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Determinants of amentoflavone interaction at the $GABA_A$ receptor

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Abstract

We investigated the recognition properties of different $GABA_A$ receptor subtypes and mutant receptors for the biflavonoid amentoflavone, a constituent of St. John's Wort. Radioligand binding studies showed that amentoflavone recognition paralleled that of the classical benzodiazepine diazepam in that it had little or no affinity for α_4- or α_6-containing receptors. Lysine and alanine substitutions at position 101 of the rat α_1 subunit resulted in a complete loss of competitive amentoflavone binding, but functional analysis of the alanine mutant expressed with β_2 and γ_2 subunits in *Xenopus* oocytes revealed no significant difference in the negative modulation of GABA-induced currents brought about by amentoflavone. Furthermore, elimination of the γ subunit had no effect on the negative modulation of these currents. This negative modulation was also observed at $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors and is therefore not likely mediated by the loreclezole site. These results suggest a complex mechanism of amentoflavone interaction at $GABA_A$ receptors.

Keywords: $GABA_A$ receptor; Benzodiazepine; Amentoflavone; Biflavonoid

1. Introduction

The flavonoids are a group of compounds found in all vascular plants and therefore constitute an important part of the human diet (Medina et al., 1997; Marder and Paladini, 2002). Although the full extent of biological activity of these compounds is largely unknown, research into flavonoid actions on the central nervous system (CNS) has begun to indicate effects at some neurotransmitter receptors (Butterweck et al., 2002). In particular, there has been increasing evidence that flavonoid effects may be mediated by the γ-aminobutyric $acid_A$ ($GABA_A$) receptor benzodiazepine ligand binding site (Nielsen et al., 1988; Medina et al., 1997; Marder and Paladini, 2002; Butterweck et al., 2002).

The $GABA_A$ receptor is the major inhibitory neurotransmitter receptor in the mammalian CNS, and is a member of the superfamily of ligand-gated ion channels (Sieghart, 1995; Barnard et al., 1998, Sieghart et al., 1999). Similar to other members of this superfamily (nicotinic acetylcholine receptor, $5HT_{3A}$ receptor, glycine receptor), the receptor is composed of five subunits arranged in a rosette conformation around a central chloride ion channel (Nayeem et al., 1994). Thus far, 19 subunits have been identified including 6α, 3β, 3γ, δ, ε, π, θ and 3ρ subunits belonging to the $GABA_C$ subtype (Barnard et al., 1998; Sieghart et al., 1999; Korpi et al., 2002). The $\alpha_1\beta_2\gamma_2$ receptor subtype is thought to be the most abundant subtype in the mammalian CNS.

The chloride current flowing through the integral ion channel of the $GABA_A$ receptor is gated by the endogenous agonist γ-aminobutyric acid (GABA), and can be modulated by a number of different classes of compounds including the barbiturates, the benzodiazepines, the cyclopyrrolones, ethanol, zinc, and the steroids (Sieghart, 1995). It has been shown that the presence of both an α and a γ subunit is necessary to form a benzodiazepine binding site (Pritchett et al., 1989; Zezula et al., 1996). Recent models based on the acetylcholine binding protein indicate that this site may best be described as a cleft or pocket within which benzodiazepine site ligands bind (Cromer et al., 2002). The large extracellular N-terminal domains of the α and γ subunits which interface are thought to contribute to formation of this

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cleft, with discrete subsets of amino acid residues from each subunit involved in forming binding contacts for ligands that interact at this site (Galzi and Changeux, 1994; Changeux and Edelstein, 1998). Specifically, loops A, B, and C of the α subunit are thought to contribute, along with loops D, E, and F of the γ subunit (Corringer et al., 2000; Cromer et al., 2002). Site-directed mutagenesis experiments involving point mutation of residues found within these loops allow for the study of subsequent effects on the affinity and efficacy of ligands that interact with the benzodiazepine binding site (Davies et al., 2001). Specifically, histidine 101 of loop A (rat α_1 subunit) is an amino acid residue which has been shown to be important to the binding of classical benzodiazepine agonists such as diazepam (Wieland et al., 1992). The α4 and α6 subunits, which have an arginine in this position, confer insensitivity to classical benzodiazepine agonists.

Amentoflavone, a biflavonoid constituent of St. John's Wort (*Hypericum perforatum*), was the first flavonoid compound shown to have very high affinity for brain benzodiazepine receptors in vitro, being able to inhibit [^{3}H]-flunitrazepam binding with an IC_{50} of 6 nM (Nielsen et al., 1988). Subsequent studies with $GABA_A$ receptors have shown similar results (Baureithel et al., 1997; Butterweck et al., 2002). A recent study used functional $GABA_A$ receptor assays to show that a semisynthetic preparation of amentoflavone acted as a negative modulator of GABA at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, and that this negative modulation was not blocked by the addition of the benzodiazepine site antagonist flumazenil (Hanrahan et al., 2003).

The present study examined the binding affinity of amentoflavone at recombinant wild type and mutant $GABA_A$ receptors. In particular, we looked at recombinant wild type receptors containing α_1, α_4, α_5, or α_6 subunits expressed in combination with β_2 and γ_2 subunits. Additionally, mutant receptors incorporating the array of histidine 101 mutant α_1 subunits constructed previously in this lab were also studied (Davies et al., 1998). Changes in affinity resulting from amino acid substitution at histidine 101, as compared to rat brain membrane $GABA_A$ receptors and wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, were examined. Functional modulation of GABA-gated chloride currents, measured from recombinant wild type and mutant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, was assessed using the two-electrode voltage-clamp technique. Our results suggest that the biflavonoid amentoflavone binds to recombinant wild type $GABA_A$ receptors expressing different α subunits in a manner resembling that of the classical benzodiazepine agonist diazepam. Despite the high affinity of amentoflavone for the benzodiazepine site, and the observed pattern of binding similar to that of diazepam, negative modulation of GABA-gated chloride flux of $GABA_A$ receptors by this biflavonoid may occur by means of a mechanism independent of both the benzodiazepine and loreclezole sites.

2. Materials and methods

2.1. Transfection of cells and preparation of membrane homogenate

Transient transfection of tsA 201 cells was performed using a calcium phosphate transient transfection protocol (Chen and Okayama, 1988; Newell and Dunn, 2002). Cells were maintained in low-glucose Dulbecco's modified Eagle's medium [Hyclone, Logan, Utah] containing 10% fetal calf or bovine growth serum and stored at 37 °C in a 7% CO_2 humidified incubator. Cells were passaged twice a week. cDNAs encoding the subunits of interest were added in equal amounts to an appropriate volume of 250 mM $CaCl_2$, followed by addition of an equal volume of *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer [50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0]. Contents were mixed and allowed to stand for 5 min at room temperature before aliquots were added dropwise to plates of cells. Cells were then incubated at 37 °C in a 3% CO_2 humidified incubator for 48 h, and media was replaced with fresh media 24 h following transfection. Cells were harvested into a Tris–HCl solution [50 mM Tris, 250 mM KCl, 0.02% NaN_3, pH 7.4, supplemented with 1 mM benzamidine, 0.1 mg/ml bacitracin, 0.01 mg/ml chicken egg white trypsin inhibitor, and 0.5 mM phenylmethylsulfonyl fluoride] and sheared using an UltraTurrax T25 homogenizer [IKA Labortechnik, Staufen, Germany] (13 500 rpm for two 10 s pulses). Homogenized cells were pelleted and the membrane homogenate resuspended and stored at −80 °C.

2.2. Radioligand binding assays

Radioligand competition binding experiments were performed as described previously (Davies et al., 1998; Newell and Dunn, 2002; Derry et al., 2004). Aliquots of cell homogenates were incubated with [^{3}H]Ro15-4513 or [^{3}H]Ro15-1788 in a Tris–HCl buffer [50 mM Tris, 250 mM KCl, 0.02% NaN_3, pH 7.4] at a final volume of 500 μl for 1 h at 4 °C. GF/B filters [Whatman, Maidstone, UK] to be used were also soaked in Tris–HCl buffer at 4 °C for 1 h. Nonspecific binding was determined in the presence of excess unlabelled ligand. The mixture was filtered using rapid filtration via a cell harvester [Brandel, Gaithersburg, MD] and washed twice with 5 ml of ice-cold Tris–HCl buffer. Filters were then dried under a heat lamp and placed into scintillation minivials. CytoScint scintillation fluid [ICN, Costa Mesa, CA, 5 ml] was added to each vial and radioactive decay was measured by liquid scintillation counting.

2.3. Data analysis

Data from competition binding assays were fit to curves using least-squares nonlinear regression analysis of GraphPad Prism 3.0 [GraphPad, San Diego, CA]. Log IC_{50} values were determined as mean ± S.E.M. of at least three independent experiments. K_i values were determined using the equation of Cheng and Prusoff (1973). One-way analysis of variance between groups (ANOVA) was used for data analysis and levels of significance were determined using Dunnett's post-test.

2.4. Transcript preparation and injection of oocytes

The histidine 101 array of mutants was created via site-directed mutagenesis as described previously (Davies et al., 1998). Briefly, the α_1 subunit was subcloned into the pAlter-1 vector using the Altered Sites kit [Promega, Madison, WI]. The presence of a

restriction site, introduced by the mutagenic oligonucleotide as a silent mutation, was used to identify potential mutants. Correct substitutions were verified by DNA sequencing, and mutant α_1 cDNAs were then subsequently subcloned into the pcDNA 3.1 expression vector [InVitrogen, San Diego, CA].

cRNA transcripts were prepared by standard protocol as described previously (Hope et al., 1993) using cDNAs in the pcDNA 3.1 vector, encoding α, β, and γ subunits, that had been linearized. cRNA transcripts were injected [50 nl of 1 mg/ml cRNA] into stage V and VI *X. laevis* oocytes that had been defolliculated by treatment with collagenase A [2 mg/ml, Beohringer-Mannheim, Indianapolis, IN] for 3 h at room temperature. Care and handling of *Xenopus* frogs was in accordance with approved guidelines by the Canadian Council on Animal Care, and the Biosciences Animal Policy and Welfare Committee of the University of Alberta (Smith et al., 2004). The oocytes were individually maintained at 14 °C for up to 14 days in 96-well plates containing 200 μl of standard ND96 solution [96 mM NaCl, 5 mM HEPES, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4] supplemented with 0.1 mg/ml gentamycin.

2.5. Electrophysiological recordings

Following a 48 h incubation period, injected oocytes were used for electrophysiology experiments for up to 14 days. Experimental method was similar to that described previously (Pistis et al., 1997; Dunn et al., 1999; Kapur et al., in press). Briefly, a GeneClamp 500 Amplifier [Axon Instruments, Inc., Foster City, CA] was used to hold the membrane potential of an oocyte in the bath chamber at −60 mV, via the two-electrode voltage-clamp mode. The chamber (0.5 ml) in which the oocytes were held was supplied with a constant flow of ND96 perfusion buffer [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4] using a gravity-flow drip system. The voltage-sensing and current-passing electrodes were filled with 3 M KCl such that they had a resistance of 0.5–3.0 MΩ as measured in ND96 perfusion buffer. The electrical signal from the amplifier was transduced to a digital signal that was fed into a computer by a Digidata 1322 A [Axon Instruments, Inc., Foster City, CA] data acquisition system. Current response to drug application over a time interval was obtained as a current trace that could be analyzed using the Axoscope 9.0 [Axon Instruments, Inc., Union City, CA] acquisition program.

For all $GABA_A$ receptor subtypes studied, the effects of amentoflavone were investigated using a GABA concentration that elicited a current approximately 50% (EC_{50}) of the maximal GABA response, with the exception of the experiment testing amentoflavone block of diazepam positive modulation of GABA-evoked current, where a 3 μM concentration of GABA was used. Amentoflavone stock concentrations were made by dissolving the compounds in dimethyl sulfoxide, and stocks were stored at room temperature under light-sensitive conditions. In all experiments, the concentration of dimethyl sulfoxide was kept constant at a concentration that produced no overt vehicle effects. Control currents using an EC_{50} concentration of GABA were first recorded until current amplitude was consistent to within ±5% over three successive challenges, with a 12 min wash with perfusion buffer allowed between each application to permit recovery. A 3 min perfusion of amentoflavone was followed by coapplication of the compound with an EC_{50} concentration of GABA. This protocol was also followed for application of diazepam and its coapplication with a 3 μM concentration of GABA. Evoked current was recorded, and a 12 min wash with perfusion buffer was again allowed before the next application of amentoflavone. Control responses to the EC_{50} concentration of GABA were recorded periodically throughout each experiment to confirm that current response remained stable, and that the effects of amentoflavone application were reversible.

2.6. Data analysis

Data analysis was performed by means of nonlinear regression techniques using GraphPad Prism 3.0 [GraphPad, San Diego, CA]. The effects of amentoflavone were fit by one-site binding analysis using the equation:

$$I = I_o + \frac{(I_{GABA} - I_o)}{(1 + 10^{x-c})}$$

where I is the measured amplitude of evoked current, I_{GABA} is the current in response to EC_{50} GABA concentration in absence of amentoflavone, I_o is the current in the presence of both EC_{50} GABA concentration and a saturating concentration of amentoflavone, X is the logarithm of amentoflavone concentration, and C is the logarithm of the IC_{50} concentration. Data are presented as log IC_{50}±S.E.M. and mean with 95% confidence intervals of observations made from at least three oocytes.

2.7. Drugs

[^{3}H]Ro15-4513 and [^{3}H]Ro15-1788 were obtained from Perkin-Elmer (Boston, MA). GABA was obtained from Sigma Chemical Co. (St. Louis, MO). Amentoflavone was obtained from Indofine Chemical Co. (Hillsborough, NJ). Diazepam was a gift from Dr. G. Baker of the Department of Psychiatry at the University of Alberta.

3. Results

3.1. Amentoflavone binding at $GABA_A$ receptors containing different α subunits

The binding affinity of amentoflavone for recombinant wild type $GABA_A$ receptors was examined. Competition binding

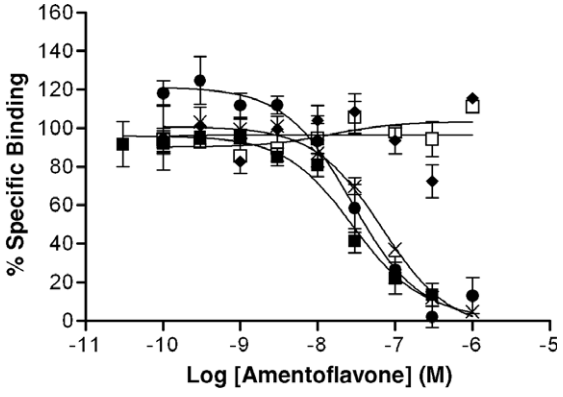

Fig. 1. Displacement of [^{3}H]Ro15-4513 by amentoflavone at $GABA_A$ receptors containing different α subunits. Competition binding curves using cell membrane expressing $\alpha_1\beta_2\gamma_2$ (■), $\alpha_4\beta_2\gamma_2$ (♦), $\alpha_5\beta_2\gamma_2$ (●), and $\alpha_6\beta_2\gamma_2$ (□) $GABA_A$ receptor subtypes. Rat brain membrane (x) is also included for comparison. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value as determined by saturation analysis. For competition assays using rat brain membrane, [^{3}H]Ro15-1788 was used as the reporter ligand. Data are from at least three separate experiments performed in duplicate.

experiments were carried out using membranes from tsA201 cells, where α_1, α_4, α_5, or α_6 $GABA_A$ receptor subunits had been transiently expressed in combination with β_2 and γ_2 subunits. [^{3}H]Ro15-4513, a benzodiazepine site inverse agonist that binds to each of these $GABA_A$ receptor subtypes with nanomolar affinity, was used as the reporter ligand. Amentoflavone was able to displace bound [^{3}H]Ro15-4513 from all of the recombinant preparations in a competitive manner with nanomolar affinity, except from the $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ receptor subtypes (Fig. 1). A slight increase in reporter ligand binding was noted for the $\alpha_5\beta_2\gamma_2$ receptor subtype at low concentrations of amentoflavone as compared to $\alpha_1\beta_2\gamma_2$; however K_i values were similar between the two subtypes. Amentoflavone also displaced [^{3}H]Ro15-1788 in competition experiments using rat brain membrane preparations. The affinity of amentoflavone for rat brain membrane and recombinant $GABA_A$ receptor subtypes are summarized in Table 1.

3.2. Amentoflavone binding at histidine 101 mutant $GABA_A$ receptors

Site-directed mutagenesis was performed previously to incorporate different amino acid substitutions at the histidine 101 site of the rat α_1 subunit (Davies et al., 1998). Four of these substitutions were examined in the present study. Amentoflavone displaced [^{3}H]Ro15-4513 for receptors containing the glutamine and tyrosine mutations, with only slight decreases in affinity observed as compared to recombinant wild type $\alpha_1\beta_2\gamma_2$ receptors (Fig. 2; Table 2). A complete loss of competitive binding by amentoflavone was revealed for receptors containing the lysine and alanine mutations, as no significant displacement of bound [^{3}H]Ro15-4513 was observed.

3.3. Functional effects of amentoflavone at $GABA_A$ receptor subtypes

Two-electrode voltage-clamp electrophysiology was used to determine functional effects of amentoflavone at recombinant $GABA_A$ receptors expressed in *X. laevis* oocytes. No effect was observed when amentoflavone was tested alone with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors. In contrast, amentoflavone displayed a significant negative modulation of GABA-evoked currents at these receptors, and was able to inhibit the EC_{50} GABA response almost completely (IC_{50}=2.23 μM, 95% CI: 0.17–29) (Fig. 3).Amentoflavone showed a comparable negative modulation of GABA-evoked currents at recombinant $\alpha_1\beta_2$ wild type $GABA_A$ receptors (IC_{50}=1.20 μM, 95% CI: 0.75–1.9) (Fig. 3B). This receptor subtype is normally insensitive to classical benzodiazepine modulation, as it lacks the required γ subunit. No effect was seen when amentoflavone was tested alone at the $\alpha_1\beta_2$ $GABA_A$ receptor subtype.

A negative modulation of GABA-evoked currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors containing the alanine mutation at position 101 of the α_1 subunit, expressed in oocytes, was observed to be comparable to that seen at recombinant wild type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, (IC_{50}=1.10 μM, 95% CI: 0.25–4.9) (Fig. 3B). Similar to the other $GABA_A$ receptor subtypes tested (above), amentoflavone produced no observable effects when given alone. Fig. 3B illustrates the inhibitory effect of amentoflavone on the EC_{50} GABA response of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype containing the alanine mutation. When data were fit to one-site binding analysis, the curve produced appeared biphasic; a significant difference was found with regards to curve fit when two-site binding analysis of the same data was performed. Data were therefore presented using two-site binding analysis, as a biphasic effect was apparent.

Recombinant $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors, expressed in oocytes, were tested to determine whether a β_1 versus β_2 subunit effect of amentoflavone could be established, similar to that seen for

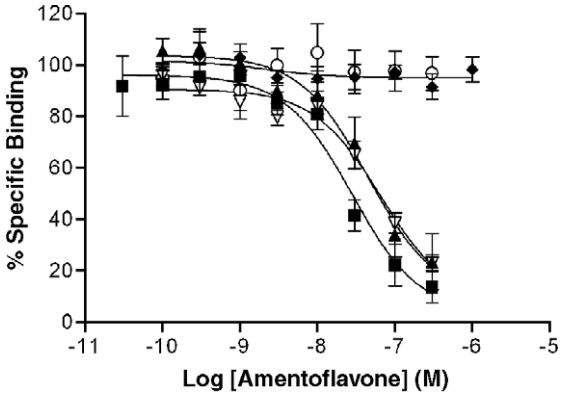

Fig. 2. Displacement of [^{3}H]Ro15-4513 by amentoflavone at $GABA_A$ receptor histidine 101 mutants. Competition binding curves using cell membrane expressing glutamine (▲), tyrosine (▽), lysine (♦), and alanine (○) mutations at position 101 of the α_1 subunit are shown. $\alpha_1\beta_2\gamma_2$ (■) is also included for comparison. [^{3}H]Ro15-4513 was present at a concentration equal to its K_d value as determined by saturation analysis. Data are from at least three separate experiments performed in duplicate.

Table 1
Effects of α subunit substitution on the affinity of amentoflavone for the $GABA_A$ receptor benzodiazepine site

α subunit	Affinity	
	Log IC_{50}±SEM (M)	K_i (nM)
$\alpha_1\beta_2\gamma_2$	−7.57±0.1	13.8
$\alpha_4\beta_2\gamma_2$	N.D.	N.D.
$\alpha_5\beta_2\gamma_2$	−7.54±0.1	15.0
$\alpha_6\beta_2\gamma_2$	N.D.	N.D.
rat[a]	−7.20±0.06	23.9

All values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equivalent to its K_d value for each receptor subtype. N.D. indicates values not determined. Data are from at least three experiments performed in duplicate.

[a] Rat brain membrane competition assays were performed as described above using [^{3}H]Ro15-1788 as the reporter ligand.

Table 2
Effects of α_1 subunit histidine 101 mutation on the affinity of amentoflavone for the $GABA_A$ receptor benzodiazepine site

α_1 subunit mutation	Affinity	
	Log IC_{50}±SEM (M)	K_i (nM)
Glutamine	−7.40±0.2	19.9
Tyrosine	−7.13±0.2	30.6
Lysine	N.D.	N.D.
Alanine	N.D.	N.D.

All values are derived from displacement of [^{3}H]Ro15-4513 at a concentration equivalent to its K_d value for each receptor subtype. N.D. indicates values not determined. Data are from at least three experiments performed in duplicate.

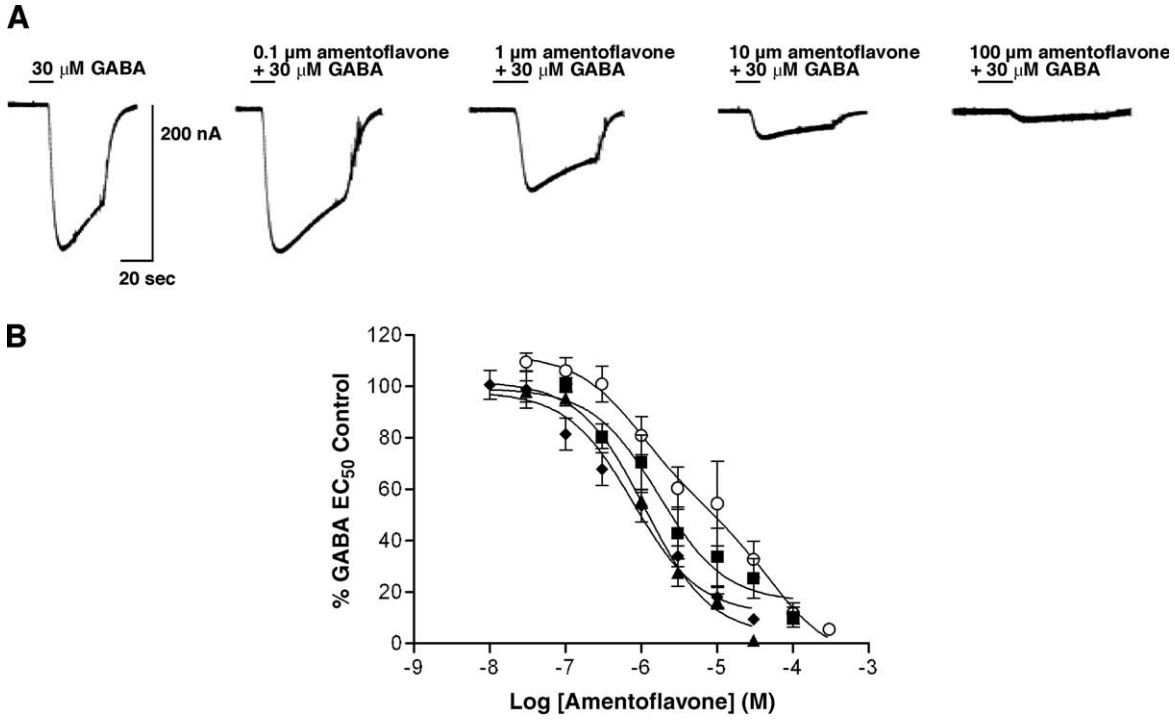

Fig. 3. Negative modulation of GABA-mediated currents by amentoflavone. A) Current traces recorded when amentoflavone was tested with oocytes expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors. A 3 min perfusion of each amentoflavone concentration (not shown) was followed by coapplication of amentoflavone with an EC_{50} concentration of GABA. B) The effects of amentoflavone on currents induced by GABA at a concentration approximately equal to its EC_{50} value in the $\alpha_1\beta_2\gamma_2$ (■), $\alpha_1\beta_2$ (▲), $\alpha_1\beta_2\gamma_2$ containing the alanine mutation (○), and $\alpha_1\beta_1\gamma_2$ (♦) receptors are shown. Data are from observations made from at least three oocytes.

loreclezole and the β-carbolines (Stevenson et al., 1995). Amentoflavone given alone produced no effect, but showed a significant negative modulation of the EC_{50} GABA response at this receptor subtype (Fig. 3B), similar to that observed for recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (IC_{50}=0.764 μM, 95% CI: 0.028–21). All electrophysiology data for amentoflavone are summarized in Table 3.

Amentoflavone was tested at a nanomolar concentration to determine whether the functional effects of the classical benzodiazepine positive modulator diazepam at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in oocytes could be blocked. Positive modulation of GABA-evoked current by diazepam at this receptor subtype was inhibited by approximately one-half upon the addition of an equivalent concentration of amentoflavone (Fig. 4).

Table 3
Negative modulation of GABA-mediated currents by amentoflavone

Subtype	Log IC_{50}±SEM (M)	IC_{50} (95% CI) (μM)
$\alpha_1\beta_2\gamma_2$	−5.65±0.4	2.23 (0.17–29)
$\alpha_1\beta_2$	−5.92±0.05	1.20 (0.75–1.9)
$\alpha_1\beta_2\gamma_2$ (alanine mutation)		
(1)[a]	−5.96±0.2	1.10 (0.25–4.9)
(2)	−3.55±0.5	285 (1.9–42000)
$\alpha_1\beta_1\gamma_2$	−6.12±0.3	0.764 (0.028–21)

Data are from observations made from at least three oocytes. Experiments were performed using GABA EC_{50} concentrations.

[a] Data recorded from the $\alpha_1\beta_2\gamma_2$ subtype containing the alanine mutation at position 101 of the α_1 subunit was analyzed using two-site binding analysis (see Results).

4. Discussion

4.1. Amentoflavone and diazepam binding at the benzodiazepine site

In the present study, amentoflavone binding was shown to differentiate between $GABA_A$ receptor subtypes containing different α subunits in a manner resembling diazepam binding. Although amentoflavone showed affinity for subtypes containing the α_1 or α_5 subunit, a loss of competitive binding was observed at $GABA_A$ receptor subtypes containing the α_4 or α_6 subunit. This suggests that amentoflavone binding at the benzodiazepine site may involve binding contacts that also play a role in the binding of classical benzodiazepine positive modulators like diazepam.

Further evidence supporting a similar manner of binding for amentoflavone and diazepam at the benzodiazepine site involves histidine 101 (rat α_1 subunit). As mentioned, the presence of a histidine or arginine residue at this position has been shown to be responsible for the diazepam-sensitive and diazepam-insensitive $GABA_A$ receptor subtypes, respectively (Wieland et al., 1992). In addition, studies involving histidine 101 mutant $GABA_A$ receptors have supported the importance of this residue to the binding and function of classical benzodiazepine positive modulators such as flunitrazepam, as well as

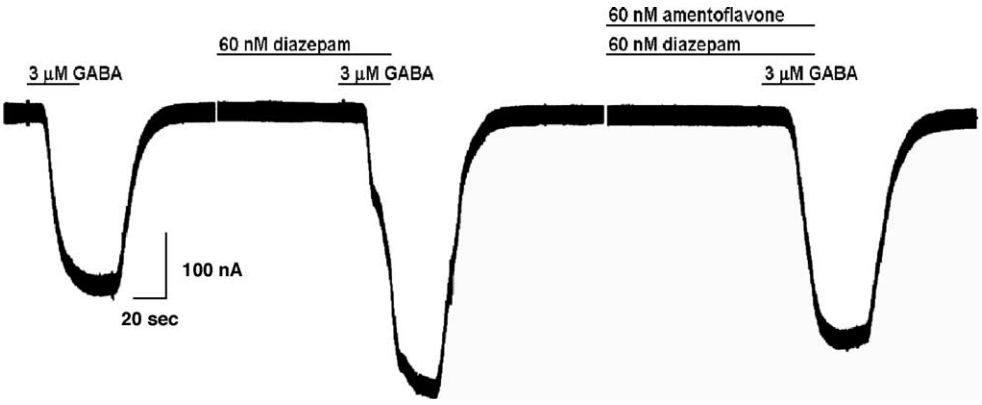

Fig. 4. Potentiation of the GABA-mediated currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by diazepam is inhibited by an equivalent concentration of amentoflavone. Current traces recorded from an oocyte expressing recombinant $\alpha_1\beta_2\gamma_2$ wild type $GABA_A$ receptors in response to 3 μM GABA. Potentiation of the GABA-mediated current upon perfusion of 60 nM diazepam followed by coapplication with 3 μM GABA is shown, which is subsequently inhibited by the addition of 60 nM amentoflavone.

other benzodiazepine site ligands such as Ro15-4513 and Ro15-1788 (Davies et al., 1998; Dunn et al., 1999). In this study, we found that competitive binding of amentoflavone was abolished by mutation of histidine 101 to either an alanine or lysine residue. This finding suggests that histidine 101 plays an important role in the ability of amentoflavone to bind to the benzodiazepine site. Differences in the structure of the residue at position 101 may be responsible for the effect seen on amentoflavone binding, albeit this is difficult to conclude. When histidine 101 is mutated, there is a loss of its imidazole side moiety. However, comparison of the side chains of the replacement residues studied provides no obvious reason for the loss of affinity for amentoflavone at two of the mutant receptors. Tyrosine and glutamine both contain side chains which are uncharged polar groups, whereas alanine and lysine have a nonpolar methyl group side chain and a positively charged polar butylammonium side chain, respectively. Interestingly, the lysine mutation was also shown to result in a complete loss of classical benzodiazepine positive modulator flunitrazepam binding at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, whereas the tyrosine and glutamine mutations only resulted in right-fold shifts in flunitrazepam binding affinity (Dunn et al., 1999). In addition, the small size of the methyl group side chain of alanine may play a role in the loss of amentoflavone affinity observed with that mutation. Therefore, we cannot exclude the possibility that changes in steric or charge–charge interactions are involved in the dramatic changes in affinity for amentoflavone seen with the mutant receptors containing the alanine or lysine residue.

4.2. Functional effects of amentoflavone distinct from benzodiazepine site binding

Previous work with amentoflavone has shown that it acts as a negative modulator of GABA-gated chloride currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Hanrahan et al., 2003). In agreement with this finding, functional analysis of amentoflavone in the current study also revealed that it acts as a negative modulator of chloride currents elicited by an EC_{50} concentration of GABA at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (IC_{50}=2.23 μM, 95% CI: 0.17–29). However, it appears that the functional effects of amentoflavone may not be mediated through the same site at which high affinity binding occurs. The functional effects of amentoflavone at this $GABA_A$ receptor subtype were observed at micromolar concentrations, whereas radioligand binding experiments showed that amentoflavone binds to the benzodiazepine binding site with nanomolar affinity (Tables 1 and 3). In addition, it was observed that amentoflavone negatively modulated EC_{50} GABA-gated chloride currents at recombinant $\alpha_1\beta_2$ $GABA_A$ receptors (IC_{50}=1.20 μM, 95% CI: 0.75–1.9). These receptors do not contain a classical benzodiazepine binding site as they lack the proposed α–γ interface. The inhibition by amentoflavone observed at these receptors was not significantly different from that seen at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. This finding is in agreement with recent studies involving other flavonoids. Goutman et al. (2003) performed the first functional experiments that directly tested the effects of flavonoid compounds on receptor-mediated ionic currents. Their findings questioned whether flavonoid actions at $GABA_A$ receptors are actually mediated by the benzodiazepine binding site, despite the strong evidence that flavonoids do competitively bind to this site (Nielsen et al., 1988; Viola et al., 1995; Baureithel et al., 1997; Medina et al., 1997; Marder and Paladini, 2002). In addition to showing that flavonoids had similar actions at ρ_1 $GABA_C$ receptors, which lack the classical benzodiazepine site, they demonstrated that specific flavonoid actions at the $GABA_A$ receptor were flumazenil-insensitive. More recently, it was shown that 6-methylflavone has actions at $\alpha_1\beta_2$ $GABA_A$ receptors (Hall et al., 2004). Therefore, the observation that amentoflavone functionally modulates GABA-gated chloride currents at both recombinant $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_A$

receptor subtypes in a similar manner concurs with previous speculation that flavonoid actions at $GABA_A$ receptors may be mediated by a site different from that which mediates classical benzodiazepine modulation (Goutman et al., 2003; Hanrahan et al., 2003; Hall et al., 2004).

Functional analysis of the effects of amentoflavone at the mutant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype containing the alanine substitution at position 101 further substantiates the idea that flavonoids are acting at the $GABA_A$ receptor via a non-benzodiazepine site-mediated mechanism. Despite the complete abolishment of benzodiazepine site competitive binding of amentoflavone observed at this mutant receptor subtype (see Fig. 2), we showed that amentoflavone displayed a functional effect at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor containing the alanine mutation comparable to that seen at the wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor. Although amentoflavone produced no effect when given alone, it was shown to be a negative modulator of GABA-gated chloride currents, with no significant difference between its inhibitory activity at the mutant receptor (IC_{50}1) compared to the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. This result suggests that even though specific binding at the benzodiazepine binding site is lost, amentoflavone retains its functional activity at the mutant $GABA_A$ receptor by means of a different mechanism.

Taken together, these data implicate a flavonoid mechanism of action at $GABA_A$ receptors that does not involve the classical benzodiazepine binding site. We sought to further characterize amentoflavone interaction at the $GABA_A$ receptor by focusing on the β subunit. The loreclezole site at the $GABA_A$ receptor is dependent upon the presence of a β_2 or β_3 subunit, and this site has also been shown to be a low affinity binding site for the β-carbolines (Wafford et al., 1994; Wingrove et al., 1994; Stevenson et al., 1995). A number of the β-carbolines are known to bind to the benzodiazepine site with high affinity, whereas low affinity binding to the loreclezole site was shown to discriminate between β_1- versus β_2- or β_3-containing $GABA_A$ receptors based on a single amino acid difference (Stevenson et al., 1995). It was observed that β-carboline low affinity binding to the loreclezole site was dependent upon the presence of an asparagine residue (β_2 and β_3; serine in β_1) in the transmembrane 2 region of the β subunit. We tested amentoflavone at recombinant $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptors to determine whether its effects at $GABA_A$ receptors are dependent upon the β subunit variant. We found no significant difference in the effects of amentoflavone at the $\alpha_1\beta_1\gamma_2$ $GABA_A$ receptor subtype compared to the wild type recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. That is, amentoflavone negatively modulated GABA-gated chloride currents in a similar manner (IC_{50}=0.764 μM, 95% CI: 0.028–21), but had no effect when given alone. This suggests that amentoflavone does not discriminate between β_1- versus β_2-containing $GABA_A$ receptor subtypes, and therefore is likely not acting via the loreclezole site.

Given that the functional effects of amentoflavone at the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype were observed at micromolar concentrations, whereas radioligand binding experiments showed that amentoflavone binds to the benzodiazepine binding site with nanomolar affinity, we tested whether a nanomolar concentration of amentoflavone could block the modulation of GABA-evoked current by the classical benzodiazepine positive modulator diazepam. We observed that the positive modulation of GABA-evoked current by diazepam was inhibited by approximately one-half upon the addition of an equivalent concentration of amentoflavone (Fig. 4). This observation supports findings from both the current study and previous studies (Nielsen et al., 1988; Baureithel et al., 1997) which conclude that amentoflavone does bind to the classical benzodiazepine binding site with high affinity, and further reveals that amentoflavone is acting as an antagonist at this site.

Numerous studies have been undertaken to create and refine a model of flavonoid binding at the benzodiazepine binding site (Dekermendjian et al., 1999; Marder et al., 2001; Kahnberg et al., 2002; Hong and Hopfinger, 2003). In addition, attempts have been made to ascertain specific substituents that may increase the affinity of flavonoid binding to this site (Huen et al., 2003). Of both naturally-occurring and synthetic flavonoids, amentoflavone has one of the highest affinities for the benzodiazepine site (Medina et al., 1997; Marder and Paladini, 2002). Amentoflavone, or I3, II8-biapigenin, is biflavonoid in structure, with its half-structure being the structure of the flavonoid apigenin. Apigenin has also been shown to bind at the benzodiazepine site, albeit with much less affinity than amentoflavone (Viola et al., 1995). Functional studies performed using apigenin have shown that it has a very modest negative modulatory effect on GABA-induced chloride currents at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Campbell et al., 2004). In contrast, the same concentration of amentoflavone has a considerably higher potency at recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. This difference may be attributable to amentoflavone being a much larger and/or more hydrophobic molecule than apigenin, being twice its size. These may be reason for an enhancement of both benzodiazepine site- and non-benzodiazepine site-mediated interactions of amentoflavone at the $GABA_A$ receptor, in comparison to apigenin.

In conclusion, these results suggest that amentoflavone interactions at $GABA_A$ receptors include binding to classical benzodiazepine binding sites, as well as functional effects not mediated by these binding sites. Interestingly, this biflavonoid binds to $GABA_A$ receptor subtypes in a manner resembling diazepam binding, and similar to diazepam, amentoflavone affinity for the benzodiazepine site is affected by changes to histidine 101. Nevertheless, our observations suggest that amentoflavone is acting as an antagonist at the classical benzodiazepine site, and that the functional effects at $GABA_A$ receptors are most likely

mediated by mechanisms distinct from the benzodiazepine and loreclezole binding sites.

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