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Flumazenil-independent positive modulation of γ-aminobutyric acid action by 6-methylflavone at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors

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Abstract

In view of the ability of flavones to displace radiolabelled benzodiazepines from brain tissue and the interesting behavioural profile of these compounds, the present study investigated the activity of 6-methylflavone at ionotropic γ-aminobutyric acid (GABA) receptors expressed in *Xenopus laevis* oocytes. 6-Methylflavone (1–100 μM) was found to be a positive allosteric modulator at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors with no significant difference between the enhancement seen at either receptor subtype. At 100 μM, 6-methylflavone enhanced the response to 5 μM GABA by 183±20% at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. The methyl substituent was important since the parent flavone was significantly weaker as a positive modulator (103±24% enhancement of 5 μM GABA by 100 μM flavone). This enhancement is not mediated via high-affinity benzodiazepine sites as it was not inhibited by the classical benzodiazepine antagonist flumazenil under conditions where flumazenil inhibits the potentiation of the GABA response to diazepam. 6-Methylflavone (60 μM) did not significantly affect the GABA dose–response curve at ρ_1 $GABA_C$ receptors. 6-Methylflavone acts as a positive modulator of recombinant $GABA_A$ receptors at sites independent of flumazenil-sensitive benzodiazepine sites.

Keywords: GABA; 6-Methylflavone; Benzodiazepine; Flavonoid; $GABA_A$ receptor

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain and it has long been known that many neuroactive drugs, such as the benzodiazepines, interact with $GABA_A$ receptors by binding to modulatory sites within the receptor complex. Benzodiazepines have been prescribed for their anxiolytic, sedative and myorelaxant effects since the 1960s; however, it is often not possible to separate these actions, thus any unwanted actions become side effects, resulting in a reduction in the therapeutic value of benzodiazepines (Fielding and Lal, 1979). Other disadvantages include amnesic-like effects (Lister, 1985), ataxia, ethanol and barbiturate potentiation as well as tolerance and dependence (Gallagher and Primus, 1992).

$GABA_A$ receptors belong to the superfamily of ligand-gated ion channels that include $GABA_C$, nicotinic acetylcholine, strychnine-sensitive glycine and $5\text{-}HT_3$ receptors. $GABA_A$ receptors are hetero-oligomeric receptors forming a pentameric structure. At least 16 human $GABA_A$ receptor subunits have been described and are classified under six subfamilies of protein subunits: α, β, γ, δ, ε, θ (Chebib and Johnston, 2000).

Classically, benzodiazepine agonists act as positive modulators at a subpopulation of $GABA_A$ receptors by increasing the frequency of chloride channel openings (Rogers et al., 1994; Chebib and Johnston, 2000). The benzodiazepine binding sites for this action, which can be blocked by flumazenil, are considered to lie between the α and γ subunits (Klausberger et al., 2001). Flumazenil-insensitive positive modulation of $GABA_A$ receptors has been described in receptors lacking a γ subunit by benzodiazepines at μM concentrations. For example, Malherbe et al. (1990) found that recombinant $\alpha_1\beta_1$ $GABA_A$ receptors from rat brain were sensitive to potentiation by benzodiazepine receptor ligands, with both diazepam and flumazenil acting as positive modulators. Walters et al. (2000) found that classical benzodiazepines produce biphasic potentiation at

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rat recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors via two distinct mechanisms. This biphasic potentiation is believed to be mediated via two sites referred to as high-affinity and low-affinity benzodiazepine sites. Both sites are present at receptors composed of $\alpha_1\beta_2\gamma_2$ $GABA_A$ subunits and low-affinity potentiation can be selectively observed at receptor combinations lacking a γ subunit such as $\alpha_1\beta_2$ $GABA_A$ receptors. Furthermore, low-affinity potentiation at both receptor combinations is insensitive to flumazenil.

Flavones belong to a class of compounds called flavonoids, which are phenylbenzopyrones. Flavonoids are known to display a variety of biological activities in the periphery (for review, see Harborne and Williams, 2000); however, there is increasing interest in their action as benzodiazepine receptor modulators. The majority of research into the action of flavones at GABA receptors has involved the use of binding studies targeted at the benzodiazepine site of $GABA_A$ receptors, as well as behavioural studies in rodents. More recently, this has been extended to include functional studies of flavonoid activity at recombinant $GABA_A$ receptors (Ai et al., 1997; Viola et al., 2000).

There have been extensive structure–activity studies aimed at developing models of benzodiazepine pharmacophores (Gupta and Paleti, 1998; Maddalena and Johnston, 1995) that have been extended to include flavonoids (Dekermendjian et al., 1999; Hong and Hopfinger, 2003; Huang et al., 2001; Kahnberg et al., 2000; Marder et al., 2001). A problem common to most of these studies is that the activity data is based on ligand binding studies to what is now known to be a mixture of benzodiazepine binding sites. Given our increased knowledge of the diversity of benzodiazepine actions arising out of studies on cloned receptors of defined subunit composition and studies on transgenic animals (Möhler et al., 2002), future structure-activity studies need to be based on data from functional studies on GABA receptors of known subunit composition.

6-Methylflavone has been described as a benzodiazepine partial agonist/antagonist on the basis of studies on diazepam and flumazenil binding to human recombinant $GABA_A$ receptors of the $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$ and $\alpha_5\beta_2\gamma_{2S}$ subtypes expressed in Sf-9 insect cells (Ai et al., 1997). The present study describes the action of 6-methylflavone as a positive modulator at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes acting at sites that appear to be independent of classical flumazenil-sensitive benzodiazepine sites.

2. Materials and methods

2.1. Drugs

Diazepam and flumazenil were gifts from Hoffman-La Roche (Nutley, NJ, USA). GABA and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Flavone was obtained from Aldrich (Milwaukee, WI, USA) and purified by silica gel chromatography to remove a 10% phenol impurity.

2.2. Synthesis of 6-methylflavone

The synthesis of 6-methylflavone was carried out by the reaction of 5′ -methyl-hydroxyacetophenone and benzoyl chloride according to previously described methods (Tietze and Eicher, 1989). Crude product was treated with activated charcoal, filtered through celite and recrystallised twice from acetone to afford 6-methylflavone (>99% by ^1H NMR). The structure was confirmed by NMR (^1H and ^{13}C), IR, mass spectrometry and X-ray crystallography (Hall et al., 2001). Elemental analysis was also carried out.

2.3. Expression of recombinant GABA receptors in X. laevis oocytes

The procedures involving the use of *X. laevis* were carried out according to those described by Huang et al. (2003) and were approved by the Animal Ethics Committee of the University of Sydney. Female *X. laevis* were anaesthetised by immersion in 0.17% 3-aminobenzoic acid ethyl ester with 0.02% NaCl for 10–15 min, and a lobe of the ovaries was surgically removed. The lobe was rinsed with oocyte releasing buffer 2 (OR2) (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$·6H2O, 5 mM HEPES, pH 7.5) and treated with Collagenase A (2 mg/ml in OR2, Bohringer Mannheim, Germany) for 2 h to separate oocytes from connective tissue and follicular cells. Released oocytes were rinsed in ND96 'wash' solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$·6H2O, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). Stages V–VI oocytes were collected and stored on an oscillator at 16 °C in ND96 'storage' solution (ND96 'wash' solution supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 μg/ml gentamycin).

Human α_1, β_2 and γ_{2L} DNA in pcDM8 were provided by Dr. Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). Human ρ_1 DNA in pcDNA1.1 (Invitrogen, San Diego, CA, USA) was provided by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA). Plasmids containing α_1, β_2 or γ_{2L} DNA and ρ_1 DNA were linearised using the restriction enzymes *NOT*1 and *Xba*1, respectively. RNA was synthesised from linearised DNA using the 'mMessage mMachine' kit from Ambion (Austin, TX, USA). A 'Nanoject' (Drummond Scientific, Broomali, PA, USA) was used to inject either $\alpha_1\beta_2\gamma_{2L}$ (20 ng/50 nl), $\alpha_1\beta_2$ (20 ng/50 nl) or ρ_1 (10 ng/50 nl) RNA dissolved in nuclease-free water into the cytoplasm of the oocytes, which were then stored at 16 °C in ND96 storage solution. For $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, the RNA for each subunit was injected in the ratio 1:1:2, respectively, to favour incorporation of the γ subunit. For $\alpha_1\beta_2$ $GABA_A$ receptors, the RNA for each subunit was injected in a 1:1 ratio. Sham-injected oocytes were prepared by injection with nuclease-free water.

2.4. Recording from oocytes

Two to seven days after injection of RNA, receptor activity was measured by two-electrode voltage clamp recording. Glass micropipettes for the electrodes were made using a micropipette puller (Narishige Scientific Instrument Lab, Tokyo, Japan) and filled with 3 M potassium chloride. Oocytes were transferred into a cell bath, impaled by two micropipettes and voltage clamped using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA). The membrane potential was clamped at −60 mV and the electrodes had resistance values between 0.5 and 2 MΩ. In the cell bath, oocytes were continuously superfused with ND96 solution and drugs were applied in the perfusate. Current traces were recorded using a Mac Lab 2e recorder (AD Instruments, Sydney, NSW, Australia) and Chart v.3.5.2.

Oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors were screened with 10 μM Zn^{2+} in the presence of two concentrations of GABA (10 and 100 μM) to ensure that the γ subunit was incorporated. $GABA_A$ receptors without a gamma subunit are sensitive to inhibition by zinc, whereas those expressing a gamma subunit are not (Hosie et al., 2003). To test for positive modulation, low GABA doses at each subtype were chosen for the control doses since benzodiazepines produce greatest enhancement of the GABA response at lower GABA doses. The 5 and 1 μM GABA were used as control GABA doses at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors, respectively, as these doses corresponded to approximately an EC_{10} response at each subtype. Stock solutions in DMSO of 100 mM (6-methylflavone, diazepam and flumazenil) concentrations were prepared. GABA stock solutions (100 mM) were prepared in milli-Q water. For experiments where any drugs were dissolved in DMSO, all drug solutions were standardised to contain 0.8% DMSO, which had no significant effect on the oocytes.

2.5. Data analysis

Standardised responses were plotted against drug concentration on a semi-logarithmic scale using GraphPad Prism version 2.0. Logarithmically transformed data were tested for significance using a linear regression fit. Provided the slope of the curve significantly deviated from zero, a nonlinear regression fit was performed using a sigmoidal dose response (variable slope), the equation of which is

$$I = I_{max}/(1 + [EC_{50}/(A)]^{n_H})$$

where [A] is the agonist concentration, I is the current and I_{max} is the maximum current. EC_{50} is the concentration of agonist that produces a response that is 50% of the maximum current and n_H is the Hill coefficient. For diazepam action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors where the data displays two components of potentiation, a sum of two sigmoidal dose response curves (variable slope) was used to fit the data, the equation of which is

$$I = I_{max_1}/(1 + [EC_{50_1}/(A)]^{nH_1}) + I_{max_2}/(1 + [EC_{50_2}/A)]^{nH_2})$$

where the values corresponding to the first and second components of the potentiation have been differentiated by 1 and 2, respectively. EC_{50} values are expressed as mean with 95% confidence intervals. Hill coefficients (n_H) are expressed as mean±S.E.M. Significant differences were determined using two-way ANOVAs producing F and P values, which have been quoted where appropriate.

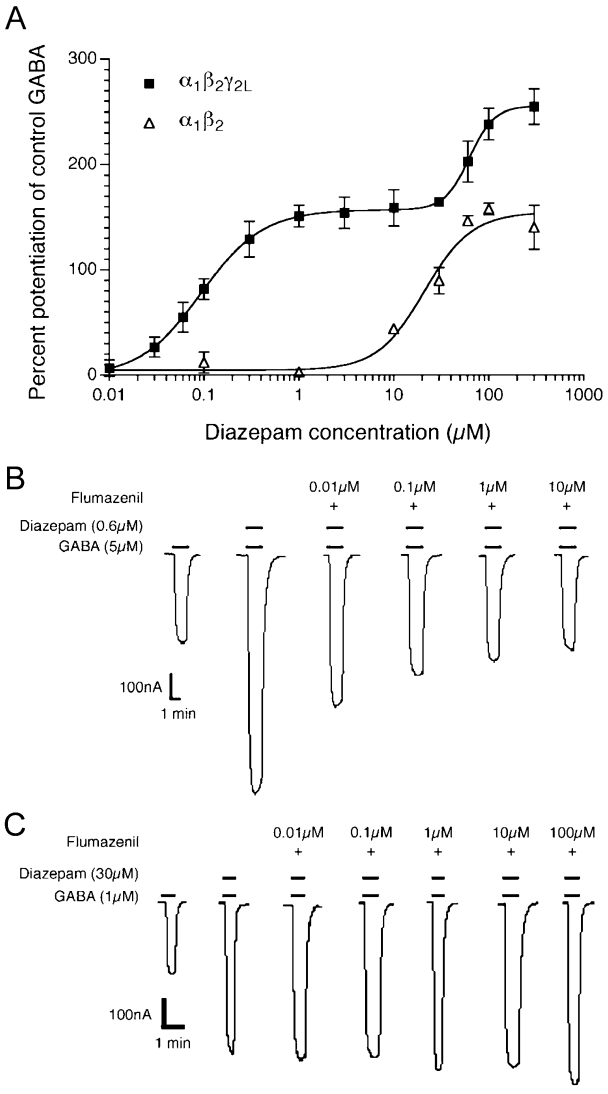

Fig. 1. (A) Diazepam potentiates the response to GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors (control GABA doses at each receptor subtype were 5 and 1 μM, respectively). Data are mean±S.E.M. (n=3–9 oocytes). Potentiation of the GABA response at (B) human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors by diazepam is inhibited by increasing concentrations of flumazenil (0.01–10 μM) (n=7), while the potentiation at (C) human recombinant $\alpha_1\beta_2$ $GABA_A$ receptors by diazepam is not inhibited by flumazenil (0.01–100 μM) (n=4). Control GABA doses used were (B) 5 and (C) 1 μM.

3. Results

3.1. Expression of GABA receptors in X. laevis oocytes

Injection of $\alpha_1\beta_2\gamma_{2L}$, $\alpha_1\beta_2$ or ρ_1 receptor mRNA into *Xenopus* oocytes resulted in expression of GABA-sensitive channels. Increasing concentrations of GABA produced a dose-dependent increase in inward whole-cell current until a maximal response was reached. Respective EC_{50} values are 37.1 μM (n=4, 95% CI: 29.8–46.2 μM), 3.92 μM (n=4, 95% CI: 3.08–5.00) and 1.25 μM (n=3, 95% CI: 1.16–1.34). Hill coefficients (n_H) are 1.19±0.07, 1.28±0.2 and 2.59±0.02, respectively. GABA did not produce any response at sham-injected oocytes (n=3).

3.2. Biphasic potentiation by diazepam at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors

Diazepam enhanced the effects of a low dose of GABA at $GABA_A$ receptors (n=3–9, F=226.4, P<0.0001). Fig. 1A shows the concentration–response relationship for diazepam in the presence of GABA at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors. Since the curve at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors had two phases of enhancement, two EC_{50} values were obtained. The EC_{50} for the high-affinity effect of diazepam was 0.09 μM (95% CI: 0.06–0.16) with a Hill coefficient of 1.3±0.16 (n=9). The EC_{50} for the low-affinity effect of diazepam was 56.5 μM (95% CI: 35.8–78.6) with a Hill coefficient of 3.3±1.2 (n=3). The low-affinity effect of diazepam could be selectively observed at $\alpha_1\beta_2$ $GABA_A$ receptors. There was a significant difference between the effect of diazepam at each receptor subtype (n≥3, F=32.33, P<0.0001). Diazepam produced monophasic potentiation at $\alpha_1\beta_2$ $GABA_A$ receptors that was similar to the second phase of potentiation observed at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors however the EC_{50} was somewhat lower. The EC_{50} for diazepam potentiation at $\alpha_1\beta_2$ $GABA_A$ receptors was 21.1 μM (95% CI: 14.2–31.3) with a Hill coefficient of 1.87±0.35 (n=3). Diazepam did not have any effect on sham-injected oocytes (n=3). While the EC_{50} and Hill coefficient at $\alpha_1\beta_2$ $GABA_A$ receptors appear to be significantly different from the EC_{50} and Hill coefficient for the low-affinity effect of diazepam at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, this difference may not be real because of the overlapping sites at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. These overlapping sites cannot be individually resolved; thus, it is difficult to provide accurate data for the low-affinity site at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

The high-affinity diazepam potentiation at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was sensitive to antagonism by flumazenil, a known benzodiazepine antagonist. At these receptors, flumazenil (10 μM) completely abolished the enhancement by 0.6 μM diazepam (EC_{50}=0.09 μM for high-affinity diazepam action) (Fig. 1B). In contrast, potentiation at $\alpha_1\beta_2$ $GABA_A$ receptors by diazepam was not inhibited by flumazenil (0.01–100 μM) (n=4, Fig. 1C).

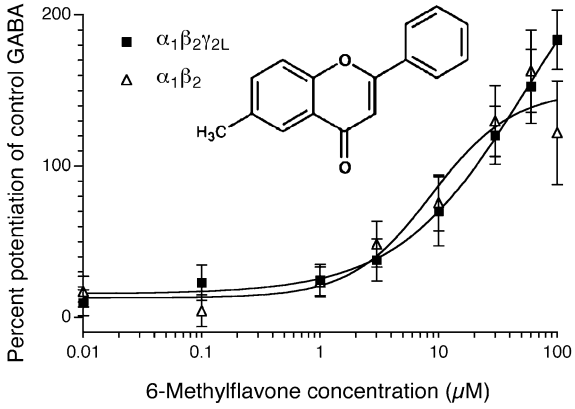

Fig. 2. 6-Methylflavone (structure shown) potentiates the response to GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors (control GABA doses at each receptor subtype were 5 and 1 μM, respectively). Data are mean±S.E.M. (n=4–7 oocytes).

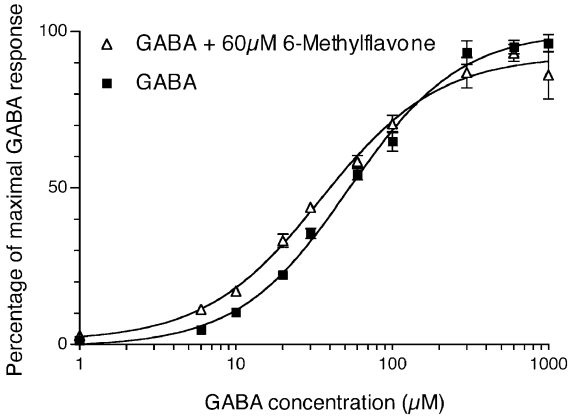

Fig. 3. 6-Methylflavone (60 μM) shifts the GABA dose response curve to the left at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, decreasing the mean EC_{50} by 33±5.4%. Data are mean±S.E.M. (n=4 oocytes).

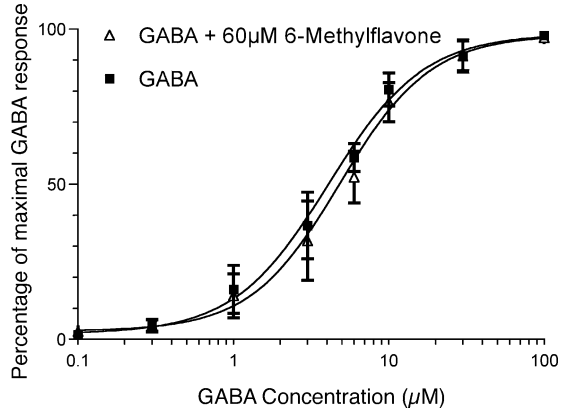

Fig. 4. 6-Methylflavone (60 μM) has no significant effect on the GABA dose–response curve at human recombinant ρ_1 $GABA_C$ receptors. Data are mean±S.E.M. (n=8 oocytes).

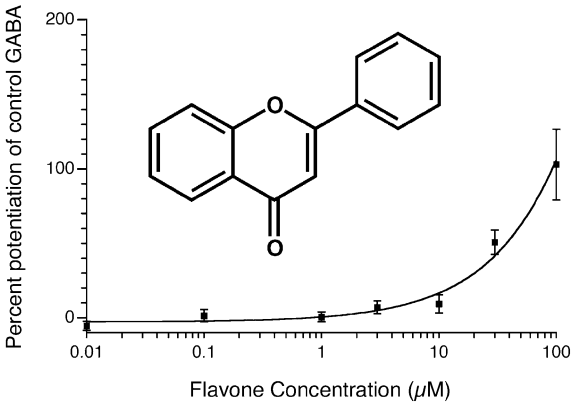

Fig. 5. Flavone (structure shown) potentiates the response to GABA at human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (control GABA dose was 5 μM) to a lesser extent than 6-methylflavone (see Fig. 2). Data are mean±S.E.M. (n=5 oocytes).

3.3. 6-Methylflavone as a positive allosteric modulator at $GABA_A$ receptors, inactive at $GABA_C$ receptors

6-Methylflavone had no activity when administered alone; however, it enhanced the normal response to GABA at recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors (Fig. 2) ($n\geq4$, F=18.79, P<0.0001) at concentrations from 1–100 μM. There was no significant difference between the enhancement produced by 6-methylflavone at either receptor subtype ($n\geq4$, F=0.22, P=0.6082). The limit of solubility for 6-methylflavone was 100 μM; thus, an accurate EC_{50} at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors could not be determined; however, at this concentration, 6-methylflavone enhanced the control response to GABA by 183±20% (n=7). At $\alpha_1\beta_2$ $GABA_A$ receptors, a maximum was reached and an EC_{50} of 8.7 μM (n=4, 95% CI: 2.6–29.7) was obtained. The Hill coefficients were 1.10±0.26 and 1.50±0.11 at $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors, respectively (Fig. 2). 6-Methylflavone (60 μM) shifted the GABA dose–response curve to the left (n=4, F=16.38, P=0.0004), decreasing the mean EC_{50} at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors by 33±5.4% (Fig. 3).

6-Methylflavone (60 μM) did not significantly affect the GABA dose–response curve at ρ_1 $GABA_C$ receptors (n=5, F=0.52, P=0.4740) (Fig. 4). Furthermore, 6-methylflavone, at concentrations below those producing significant enhancement (i.e. 0.01–10 μM), had no effect on the enhancement produced by 0.1 μM diazepam in the presence of 5 μM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (n=7, F=1.084, P=0.3032). 6-Methylflavone did not have any effect at sham-injected oocytes (n=3).

Flavone also potentiated the response to 5 μM GABA at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors; however, a lower level of enhancement was observed. It enhanced the control response to GABA by 103±24% at a concentration of 100 μM (n=5) (Fig. 5).

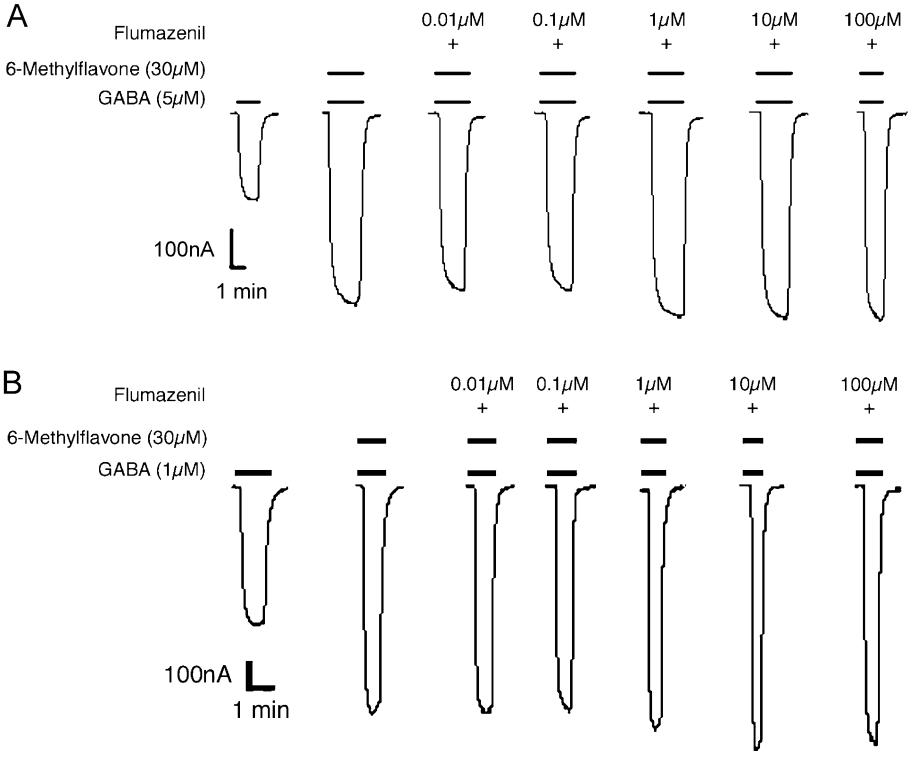

Fig. 6. Potentiation of the GABA response at human recombinant (A) $\alpha_1\beta_2\gamma_{2L}$ and (B) $\alpha_1\beta_2$ $GABA_A$ receptors by 6-methylflavone (30 μM) is not inhibited by flumazenil (0.01–100 μM) (n=5 and 4, respectively). Control GABA doses used were (A) 5 and (B) 1 μM.

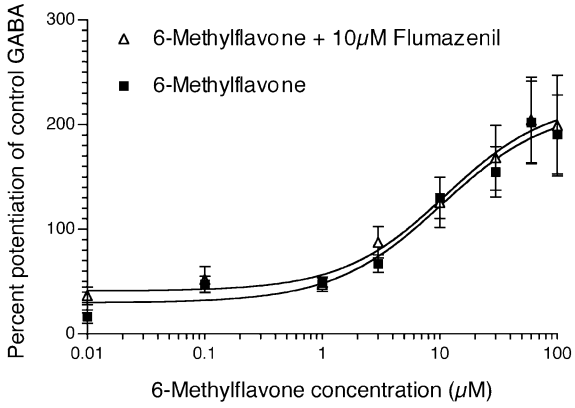

Fig. 7. Flumazenil (10 μM) has no significant effect on the dose response curve for 6-methylflavone at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Data are mean±S.E.M. (n=3 oocytes).

3.4. Flumazenil insensitive action of 6-Methylflavone

Flumazenil (0.01–100 μM) did not block the enhancement produced by 30 μM 6-methylflavone at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 6A), whereas under the same conditions 10 μM flumazenil completely blocked the response to 0.6 μM diazepam (Fig. 1B). Flumazenil (10 μM) did not have any significant effect on the dose response curve for 6-methylflavone in the presence of 5 μM GABA (n=3, F=0.37, P=0.5453) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 7). As is the case for diazepam, flumazenil (0.01–100 μM) did not inhibit potentiation by 6-methylflavone at $\alpha_1\beta_2$ $GABA_A$ receptors (n=4, Fig. 6B).

4. Discussion

To date, much of the flavonoid research related to GABA receptors has focused on the ability of these compounds to displace radiolabelled benzodiazepines in binding assays, and also on the behavioural effects exhibited in rodents. Many studies have shown that there are definite links between the ability of flavones to bind to the benzodiazepine binding site and the many behavioural effects that they induce; however, little has been done to investigate the molecular mechanisms underlying flavone action at the GABA receptor in functional studies.

The current study showed that 6-methylflavone acted in a similar way to a benzodiazepine agonist by positively modulating the response to a low dose of GABA (5 μM) at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. It may be predicted that this enhancement was mediated through the benzodiazepine site, as 6-methylflavone has been shown to inhibit the binding of [^{3}H]flumazenil and [^{3}H]diazepam to synaptosomes (Ai et al., 1997). However, the positive modulation by 6-methylflavone was not blocked by flumazenil, under conditions where flumazenil inhibited positive modulation by diazepam.

Furthermore, 6-methylflavone displayed potentiation at $GABA_A$ receptors both with and without a γ subunit (i.e. $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$). In both cases, this potentiation was insensitive to flumazenil. In contrast, diazepam displayed biphasic potentiation at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors and monophasic low-affinity potentiation at $\alpha_1\beta_2$ $GABA_A$ receptors as described by Walters et al. (2000). The high-affinity component of diazepam action at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was sensitive to antagonism by flumazenil, whereas the low-affinity component at both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors was insensitive to flumazenil (Walters et al., 2000), which was also shown in this study for $\alpha_1\beta_2$ $GABA_A$ receptors. Although flumazenil enhanced the response to 5 μM GABA at higher doses, this did not appear to influence it's antagonist activity at lower doses. Flumazenil enhancement of the GABA response appeared to occur most significantly at flumazenil concentrations above 100 μM, which was well above the concentration required to antagonise the response to diazepam. Thus, it is unlikely that the apparent flumazenil insensitivity of 6-methylflavone-induced positive modulation was due to any positive modulation by flumazenil itself.

A complete dose–response curve displaying 6-methylflavone-induced enhancement was not obtained since a maximal response to 6-methylflavone was not achieved at the limit of solubility for this compound. Thus, the enhancement of the GABA response has been expressed as a percentage of the response to 5 μM GABA at the dose of 6-methylflavone producing the greatest enhancement (100 μM). In contrast, at $\alpha_1\beta_2$ $GABA_A$ receptors, it appears that the potentiation by 6-methylflavone reached a maximal response at a concentration of 60 μM; thus, it was possible to establish an EC_{50} value. Despite this discrepancy, there was no significant difference between the enhancement produced at either receptor subtype.

Although we have shown that potentiation of the GABA response by 6-methylflavone is independent of high-affinity benzodiazepine sites, it is possible that 6-methylflavone is interacting with high-affinity benzodiazepine sites as an antagonist. It has been reported that 100 μM 6-methylflavone has an antagonistic effect on the diazepam-induced enhancement of GABA currents at human $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in Sf-9 insect cells (Ai et al., 1997). At concentrations (0.01–10 μM) below those that produce enhancement, 6-methylflavone had no effect in our oocyte expression system on the response to 5 μM GABA in the presence of 0.1 μM diazepam at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors.

Ai et al. (1997) characterised the actions of 6-methylflavone using radiolabelled binding assays on both rat brain membranes and human recombinant $GABA_A$ receptors expressed in Sf-9 insect cells, as well as functional studies using the whole-cell patch clamp technique. On the basis of GABA ratios in these binding studies, 6-methylflavone was found to act as a weak partial agonist at $\alpha_1\beta_2\gamma_{2S}$, an antagonist at $\alpha_2\beta_2\gamma_{2S}$, and $\alpha_3\beta_2\gamma_{2S}$, and a partial inverse agonist at $\alpha_5\beta_2\gamma_{2S}$ $GABA_A$ receptors, while functional

studies characterised 6-methylflavone as a weak benzodiazepine partial agonist/antagonist at these receptors (Ai et al., 1997). Using Sf-9 insect cells, it was found that 6-methylflavone (25 μM) showed a similar weak positive modulation of GABA-induced chloride currents in all of the four $GABA_A$ receptor subtype combinations tested ($\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$). Furthermore, 6-methylflavone (100 μM) antagonised the potentiating effect of diazepam (2.5 μM) on GABA-induced chloride currents at recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors.

In the present study, any diazepam antagonist action of 6-methylflavone may have been masked by the observed positive modulatory action on GABA responses. It may be that the *Xenopus* oocyte expression system used in the present study is more sensitive to the positive modulatory action of 6-methylflavone on GABA responses than is the insect expression used by Ai et al. (1997). While the current study used a different γ_2 splice variant to that used by Ai et al. (1997) to form $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, it is unlikely that this is significant since the positive modulation by 6-methylflavone observed in the current study is independent of the γ subunit. The extent of phosphorylation mediated by endogenous protein kinases in the two expression systems may be sufficiently different to favour 6-methylflavone having a predominately flumazenil-like action in the insect cells and a predominately flumazenil-independent positive modulation action in the oocytes. Protein kinases are known to be very important in $GABA_A$ receptor function and cell surface expression (Brandon et al., 2002). The importance of protein kinases in this action of 6-methylflavone at $GABA_A$ receptors could be tested using specific protein kinase inhibitors.

6-Methylflavone did not affect the response to GABA at ρ_1 $GABA_C$ receptors; thus, it appears that the modulating action of 6-methylflavone is selective for $GABA_A$ receptors. Furthermore, the methyl group seems to be important since flavone produces a lower level of potentiation than 6-methylflavone.

There are several reports in the literature of flavonoids having flumazenil-insensitive effects that may be associated with $GABA_A$ receptors. Thus, Avallone et al. (2000) and Zanoli et al. (2000) described sedative and locomotor effects of apigenin that are flumazenil-insensitive. Goutman et al. (2003) have recently described flumazenil-insensitive effects of apigenin and related flavonoids on recombinant $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ and ρ_1 $GABA_C$ receptors, while we have reported that amentoflavone is a flumazenil-insensitive negative modulator at recombinant $\alpha_1\beta_1\gamma_{2L}$ $GABA_A$ receptors (Hanrahan et al., 2003). Some of these flumazenil-independent effects of flavonoids may be mediated through effects on protein kinases associated with GABA receptors (Huang et al., 1999, 2001).

The current study shows that 6-methylflavone is a positive modulator at recombinant $GABA_A$ receptors of $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ subtypes expressed in *Xenopus* oocytes. This action is not sensitive to antagonism by flumazenil thus it is not mediated via high-affinity benzodiazepine sites. Since 6-methylflavone appears to act as a partial agonist/antagonist at the benzodiazepine sites of some $GABA_A$ receptor subtype combinations (Ai et al., 1997), and it has been demonstrated in this study to produce positive modulation at $GABA_A$ receptors through an additional site, it is likely that this compound binds to more than one site at $GABA_A$ receptors.

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References

Ai, J., Dekermendjian, K., Wang, X., Nielsen, M., Witt, M., 1997. 6-Methylflavone, a benzodiazepine receptor ligand with antagonistic properties on rat brain and human recombinant $GABA_A$ receptors in vitro. Drug Dev. Res. 41, 99–106.

Avallone, R., Zanoli, P., Puia, G., Kleinschnitz, M., Schreier, P., Baraldi, M., 2000. Pharmacological profile of apigenin, a flavonoid isolated from matricaria chamomilla. Biochem. Pharmacol. 59, 1387–1394.

Brandon, N.J., Jovanovic, J.N., Moss, S.J., 2002. Multiple roles of protein kinases in the modulation of gamma-aminobutyric $acid_A$ receptor function and cell surface expression. J. Pharmacol. Ther. 94, 113–122.

Chebib, M., Johnston, G.A.R., 2000. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. J. Med. Chem. 43, 1–21.

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. J. Med. Chem. 42, 4343–4350.

Fielding, S., Lal, H., 1979. Anxiolytics. An introduction. In: Fielding, S., Lal, H. (Eds.), Anxiolytics. Futura, New York, NY, pp. 23–26.

Gallagher, D., Primus, R.J., 1992. Benzodiazepine tolerance and dependence: $GABA_A$ receptor complex locus of change. Biochem. Soc. Symp. 59, 135–151.

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. Eur. J. Pharmacol. 461, 79–87.

Gupta, S.P., Paleti, A., 1998. Quantitative structure–activity relationship studies on some nonbenzodiazepine series of compounds acting at the benzodiazepine receptor. Bioorg. Med. Chem. 6, 2213–2218.

Hall, B.J., Hanrahan, J.R., Johnston, G.A.R., Hambley, T.W., Hibbs, D.E., 2001. 6-Methylflavone. Acta. Crystallogr., o592–o593.

Hanrahan, J.R., Chebib, M., Davucheron, N.L.M., Hall, B.J., Johnston, G.A.R., 2003. Semisynthetic preparation of amentoflavone: a negative modulator at $GABA_A$ receptors. Bioorg. Med. Chem. Lett. 13, 2281–2284.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Hong, X., Hopfinger, A.J., 2003. 3D-pharmacophores of flavonoid bind-

ing at the benzodiazepine $GABA_A$ receptor site using 4D-QSAR analysis. J. Chem. Inf. Comput. Sci. 43, 324–336.

Hosie, A.M., Dunne, E.L., Harvey, R.J., Smart, T.G., 2003. Zinc-mediated inhibition of $GABA_A$ receptors: discrete binding sites underlie subtype specificity. Nat. Neurosci. 6, 362–369.

Huang, R.-Q., Fang, M.-J., Dillon, G.H., 1999. The tyrosine kinase inhibitor genistein directly inhibits $GABA_A$ receptors. Mol. Brain Res. 67, 177–183.

Huang, X.Q., Liu, T., Gu, J.D., Luo, X.M., Ji, R.Y., Cao, Y., Xue, H., Wong, J.T.F., Wong, B.L., Pei, G., Jiang, H.L., Chen, K.X., 2001. 3D-QSAR model of flavonoids binding at benzodiazepine site in $GABA_A$ receptors. J. Med. Chem. 44, 1883–1891.

Huang, S.H., Duke, R.K., Chebib, M., Sasaki, K., Wada, K., Johnston, G.A.R., 2003. Bilobalide, a sesquiterpene trilactone from Ginkgo biloba, is an antagonist at recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Eur. J. Pharmacol. 464, 1–8.

Kahnberg, P., Lager, E., Rosenberg, C., Schougaard, J., Camet, L., Sterner, O., Nielsen, E.O., Nielsen, M., Liljefors, T., 2000. Refinement and evaluation of a pharmacophore model for flavone derivatives binding to the benzodiazepine site of the $GABA_A$ receptor. J. Med. Chem. 45, 4188–4201.

Klausberger, T., Sarto, I., Ehya, N., Fuchs, K., Furtmuller, R., Mayer, B., Huck, S., Sieghart, W., 2001. Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. J. Neurosci. 21, 9124–9133.

Lister, R.G., 1985. The amnesic action of benzodiazepines in man. Neurosci. Biobehav. Rev. 9, 87–94.

Maddalena, D.J., Johnston, G.A.R., 1995. Prediction of receptor properties and binding affinity of ligands to benzodiazepine/$GABA_A$ receptors using artificial neural networks. J. Med. Chem. 38, 715–724.

Malherbe, P., Draguhn, A., Multhaup, G., Beyreuther, K., Möhler, H., 1990. $GABA_A$-receptor expressed from rat brain α- and β-subunit cDNAs displays potentiation by benzodiazepine receptor ligands. Mol. Brain Res. 8, 199–208.

Marder, M., Estiu, G., Blanch, L.B., Viola, H., Wasowski, C., Medina, J.H., Paladini, A.C., 2001. Molecular modelling and QSAR analysis of the interaction of flavone derivatives with the benzodiazepine site of $GABA_A$ receptor complex. J. Biorg. Med. Chem. 9, 323–335.

Möhler, H., Fritschy, J.M., Rudolph, U., 2002. A new benzodiazepine pharmacology. J. Pharmacol. Exp. Ther. 300, 2–8.

Rogers, C.J., Twyman, R.E., Macdonald, R.L., 1994. Benzodiazepine and β-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurons in culture. J. Physiol. 475, 69–82.

Tietze, L.F., Eicher, T., 1989. Reactions and Synthesis in the Organic Chemistry Laboratory, vol. 122. University Science Books, Sausalito, CA, USA, pp. 139–140.

Viola, H., Wolfman, C., Marder, M., Goutman, J.D., Bianchin, M., Wasowski, C., Calvo, D.J., Izquierdo, I., Paladini, A.C., Medina, J.H., 2000. 6-Chloro-3′ -nitroflavone is a potent ligand for the benzodiazepine binding site of the $GABA_A$ receptor devoid of intrinsic activity. Pharmacol. Biochem. Behav. 65, 313–320.

Walters, R.J., Hadley, S.H., Morris, D.W., Amin, J., 2000. Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms. Nat. Neurosci. 3, 1274–1281.

Zanoli, P., Avallone, R., Baraldi, M., 2000. Behavioural characterisation of the flavonoids apigenin and chrysin. Fitoterapia 71, S117–S123.