

Flavonoid modulation of ionic currents mediated by GABA_A and GABA_C receptors

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Abstract

The modulation of ionotropic γ -aminobutyric acid (GABA) receptors (GABA-gated Cl[−] channels) by a group of natural and synthetic flavonoids was studied in electrophysiological experiments. Quercetin, apigenin, morine, chrysin and flavone inhibited ionic currents mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors expressed in *Xenopus laevis* oocytes in the micromolar range. $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors differ largely in their sensitivity to benzodiazepines, but they were similarly modulated by different flavonoids. Quercetin produced comparable actions on currents mediated by $\alpha_4\beta_2$ neuronal nicotinic acetylcholine, serotonin 5-HT_{3A} and glutamate AMPA/kainate receptors. Sedative and anxiolytic flavonoids, like chrysin or apigenin, failed to potentiate but antagonized $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors. Effects of apigenin and quercetin on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors were insensitive to the benzodiazepine antagonist flumazenil. Results indicate that mechanism/s underlying the modulation of ionotropic GABA receptors by some flavonoids differs from that described for classic benzodiazepine modulation.

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1. Introduction

Flavonoids are substances of low molecular weight found in vascular plants (Bohm, 1998; Harborne and Williams, 2000). They show a wide range of biological activities (Miksicek, 1993; Middleton and Kandaswami, 1994; Harborne and Williams, 2000), with neuropharmacological actions such as analgesia, effects on motility and sleep (Speroni and Minghetti, 1988; Picq et al., 1991), modulation of neuronal oxidative metabolism (Oyama et al., 1994), proconvulsant, anticonvulsant, sedative and anxiolytic effects (Speroni and Minghetti, 1988; Medina et al.,

1990; Häberlein et al., 1994; Viola et al., 1995; Marder et al., 1995; Medina et al., 1998; Griebel et al., 1999), etc.

Effects of flavonoids on the central nervous system (CNS) are complex and involve different mechanisms (Vohora et al., 1980; Chakravarthy et al., 1981; Medina et al., 1998), including actions on synaptic receptors or ionic channels (Nielsen et al., 1988; Koh et al., 1994; Ji et al., 1996; Medina et al., 1998; Simmen et al., 1998; Dekermendjian et al., 1999; Avallone et al., 2000; Calvo et al., 2000; Mall et al., 2000; Saponara et al., 2002).

Many studies correlated the affinities of diverse natural and synthetic flavonoids for the benzodiazepine binding sites located to the GABA_A receptors (γ -aminobutyric acid: GABA) to their pharmacological properties shown in vivo (Medina et al., 1998; Griebel et al., 1999; Avallone et al., 2000; Viola et al., 2000). Based on these and other evidences, a benzodiazepine-like mechanism was proposed for flavonoid modulation of ionotropic GABA receptors (Medina et al., 1998; Dekermendjian et al., 1999; Marder and Paladini, 2002). However, experimental support for a ben-

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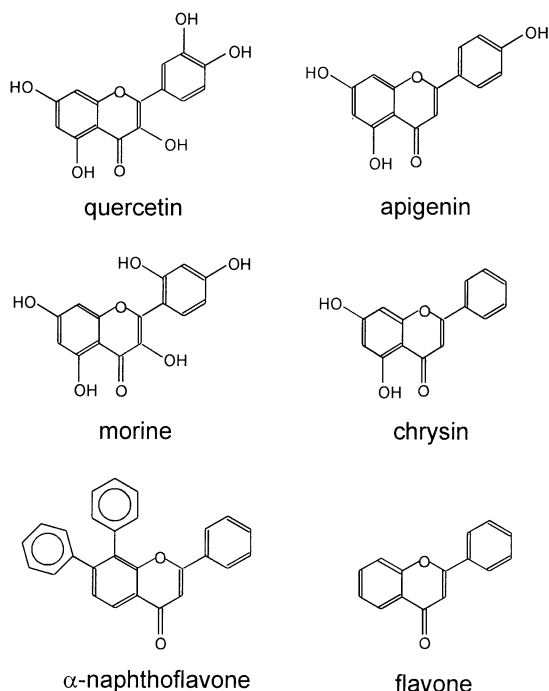


Fig. 1. Chemical structures of the flavonoids used in this study.

zodiazepine-like modulation of the GABA receptor function by flavonoids is still lacking. Now we tested this hypothesis by studying the effects of a group of flavonoids (chemical structures illustrated in Fig. 1) on ionic currents mediated by two common ionotropic GABA receptor subtypes.

GABA_A receptors are heteromeric proteins forming pentameric structures assembled from diverse subunit types (α_1 – α_6 , β_1 – β_4 , γ_1 – γ_3 , δ , ϵ and π) (Barnard et al., 1998; Hevers and Luddens, 1998). In contrast, GABA_C receptors would be homomeric, exclusively composed by ρ subunits (Enz and Cutting, 1998). $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors are highly expressed in the brain and represent a quite common GABA_A receptor subtype (in cerebral cortex, thalamus, etc.) that is strongly potentiated by benzodiazepines (Hevers and Luddens, 1998). ρ_1 GABA_C receptors are mainly expressed in the retina and mediate benzodiazepine-insensitive responses (Zhang et al., 2001).

We studied the effects of flavonoids on $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors expressed in *Xenopus* oocytes. Results indicated that diverse flavonoids antagonized ionic currents mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors in a similar way and did not behave as benzodiazepine-like modulators.

2. Materials and methods

2.1. RNA preparation for oocyte injections

Full-length cDNAs, encoding the different receptor subunits, cloned in vectors suitable for in vitro transcription,

were provided by colleagues (see acknowledgments). Rat GABA_A receptor subunits: α_1 and β_1 in pBluescript SK[−] (Promega, Madison, WI, USA), γ_{2s} in pSP6 (Invitrogen, Groningen, The Netherlands). Human GABA_A receptor subunits: α_1 , β_1 and γ_{2s} in pCDM8 (Invitrogen). Human GABA_C receptor subunit: ρ_1 in pBluescript SK[−] (Promega). Rat neuronal nicotinic acetylcholine receptor subunits α_4 , β_2 and rat 5-HT_{3A} (serotonin: 5-HT) receptor subunit were cloned in pGEMHE. cRNAs were synthesized using the mMessage mMachine transcription kit (Ambion, Austin, TX, USA). Isolation of native mRNA from rat cerebral cortex was carried out as previously described (Miledi et al., 1989).

2.2. Voltage-clamp recording in oocytes

Xenopus laevis oocytes at stages V and VI were used for injection and expression of exogenous mRNAs (Miledi et al., 1989). Briefly, frogs were anesthetized with 3-amino-benzoic ethylester (~1 mg/ml) and ovaries were surgically removed. After treating the cells with collagenase (230 units/ml, 50 min, room temperature), they were maintained in an incubator at 17 °C in Barth's medium (in mM: 88 NaCl; 0.33 Ca(NO₃)₂; 0.41 CaCl₂; 1 KCl; 0.82 MgSO₄; 2.4 NaHCO₃; 10 HEPES; pH adjusted to 7.4 with NaOH, with 0.1 mg/ml gentamycin). One day later, each oocyte was manually microinjected (microinjector Drummond Sci., Broomall, PA, USA) with 50 nl of 5 to 50 ng of cRNA dissolved in RNase-free water.

Recordings were performed, 3 to 7 days after injection, with an Axoclamp 2B two-electrode voltage-clamp (Axon Instruments, Foster City, CA, USA), placing the cells in a chamber (approx. 0.1-ml volume) continuously superfused (10 ml/min) with frog Ringer's solution (in mM: 115 NaCl; 2 KCl; 1.8 CaCl₂; 5 HEPES; pH 7.0). Agonists and modulators were applied through the perfusion system. Standard glass recording electrodes were filled with 3 M KCl, and resistance values were approximately of 1 MΩ. Holding potential was set to −70 mV and current traces were acquired in a PC through a Labmaster TL-1 DMA interface (Scientific Solutions, Solon, OH, USA) using AXOTAPE software (Axon Instruments). Current recordings for drug testing were always flanked by control responses. All the experiments were carried out at room temperature 23–24 °C.

A set of experiments was performed incubating the oocytes for 3 h in frog Ringer's solution with 1,2-bis (2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid/acetoxymethyl ester (BAPTA-AM) 0.1 mM to avoid interference of the endogenous oocyte Cl[−] current, which can potentially be activated in response to the entrance of Ca²⁺ through neuronal nicotinic acetylcholine, 5-HT or glutamate AMPA/kainate receptors (Miledi and Parker, 1984; Gundersen et al., 1984; Buisson et al., 1996; Blaschke et al., 1997; van Hooft and Vijverberg, 2000; Frerking and Nicoll, 2000).

Data were illustrated and analyzed using Origin v. 6.0 (MicroCal, Northampton, MA, USA). Results were pre-

sented as the mean \pm S.E.M. IC_{50} represents the concentration of flavonoid that blocks half-maximal current response. IC_{50s} for quercetin were calculated from inhibition curves, fitting normalized data points to the equation $\% I_{max} = 100[1/(1+(X/IC_{50})^n)]$, where I_{max} is the maximal ionic current elicited by 300 μ M GABA for $GABA_A$ receptors and 30 μ M for $GABA_C$ receptors in the absence of quercetin, X is the quercetin concentration, and n is the Hill coefficient. Two-tailed Student's t -test was used to determine statistical significant difference between two sample groups. In this study, independent sampling and comparison were used, and $p < 0.05$ was set as a significant level.

2.3. Materials

Different classes of flavonoids were used, natural: quercetin (3,3',4',5,7-pentahydroxyflavone), chrysin (5,7-dihydroxyflavone), apigenin (4',5,7-trihydroxyflavone), morine (2',3,4',5,7-pentahydroxyflavone), flavone (2-phenyl-4H-1-benzopyran-4-one); or synthetic: α -naphthoflavone (7,8-benzoflavone) (Fig. 1). Flavonoids and flumazenil solutions were made up freshly each day in normal Ringer's containing dimethyl sulfoxide (DMSO) to a maximal concentration of 0.3%. Up to this value, neither alterations in the oocyte properties nor direct actions of the solvent on responses tested were found over the recording time. pH was always adjusted to 7.0. Flavonoids were applied 30 s before their co-application with GABA, and both were washed at the same time after responses reached a plateau. All chemicals were purchased from Sigma (St. Louis, MO, USA) with the exception of collagenase (type I or II) from Worthington Biochemical (Lakewood, NJ, USA), *trans*- and *cis*-amino-crotonic acid from Tocris Cookson (Bristol, UK) and flumazenil (RO 15-1788, 8-fluoro-3-carboethoxy-5,6-dihydro-5-methyl-oxo-4H imidazol[1,5- α] 1,4 benzodiazepine) from Hoffmann-La Roche (NJ, USA).

3. Results

The modulation of ionic currents mediated by $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors by quercetin, chrysin, apigenin, morine, flavone and α -naphthoflavone was examined in electrophysiological experiments. Selectivity of the flavonoid actions was also analyzed by studying in a similar way the effects of quercetin on other ionotropic neurotransmitter receptors, namely, $\alpha_4\beta_2$ neuronal nicotinic acetylcholine, 5-HT $_{3A}$ and glutamate AMPA/kainate receptors.

3.1. Effects of flavonoids on $GABA_A$ and $GABA_C$ receptors expressed in *Xenopus* oocytes

3.1.1. Effects of quercetin on $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors expressed in *Xenopus* oocytes

Quercetin, an anthoxanthin first isolated from oaks (genus *Quercus*) and widely distributed in nature (in lemon, aspar-

agus, etc), inhibited GABA-mediated ionic currents on both $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors expressed in oocytes (Fig. 2A and B). Quercetin 30 μ M inhibited $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ receptor responses in $84.5 \pm 6.1\%$ ($n = 3$) and ρ_1 $GABA_C$ receptor responses in $98.9 \pm 1.1\%$ ($n = 3$). Analysis of inhibition curves of $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors by quercetin yielded IC_{50s} of $4.8 \pm 0.5 \mu$ M (n Hill = 1.4 ± 0.2) and $4.4 \pm 0.4 \mu$ M (n Hill = 1.5 ± 0.2), respectively ($n = 4$) (insets Fig. 2A and B). No significant differences were found between these IC_{50s} ($p > 0.05$).

Quercetin actions on responses mediated by $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors were not surmountable by increases in the GABA concentration up to 1 mM, a concentration that yields maximal responses on both GABA

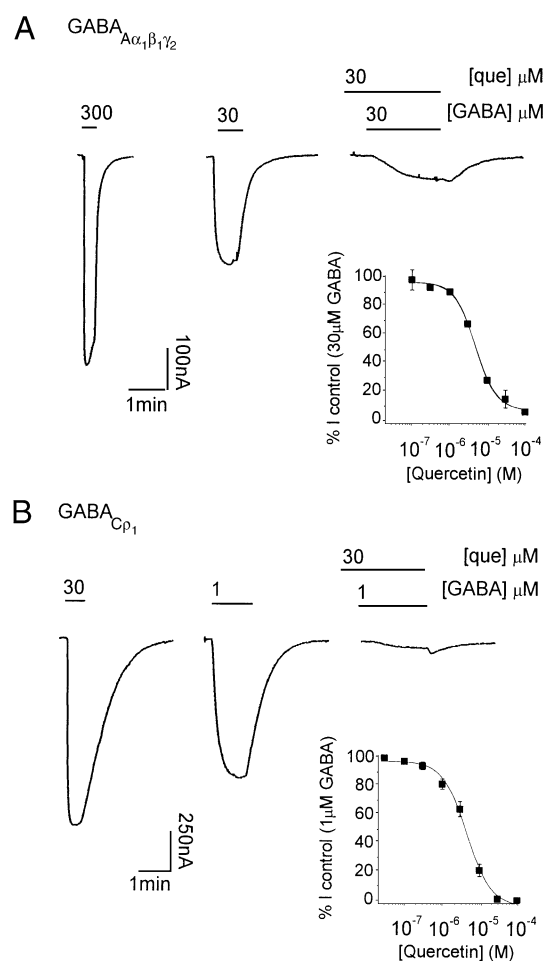


Fig. 2. Effects of the natural flavonoid quercetin on $GABA_A$ and $GABA_C$ receptors expressed in *Xenopus* oocytes. (A) Representative responses (Cl^- currents) mediated by rat $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ receptors ($GABA_{A\alpha_1\beta_1\gamma_{2s}}$), control or after exposure to quercetin (que). Flavonoid (30 μ M) was added 30 s before and during the application of GABA 30 μ M. On the left, maximal response to GABA 300 μ M. Inset: curve of inhibition for quercetin made at the GABA EC_{50} (30 μ M). (B) Same as in (A), but responses (Cl^- currents) mediated by ρ_1 $GABA_C$ receptors from human retina were activated by GABA 1 μ M. On the left, maximal response to GABA 30 μ M. Inset: curve of inhibition for quercetin made at the GABA EC_{50} (1 μ M). (A) and (B) ($V_{hold} = -70$ mV). Scale bars indicate current amplitudes (y axis) and time (x axis).

receptors (not shown). Effects of quercetin were fast and reversible and this can be appreciated in Fig. 2 as rapid deflections of the current trace at the end of drug applications. Small peaks seen in the records were due to partial recovery of the GABA response during quercetin washout. Ionic currents evoked by applications of GABA alone at maximal concentrations (Fig. 2A and B, left) were also included as a reference.

As the efficacy of GABA for eliciting currents through $\alpha_1\beta_1\gamma_{2s}$ GABA_A or ρ_1 GABA_C receptors differs, experiments were done at the corresponding EC₅₀ values (calculated from dose–response curves, not shown). Desensitization of $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors, due to repeated stimulation with GABA, was not marked for the used agonist concentration. Nevertheless, decay of GABA responses was circumvented doing short (30 s) GABA applications separated by recovery intervals of 12 min. Under the same experimental conditions, ρ_1 GABA_C receptors underwent negligible desensitization.

3.1.2. Effects of different flavonoids on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors

Fig. 3A illustrates representative responses evoked by GABA 30 μ M in oocytes expressing rat $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors. Recordings were obtained in the presence (thin line) or absence (thick line) of increasing concentrations of flavonoids. Superimposed ionic current traces were composed for simplicity (see explanation in Fig. 3 legend).

All the flavonoids tested were inactive at nanomolar concentrations but in the micromolar range exhibited different degrees of inhibition. Dose-dependent actions were observed on $\alpha_1\beta_1\gamma_{2s}$ GABA_A responses for apigenin, mor-

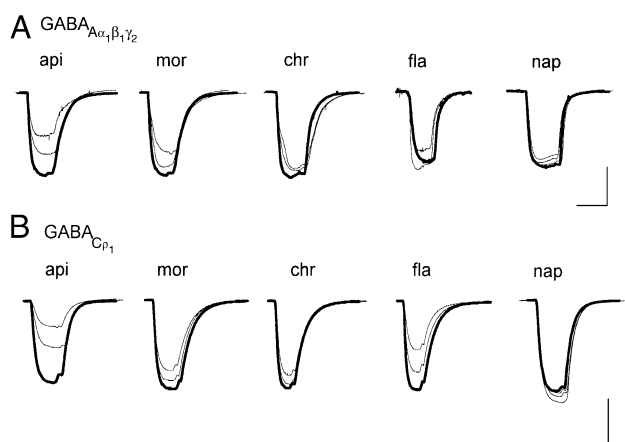


Fig. 3. Effects of different flavonoids on $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors expressed in *Xenopus* oocytes. (A) Representative responses mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors elicited by GABA 30 μ M either alone (thick line) or in the presence (thin line) of apigenin (api), morine (mor), chrysin (chr) or flavone (fla) (10 and 30 μ M), or α -naphthoflavone (nap) (1, 3 and 10 μ M). (B) Same as in (A) but responses to GABA 1 μ M were mediated by ρ_1 GABA_C receptors. (A) and (B) ($V_{\text{hold}} = -70$ mV). Flavonoids were added 30 s before and during the application of GABA. Scale bars indicate current amplitudes (y axis, 50 nA) and time (x axis, 30 s).

Table 1

Modulation of GABA_A and GABA_C receptors by natural and synthetic flavonoids

| Flavonoid | () μ M | GABA _{A$\alpha_1\beta_1\gamma_{2s}$} % of change (mean \pm S.E.) | n | GABA _{Cρ_1} % of change (mean \pm S.E.) | n |
|-----------|----------------|--|---|--|---|
| que | 10 | -71.9 ± 1.5 | 3 | -82.7 ± 2.7 | 3 |
| | 30 | -84.5 ± 4.9 | 3 | -98.9 ± 1.1 | 3 |
| api | 10 | -27.4 ± 1.1 | 3 | -39.6 ± 2.1 | 2 |
| | 30 | -50.6 ± 0.8 | 3 | -69.5 ± 4.2 | 4 |
| mor | 10 | -14.6 ± 0.8 | 4 | -16.9 ± 3.0 | 3 |
| | 30 | -38.4 ± 4.8 | 5 | -41.3 ± 4.0 | 3 |
| chr | 10 | -9.7 ± 0.4 | 2 | -6.9 ± 0.3 | 4 |
| | 30 | -12.1 ± 0.5 | 2 | -23.3 ± 5.1 | 3 |
| fla | 10 | $+6.7 \pm 1.0$ | 4 | -26.9 ± 3.9 | 3 |
| | 30 | -14.8 ± 0.8 | 3 | -55.8 ± 6.2 | 3 |
| nap | 1 | -3.7 ± 0.1 | 2 | $+3.7 \pm 0.1$ | 2 |
| | 3 | -6.9 ± 0.2 | 2 | $+2.9 \pm 0.9$ | 3 |
| | 10 | -17.2 ± 3.2 | 5 | $+10.2 \pm 1.1$ | 5 |

Data express changes (in percentage of control values) observed in GABA_A and GABA_C receptor-mediated responses in the presence of different flavonoids at the concentrations indicated. Natural flavonoids: quercetin (que), apigenin (api), morine (mor) chrysin (chr), flavone (fla); synthetic flavonoid: naphthoflavone (nap). Control responses were elicited by GABA at ED₅₀ values: 30 μ M for GABA_A or 1 μ M for GABA_C receptors. Oocytes were voltage-clamped at -70 mV.

ine, chrysin and flavone. Fig. 3A and Table 1 show results obtained for flavonoid applications of 10 and 30 μ M (with the exception of α -naphthoflavone, see below).

Apigenin (a compound found in parsley and camomile flowers) and morine (present in old fustic, *Moraceae*, whose common names are Cuba wood or yellow Brazil wood) behaved similarly to quercetin (Figs. 2A and 3A and Table 1), but inhibitory effects were smaller. Apigenin 30 μ M inhibited GABA responses on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors a $56.5 \pm 5.5\%$ ($n=3$), while morine 30 μ M a $37.7 \pm 4.3\%$ ($n=5$). Under similar conditions, α -naphthoflavone (synthetic) and chrysin (found in passionflowers and in the buds of poplar trees) produced little effect on GABA responses (Table 1). α -Naphthoflavone was only assayed up to 10 μ M because this concentration is about its limit of solubility in Ringer's buffer (Table 1). α -Naphthoflavone 10 μ M inhibited GABA responses on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors $17.2 \pm 3.6\%$ ($n=5$), while inhibitory effect of chrysin 30 μ M was $12.1 \pm 0.7\%$ ($n=3$). Flavone, a nonsubstituted flavonoid analog (see Fig. 1) found in numerous plants, produced little but statistically significant effects of potentiation or inhibition of $\alpha_1\beta_1\gamma_{2s}$ GABA_A responses according to the concentration used (Table 1). Flavone 10 μ M potentiated GABA_{A $\alpha_1\beta_1\gamma_{2s}$} responses $6.8 \pm 1.2\%$ ($n=3$) and at 30 μ M inhibited them $14.9 \pm 1.1\%$ ($n=3$). The grade of antagonism for these flavonoids (30 μ M) on GABA responses mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors was: quercetin>apigenin>morine>flavone \approx chrysin (Student's *t*-test, $p<0.05$). In preliminary experiments, identical results were obtained for human $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors (data not shown). Quercetin, apigenin, morine and chrysin were also assayed on ionic currents mediated by native bicul-

line-sensitive GABA_A receptors recorded from oocytes injected with polyA⁺ mRNA from rat cerebral cortex, and all the flavonoids showed identical effects to those described for recombinant receptors (data not shown).

Effects of quercetin and apigenin on the $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors were insensitive to the benzodiazepine antagonist flumazenil at concentrations between 0.1 and 1 μ M. Fig. 4 illustrates some of these results. Inhibition of $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptor-mediated Cl[−] currents by quercetin 10 μ M or apigenin 30 μ M (values near IC_{50s}) was not prevented by flumazenil 1 μ M ($n=3$) (Fig. 4). At this concentration, flumazenil alone produced a slight increase ($8.9 \pm 1.0\%$, $n=6$) in the receptor responses elicited by GABA 30 μ M but effectively precluded diazepam (100 nM)-induced potentiation (Fig. 4C). These results would

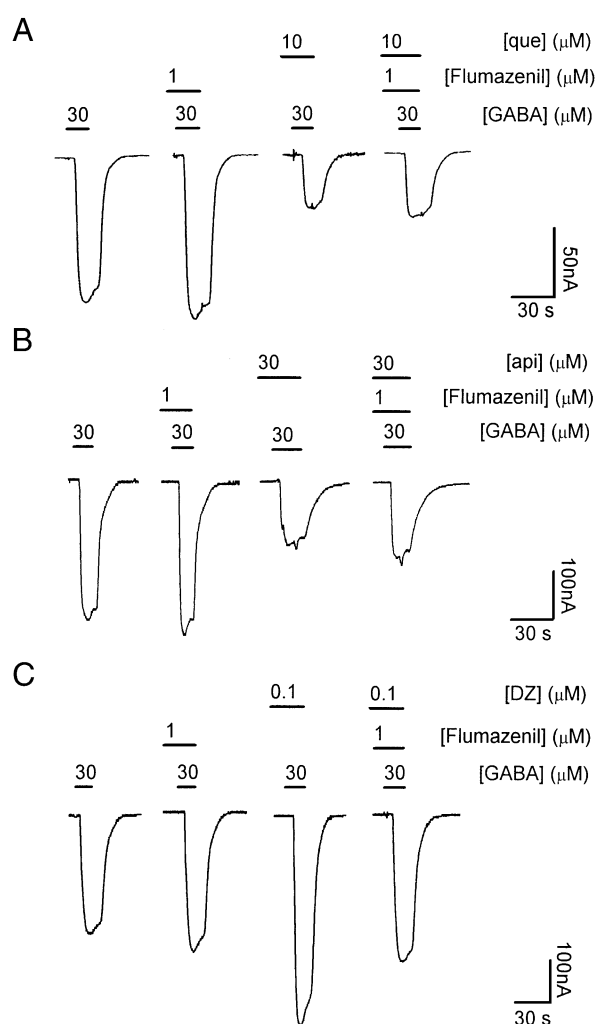


Fig. 4. Effects of quercetin and apigenin on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors were not prevented by the benzodiazepine antagonist flumazenil. (A) Representative responses mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors elicited by GABA 30 μ M either alone or in the presence of quercetin 10 μ M (que), or quercetin 10 μ M and flumazenil 1 μ M. (B) Same as in (A) but apigenin 30 μ M (api) was tested. (C) Representative control experiment showing diazepam 0.1 μ M (DZ) potentiation of a GABA-elicited current which was prevented by flumazenil 1 μ M ($V_{\text{hold}} = -70$ mV).

indicate that apigenin and quercetin do not exert their inhibitory action through the benzodiazepine site at this GABA_A receptor subtype.

No appreciable effects on the oocyte (injected or sham) properties, such as membrane potential, membrane resistance or current baseline under voltage-clamp, were observed even when relatively high concentrations of flavonoids alone were applied (quercetin, apigenin and chrysin, up to 100 μ M).

3.1.3. Effects of different flavonoids on ρ_1 GABA_C receptors

The same group of flavonoids was tested for effects on ρ_1 GABA_C receptors from human retina. Results are summarized in Fig. 3B and Table 1. Flavonoids showed a similar antagonistic profile to that observed for $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors (Fig. 3A and B), although some differences should be pointed out.

It was shown that quercetin was a relatively potent ρ_1 GABA_C receptor antagonist (Figs. 2B and 3B). Quercetin inhibition was independent on the agonist used. GABA, *trans*-aminocrotonic acid, *cis*-aminocrotonic acid or muscimol gave equivalent results (data not shown).

Inhibitory effects caused by quercetin, morine and apigenin (30 μ M) on responses mediated by ρ_1 GABA_C or $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors were equivalent (Table 1) (Student's *t*-test $p > 0.05$). Morine inhibited ρ_1 GABA_C receptor responses in a $41.3 \pm 4.0\%$, $n=3$, while apigenin in a $69.6 \pm 4.2\%$, ($n=4$). In contrast, chrysin and flavone were consistently more effective on ρ_1 GABA_C than on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors (Fig. 3A and B) (Student's *t*-test $p < 0.05$). Chrysin 30 μ M inhibited ρ_1 GABA_C receptor-mediated responses in a $24.3 \pm 4.3\%$ ($n=3$) and flavone 30 μ M in a $55.9 \pm 6.2\%$ ($n=3$).

Flavone and α -naphthoflavone displayed different actions on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors than those observed on ρ_1 GABA_C receptors (see records illustrated in Fig. 3A and B). It is worth to note that in contrast to the mild inhibitory effect exerted on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors by α -naphthoflavone, its effect on ρ_1 GABA_C receptors showed a very slight but consistent potentiation. α -Naphthoflavone 10 μ M significantly (Student's *t*-test $p < 0.05$) increased ρ_1 GABA_C receptor-mediated responses in a $10.2 \pm 1.1\%$ ($n=5$). Effects lasted throughout the application and were easily removed by washing with Ringer's solution. Ranking of antagonism by flavonoids (30 μ M) on ρ_1 GABA_C receptors was quercetin > apigenin \cong flavone \cong morine > chrysin.

3.2. Effects of quercetin on other neurotransmitter receptors

Given the fact that quercetin was the most effective modulator of the GABA_A and GABA_C receptors studied here, we tested its effects on other ionotropic neurotransmitter receptors to assess the selectivity of these actions. Fig. 5 depicts current traces corresponding to responses mediated by native and recombinant receptors from rat brain. Ionic currents mediated by cloned $\alpha_4\beta_2$ neuronal nicotinic acetylcholine and 5-HT_{3A} receptors and native

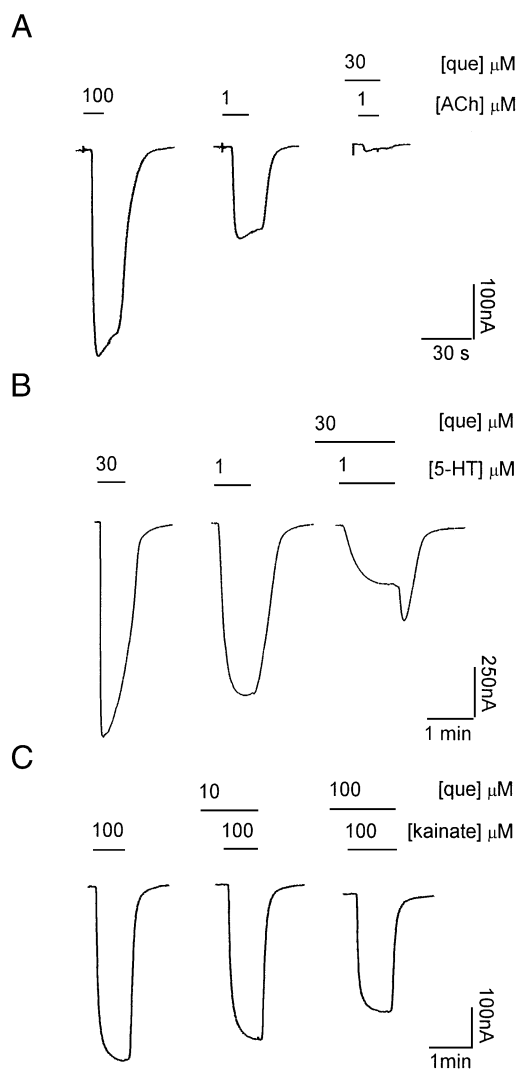


Fig. 5. Effects of quercetin on neuronal nicotinic acetylcholine, 5-HT and glutamate AMPA/kainate receptors expressed in *Xenopus* oocytes. Representative responses (cationic currents, see text) mediated by rat $\alpha_4\beta_2$ neuronal nicotinic acetylcholine (A), 5-HT_{3A} (B) and glutamate AMPA/kainate receptors (C) expressed in *Xenopus* oocytes. Experiments carried out as in Fig. 2. Acetylcholine 1 μ M, 5-HT 1 μ M and kainate 100 μ M were used to activate responses. (A), (B) and (C) ($V_{\text{hold}} = -70$ mV).

glutamate AMPA/kainate receptors were evaluated in the absence or the presence of quercetin in oocytes pre-incubated with BAPTA-AM. It is necessary to point out that pre-incubation of the oocytes with this chelator (see Materials and methods) produced no changes in the responses recorded in normal frog Ringer's solution, neither in magnitude nor in kinetics (not shown). This excluded the chance that a component due to activation of native Ca^{2+} -activated Cl^- conductances (Miledi and Parker, 1984) was present in this set of experiments. Thus, responses evoked by acetylcholine and 5-HT were entirely carried by $\text{Na}^+/\text{K}^+/\text{Ca}^{2+}$ ions permeating through these channels (Buisson et al., 1996; van Hooft and Vijverberg, 2000). On the other hand, responses mediated by native glutamate AMPA/kainate receptors (Gundersen et al., 1984; Blaschke et al., 1997)

were studied by expressing mRNA from rat cerebral cortex (see Materials and methods). Kainic acid (10 μ M to 1 mM) elicited inward membrane currents that were also carried by $\text{Na}^+/\text{K}^+/\text{Ca}^{2+}$ ions (Gundersen et al., 1984; Blaschke et al., 1997; Frerking and Nicoll, 2000). The slow time course of onset and lack of desensitization showed by these responses verified that native Cl^- conductance was not present or activated (Miledi and Parker, 1984).

It is interesting to note that quercetin also behaved as an antagonist at receptors mediating cationic currents. $\alpha_4\beta_2$ neuronal nicotinic acetylcholine receptors were also very sensitive to quercetin modulation (Fig. 5A). Quercetin 10 μ M inhibited a $48.1 \pm 5.1\%$ ($n=4$) responses elicited by acetylcholine 1 μ M ($n=4$) and quercetin 30 μ M inhibited them $79.9 \pm 4.9\%$ ($n=4$). 5-HT_{3A} receptors were inhibited by quercetin in a lesser extent (Fig. 5B), quercetin 10 μ M inhibited responses to 5-HT 1 μ M a $10.5 \pm 3.7\%$ ($n=3$) and for quercetin 30 μ M, the effect reached a $54.9 \pm 7.0\%$ ($n=6$). A rebound was observed at the end of the 5-HT-evoked response due to quercetin washout, similarly to that seen for GABA receptors (Fig. 2). Ionic currents elicited by kainate, in oocytes expressing rat cerebral cortex mRNA, were inhibited by quercetin in a much lesser degree than those mediated by the other receptors studied (Fig. 5C). Quercetin 100 μ M inhibited a $31.2 \pm 0.6\%$ ($n=4$) responses to kainate 100 μ M, quercetin 10 μ M inhibited them only $11.2 \pm 1.5\%$ ($n=4$) and no effect was noticed during quercetin 1 μ M applications ($n=4$). Kainate responses were insensitive to chrysin (100 μ M, $n=2$), in sharp contrast to GABA_A and GABA_C receptor-mediated responses which were approximately inhibited in a 40% (both of them) under identical conditions.

4. Discussion

We have characterized the pharmacological actions of a group of flavonoids on ionotropic GABA receptors through electrophysiological studies. A number of naturally occurring and synthetic compounds were selected based on their previously reported effects in binding assays and in vivo pharmacological experiments. The main contribution of the present work is the demonstration that diverse flavonoids modulate the function of GABA_A and GABA_C receptors and also of other ionotropic receptors expressed in *Xenopus* oocytes. We found that flavonoids produce inhibitory effects on $\alpha_1\beta_1\gamma_{2s}$ GABA_A and homomeric ρ_1 GABA_C receptors and also on $\alpha_4\beta_2$ neuronal nicotinic acetylcholine, 5-HT_{3A} and glutamate AMPA/kainate receptors.

4.1. Modulation of GABA_A and GABA_C receptors by flavonoids

Nearly all flavonoids analyzed acted as $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptor antagonists. Quercetin was the most effective, but other flavonoids, e.g. apigenin, morine

and chrysin, showed similar effects, whereas the synthetic flavonoid α -naphthoflavone was almost inactive.

It is known that chrysin and apigenin bind to the benzodiazepine sites located on GABA_A receptors (Medina et al., 1990, 1998; Viola et al., 1995; Avallone et al., 2000). Chrysin and apigenin also show sedative, anxiolytic or anticonvulsant properties in rodents (Medina et al., 1990, 1998; Wolfman et al., 1994; Viola et al., 1995). These and other data lead to propose a benzodiazepine-like action for flavonoids, with compounds acting as agonists, antagonists or inverse agonist on those benzodiazepine sites (Medina et al., 1990, 1998; Dekermendjian et al., 1999; Marder and Paladini, 2002). However, studies about the effects of flavonoids on ionic currents mediated by GABA_A receptors are scarce (Avallone et al., 2000). In addition, the selectivity of flavonoid actions for specific GABA receptor subtypes and their putative mechanism/s of action still are not known in detail.

It was reported that apigenin competitively inhibits the binding of flunitrazepam to brain GABA_A receptors with a K_i of 4 μ M and has anxiolytic activity in mice (3–10 mg/kg i.p.) (Viola et al., 1995). It was shown that chrysin has similar effects (K_i =3 μ M, anxiolytic at 1–10 mg/kg i.p.) (Medina et al., 1990; Wolfman et al., 1994). Meanwhile, their chemical analog quercetin shares none of these effects (Salgueiro et al., 1997). Conversely to a typical benzodiazepine action, we found that chrysin and apigenin did not potentiate ionic currents mediated by $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors. This was noticeable by a direct comparison of the effects produced by these two flavonoids and those produced by the benzodiazepine diazepam. Chrysin and apigenin, as well as quercetin, were inactive at nanomolar concentrations and acted as $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptor antagonists in the micromolar range. In addition, chrysin and apigenin displayed similar effects on $\alpha_1\beta_1\gamma_{2s}$ GABA_A and ρ_1 GABA_C receptors, opposite to benzodiazepines that only modulate GABA_A receptors. We have also found that the effects of apigenin and quercetin on GABA-induced ionic currents were not prevented by the benzodiazepine antagonist flumazenil (0.1–1 μ M).

Inhibitory effects observed for apigenin on $\alpha_1\beta_1\gamma_{2s}$ GABA_A receptors were in agreement with previous reports. Avallone et al. observed that apigenin (1–10 μ M) antagonized responses mediated by native GABA_A receptors in cerebellar granule cells. Moreover, and in contrast to previous results, they postulated that the sedative effect exerted by this flavonoid (25–50 mg/kg i.p.) was not mediated by benzodiazepine receptors, and that apigenin at doses of 0.5–10 mg/kg i.p. was not anxiolytic (Avallone et al., 2000).

On the other hand, flavone showed the most pronounced differences for actions observed on $\alpha_1\beta_1\gamma_{2s}$ GABA_A vs. ρ_1 GABA_C receptors. Further experiments will be necessary to determine the occurrence of flavonoids capable to discriminate among different GABA receptor subtypes.

Thus, GABA_A and GABA_C receptors studied here were antagonized by different flavonoids despite their differential benzodiazepine sensitivity. Potentiation of the GABA_A

receptor responses by flavonoids was not observed. Our results suggest that flavonoid actions on GABA_A receptors might differ from that described for a classic benzodiazepine modulation.

4.2. Modulation of other ionotropic neurotransmitter receptors by flavonoids

We have also examined the effects of quercetin on ionic currents mediated by other ionotropic neurotransmitter receptors. Interestingly, quercetin affected not only (ligand-gated) anionic channels, but also cationic channels activity, since it showed inhibitory effects on $\alpha_4\beta_2$ neuronal nicotinic acetylcholine, 5-HT_{3A} and glutamate AMPA/kainate receptors expressed in oocytes. These results are in agreement to previous evidences supporting multiple, instead of specific, mechanisms of action underlying flavonoid's effects on the CNS. For example, flavonoids can additionally alter the activity of metabotropic receptors (i.e. adenosine receptors: Ji et al., 1996; opioid receptors: Simmen et al., 1998), K^+ and Ca^{2+} channel gating (Koh et al., 1994; Saponara et al., 2002) and catecholamine uptake (Ramassamy et al., 1992). Therefore, CNS effects for one particular flavonoid could be triggered by alterations in different neurotransmission systems, changes in the neuronal excitability through modulation of voltage-activated ionic channels and/or many other mechanisms. A careful analysis will be needed in order to properly evaluate underlying mechanisms for pharmacological actions of flavonoids in the CNS.

Flavonoid modulation of neuronal nicotinic acetylcholine, 5-HT and glutamate AMPA/kainate receptors had not been reported previously and could be relevant due to the implication of these receptors in normal and pathological physiology; i.e. effects of nicotine in human brain and neuropathological disorders (Buisson et al., 1996); 5-HT roles in cerebral microcirculation, blood–brain barrier permeability, brain metabolism and migraine (Pattichis et al., 1995; Cohen et al., 1996); and excitatory amino acids receptor roles in neural plasticity and toxicity (Frerking and Nicoll, 2000). More experiments will be necessary to establish the sensitivity of diverse nicotinic, 5-HT and glutamate receptor subtypes to the different flavonoids and to find selective compounds.

4.3. Mechanisms of action for flavonoid modulation of the ionotropic neurotransmitter receptors

The pleiotropic character of flavonoid actions could be attributed to their capacity to interact with lipidic membranes (Movileanu et al., 2000). However, this simple explanation does not seem to be the case because effects of quercetin and other flavonoids on responses mediated by neurotransmitter receptors expressed in oocytes were rapid, easy reversible and developed at relatively low concentra-

tions (1–100 μM). Thus, it is unlikely that quercetin effects involve its insertion or penetration into the bilayer, which usually takes more than 1 h to develop (Movileanu et al., 2000). Thus, a direct interaction with amino acids in the receptor subunits or with the lipidic milieu could be plausible. We cannot discard dissimilar mechanisms taking place for different flavonoids.

Quercetin $\text{IC}_{50\text{s}}$ for ionotropic GABA receptors studied here were identical and of about 4 μM , with Hill coefficient values of approximately 1.5, suggesting at least two binding sites. A direct chemical interaction between quercetin and GABA is unlikely because similar antagonistic effects were observed with other structurally nonrelated agonists. Additionally, antagonism of ionotropic GABA receptors by quercetin was noncompetitive, which also suggests that this flavonoid does not bind to the agonist site.

We observed that flavonols (i.e. quercetin, apigenin, morine and chrysin, see Fig. 1) presented similar properties on $\alpha_1\beta_1\gamma_{2\text{s}}$ GABA_A or ρ_1 GABA_C receptors, while non-substituted analogs like flavone, or α -naphthoflavone, whose radicals are phenol groups, showed a different profile. As those effects were complex, and varied with the flavonoid concentration, additional experiments must be carried out to accurately establish mechanisms and structure–function relations for flavonoid actions.

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