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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)

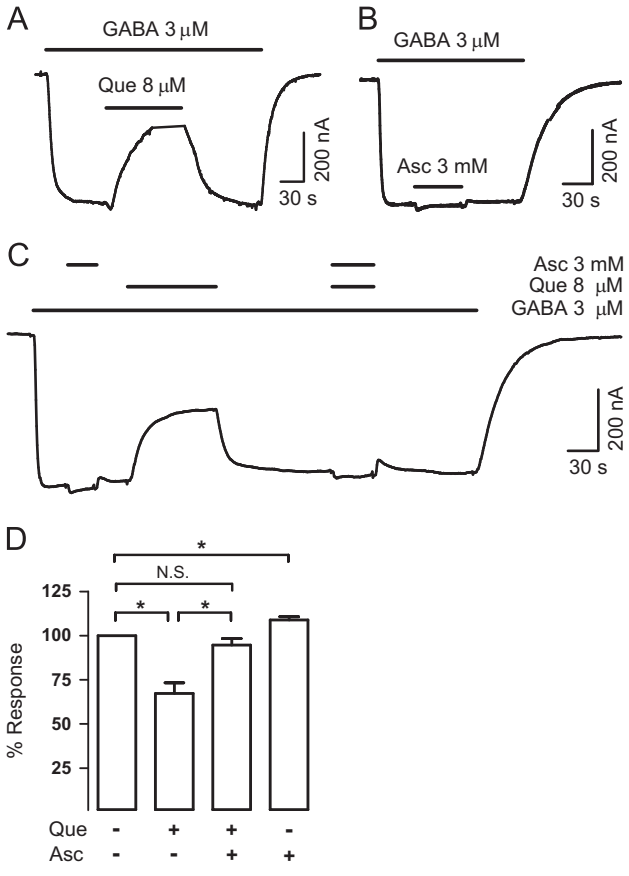

Fig. 1. Ascorbic acid prevents quercetin inhibition of $GABA_{A\rho1}$ receptors expressed in *Xenopus laevis* oocytes. (A, B, C) Representative traces of $GABA_{A\rho1}$ responses (Cl^- currents) elicited by GABA (3 µM). Bars indicate *on-top* applications of (A) quercetin (Que = 8 µM) or (B) ascorbic 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).