Medicine 36, 838–849.
Woodward, R.M., Polenzani, L., Miledi, R., 1993. Characterization of bicuculline/baclofen-insensitive (rho-like) gamma-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of gamma-aminobutyric acidA and gamma-aminobutyric acidB receptor agonists and antagonists. Molecular Pharmacology 43, 609–625.
Zhang, D., Pan, Z.H., Awobuluyi, M., Lipton, S.A., 2001. Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. Trends in Pharmacological Sciences 22, 121–132.