Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 495 (2004) 87–96

www.elsevier.com/locate/ejphar

Characterization of a new synthetic isoflavonoid with inverse agonist activity at the central benzodiazepine receptor

Daniele V.S. Lopes[a], Rodrigo R.B. Caruso[a], Newton G. Castro[a], Paulo R.R. Costa[b], Alcides J.M. da Silva[b], François Noël[a,*]

[a] *Departamento de Farmacologia Básica e Clínica, Instituto de Ciências Biomédicas, Bloco J, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha Do Fundao, 21941-590 Rio de Janeiro, Brazil*

[b] *Núcleo de Pesquisas de Produtos Naturais, Bloco, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21941-590 Rio de Janeiro, Brazil*

Received 4 February 2004; received in revised form 10 May 2004; accepted 14 May 2004

Abstract

Research aimed at developing selective drugs acting on γ-aminobutyric acid $(GABA)_A$ receptors introduced compounds from diverse chemical classes unrelated to the 1,4-benzodiazepines, including flavonoids. These studies also revealed the potential use of inverse agonists as cognition-enhancing agents. Here we report pharmacological properties of the novel synthetic isoflavonoid 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36). PCALC36 displaced [^{3}H]flunitrazepam binding to rat brain synaptosomes with an IC_{50} of 13.8 μM. Scatchard analysis of the effect of PCALC36 showed a concentration-dependent reduction of the B_{max} of [^{3}H]flunitrazepam, without a marked change in K_d. This effect could be reversed by diluting and washing the preparation. Addition of 20-μM GABA shifted to the right the inhibition curve of PCALC36 on [^{3}H]flunitrazepam binding (IC_{50} ratio of 0.68), which is characteristic for inverse agonists. PCALC36 produced little change in the GABAergic tonic currents recorded by whole-cell patch clamp in cultured rat hippocampal neurones, but it caused a 20% reduction in miniature inhibitory postsynaptic current amplitude and completely antagonised the full (direct) agonist midazolam in a quickly reversible manner. The data suggest that the coumestan backbone can be useful for developing novel ligands at the $GABA_A$ receptor.

Keywords: Benzodiazepine; GABA; Flavonoid; Coumestan; Flunitrazepam

1. Introduction

Since their discovery in the 1960s, 1,4-benzodiazepines have been utilized with great success in therapy, mainly as anxiolytics. Benzodiazepines exert their anxiolytic, sedative/hypnotic, anticonvulsant, and myorelaxant effects by enhancing the inhibitory neurotransmission in the central nervous system. In fact, benzodiazepines are allosteric modulators of the γ-aminobutyric acid $(GABA)_A$ receptor complex, a ligand-gated ion channel that mediates the flux of chloride ions, usually leading to neuronal membrane hyperpolarization. Benzodiazepines are thought to bind at the interface between the α and γ subunits of the heteropentameric protein composed of subunits from different families, namely α1-6, β1-3, γ1-3, ε, δ, π, and θ (Korpi et al., 2002). Modulation of chloride conductance is also possible through the action of drugs binding at distinct allosteric sites, as is the case for barbiturates, general anaesthetics, neurosteroids, picrotoxin and other convulsants, ethanol, and some divalent cations (Korpi et al., 2002; Gordon, 2002). In spite of being among the most frequently prescribed drugs, benzodiazepines have limitations particularly in relation to long-term use, such as development of tolerance and dependence, rebound symptoms upon withdrawal, and amnesic effects (Korpi et al., 1997). The need for a more restricted activity profile (anxiolytic effect without sedation, for example) is another stimulus for the search of new compounds acting on the $GABA_A$ receptor complex. Recent advances in the molecular biology of the $GABA_A$ receptor indicate that the

* Corresponding author. Tel./fax: +55-21-2562-6732.
E-mail address: fnoel@pharma.ufrj.br (F. Noël).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.05.026

various behavioural responses to benzodiazepines are mediated by specific receptor subtypes in distinct neuronal circuits (Rudolph et al., 1999; Möhler et al., 2002). Data from gene knock-in experiments suggest that receptors containing the α1 subunit may be responsible for anticonvulsant, sedative, and amnesic effects; those containing the α2 subunit for anxiolytic effects, while those with α2, α3, and α5 subunits would be implicated in myorelaxation and ethanol potentiation (Rudolph et al., 1999; Möhler et al., 2002). As a consequence of an improved definition of the appropriate targets, the development of tranquilizers without the undesirable effects of classical benzodiazepines is currently a hot topic in medicinal chemistry (Marder et al., 2001; Huang et al., 2000). This search is not limited to modifications of the benzodiazepine backbone because a variety of compounds structurally unrelated to 1,4-benzodiazepines bind with high affinity to the same site at $GABA_A$ receptors, such as β-carbolines (Braestrup et al., 1984), triazolopyridazines, cyclopyrrolones, quinolines (Gardner et al., 1993), and flavonoids (Hui et al., 2002; Medina et al., 1990; Viola et al., 1994; Wolfman et al., 1998; Salgueiro et al., 1997; Viola et al., 2000; Kahnberg et al., 2002; Nielsen et al., 1988; Dekermendjian et al., 1999).

Recent electrophysiological evidence established the functional diversity of $GABA_A$ receptors at the single-cell level. Mature neurones from several brain regions seem to express two $GABA_A$ response types: the ubiquitous phasic currents, comprised of large (multiquantal) and miniature synaptic currents, and also the tonic currents (I_T) associated with low-level, persistent activation of $GABA_A$ receptors (Valeyev et al., 1993; Brickley et al., 1996). Once action potential activity has been halted by a sodium channel blocker, the presence of I_T can be revealed by its disappearance upon application of a $GABA_A$ receptor antagonist, like bicuculline, as seen in neurones from the cerebellum (Kaneda et al., 1995; Wall and Usowicz, 1997; Leão et al., 2000; Rossi et al., 2003) and the hippocampus (Bai et al., 2001; da Silva et al., 2002; Stell and Mody, 2002; Valeyev et al., 1993; Yeung et al., 2003). The $GABA_A$ receptors underlying the tonic and phasic responses show differences in biophysical and pharmacological properties, suggesting that they may be of distinct subtypes. In hippocampal neurones, I_T is effectively potentiated by benzodiazepine agonists (Bai et al., 2001; da Silva et al., 2002; Yeung et al., 2003), raising the possibility that it may be an important target for drugs affecting limbic seizure threshold, anxiety, or cognition.

As part of a program aimed at synthesizing biologically active flavonoids, we recently reported the synthesis of five coumestans structurally related to the naturally occurring wedelolactone, a constituent of the plant *Eclipta prostrata* L. (Asteraceae; da Silva et al., 2001). Because wedelolactone inhibited the binding of [^{3}H]flunitrazepam to rat brain synaptosomes, we have selected one of the more recently synthesized coumestans, namely, 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36), for studying in detail its molecular interaction with the benzodiazepine site at the $GABA_A$ receptor in preparations from rat brain. Binding assays were indicative of a negative allosteric modulator profile of PCALC36 at $GABA_A$ receptors. Drugs with such activity may have many undesirable effects, but they can also improve learning and memory (Eid and Rose, 1999; Maubach, 2003). Therefore, we have investigated the effects of PCALC36 in the GABAergic, benzodiazepine-sensitive I_T in neurones from the rat hippocampus. Present results indicate that this original synthetic isoflavonoid, whose structure is shown here for the first time, acts as a low-efficacy inverse agonist at the central benzodiazepine receptor.

2. Materials and methods

2.1. Synthesis of 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36)

The compound PCALC36 (Fig. 1) was prepared according to the methodology described previously for the synthesis of five coumestans with different patterns of oxygenation in rings A and D, using available benzaldehydes, isovanillin, and piperonal as starting materials (da Silva et al., 2001). The purity of PCALC36 crystals was >95% as assessed by ^{1}H NMR and ^{13}C spectroscopy. PCALC36 was dissolved in dimethylsulphoxide (DMSO) as a 30-mM stock solution stored at −15 °C for no more than 2 weeks. Just before its use, the stock solution was diluted with Tris–HCl buffered Krebs solution (pH 7.4 at 4 °C) for binding experiments or with extracellular solution (see below) for electrophysiological recordings.

2.2. Tissue preparation

Brains, without cerebellum and brainstem, were obtained from male Wistar rats sacrificed by decapitation. Briefly, tissues were homogenized in a Potter apparatus with a motor-driven Teflon pestle at 4 °C in 15 V of ice-cold 0.32-M buffered sucrose (pH 7.4) per gram of organ. After centrifuging at $1000 \times g_{max}$ for 10 min, the supernatant was centrifuged at $48{,}000 \times g_{av}$ for 20 min to obtain the crude synaptosomes that were resuspended in a buffered Krebs solution and stored at −80 °C until use. Alternatively, in order to eliminate the endogenous GABA, the synaptosomal preparation was subjected to an osmotic shock at 25 °C

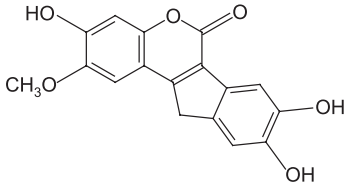

Fig. 1. Structure of PCALC36.

before being recentrifuged at 48,000 × g_{av} for 20 min to pick up the pellet that was resuspended in 50-mM Tris–HCl (pH 7.4 to 4 °C) and stored at − 80 °C (washed preparation).

2.3. [^{3}H]flunitrazepam binding assay

Synaptosomes (200 μg protein) were incubated at 4 °C for 90 min in a buffered Krebs solution containing 0.2 nM [^{3}H]flunitrazepam (85 Ci/mmol, New England Nuclear Life Science Products, USA). After incubation, samples were rapidly diluted with 3 ml of ice-cold Krebs buffer and immediately filtered on glass fibre filters (GMF 3, Filtrak, Germany) under vacuum. Filters were then washed once more with 3 ml buffer, dried, and immersed in a scintillation cocktail (0.1 g/l of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene [POPOP] and 4.0 g/l 2,5-diphenyloxazole [PPO] in toluene), and the radioactivity retained in the filters was counted with a Packard Tri-Carb 1600 TR liquid scintillation analyser. Saturation experiments were performed by the addition of increasing concentrations of unlabeled flunitrazepam (0.1 nM to 5.0 nM) to 0.2 nM [^{3}H]flunitrazepam. Nonspecific binding was estimated in the presence of 5-μM unlabeled flunitrazepam.

In another experiment (*reversibility assay*), crude synaptosomes were preincubated at 4 °C in 5 ml of buffered Krebs solution for 90 min in the absence (control) or presence of 30-μM PCALC36. After this period, the synaptosomes were diluted to 20 ml with Krebs Tris–HCl buffer and centrifuged for 30 min at 48,000 × g. The pellets were washed again by resuspension and incubated in 20 ml of buffered Krebs solution for 30 min at room temperature followed by a second centrifugation under the same conditions described above, using a protocol similar to the one described previously (Silva et al., 1996). Final pellets were used to measure the binding of [^{3}H]flunitrazepam as previously described.

To investigate the kinetics of flunitrazepam dissociation, synaptosomes (200-μg protein) were first incubated at 4 °C in 5-ml buffered Krebs solution containing 0.2 nM [^{3}H]flunitrazepam. After 90 min, either 5 μl of unlabeled flunitrazepam alone (final concentration: 300 nM) or 5 μl of flunitrazepam plus 5 μl of PCALC36 (final concentrations: 300 nM and 30 μM, respectively) was added. At various time intervals, 500-μl samples were filtered as described above. All values were corrected for nonspecific binding, defined as binding in the presence of 5-μM unlabeled flunitrazepam.

2.4. Electrophysiology

Pregnant Wistar rats at 18–20 days of gestation were sacrificed under CO_2 narcosis and hippocampal cells from the foetuses were isolated and cultured by standard methods (Goslin and Banker, 1991). About 7×10^5 cells (neurones and glia) were plated per 35-mm poly-L-lysine-coated dish and were used 24–44 days after plating, when neurones showed mature morphology and abundant synaptic activity. Maintenance medium was modified Eagle's medium (MEM) with Earle's salts supplemented with 5 g/l of D-glucose, 2-mM glutamine, and 10% horse serum. Whole-cell membrane currents were recorded with an Axopatch 200A patch clamp (Axon Instruments, USA) using a fast-switching perfusion system to apply drug pulses (Castro et al., 1999). The extracelullar solution was (in mM): NaCl 165, KCl 5, $CaCl_2$ 2, D-glucose 10, HEPES 5, and NaOH ~ 2 (pH 7.3). For tonic current recordings, drugs were dissolved in extracellular solution with added tetrodotoxin 0.6 μM (Alomone Labs, Israel) to block voltage-gated sodium channels, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 10 μM to block non-NMDA receptor channels, and $MgCl_2$ 1 mM to partially block NMDA receptor channels. The intracellular solution was (in mM): $CsCH_3SO_3$ 115, CsCl 20, 1,2-bis(2-aminophenoxy)ethane-*N*-*N*-*N*=-*N*=-tetraacetic acid (BAPTA) 10, HEPES 10, $Mg_{1.5}$ATP 4, and di-Tris-phosphocreatine 10 (pH 7.3 with CsOH). Under these conditions, outward currents evoked by GABA could be isolated from spontaneous cationic currents due to endogenous glutamate by clamping the membrane potential at 0 mV. Recordings were made at room temperature.

2.5. Data analysis

Data from saturation experiments at equilibrium were graphically represented using Scatchard plots. The model parameters (IC_{50}, B_{max} and K_d, and rate constants) were estimated using a computerized nonlinear regression analysis of the untransformed data (Prism, GraphPad Software), assuming a single population of binding sites, where applicable. The data of the dissociation assay were analysed using the model of one-phase exponential decay to a certain plateau. The time course of flunitrazepam binding was analysed using the model of one-phase exponential association (Prism, GraphPad Software). The association rate constant (k_{+1}) was calculated according to Eq. (1) (Bennett, 1978):

$$k_{+1} = (k_{obs} - k_{-1})/[L] \quad (1)$$

where k_{obs} is the slope of the line obtained by plotting Ln $[B_{eq}/(B_{eq} - B_t)]$ versus time where B_t is the amount of specifically bound ligand at time t, B_{eq} is the bound ligand at the steady state, k_{-1} is the dissociation rate constant and $[L]$ is the (free) ligand concentration. In the GABA shift assay, the GABA ratio was calculated dividing the IC_{50} value of the test compound measured in the absence of GABA by the IC_{50} value measured in the presence of 20-μM GABA.

Tonic current recordings were digitised and analysed using the EDR program (J. Dempster, Strathclyde University, Scotland). Drug effects were expressed as percent changes of the average tonic GABAergic current, which

was determined (in the presence of tetrodotoxin) as the difference between the average spontaneous current level and the true baseline level revealed by application of the $GABA_A$ receptor antagonist bicuculline. Miniature inhibitory postsynaptic currents (IPSC) were low-pass filtered at 1 kHz (Bessel) and digitised at 4 kHz for processing with a Mini Analysis software (Synaptosoft, USA). The root-mean-square (RMS) value of the current fluctuations was estimated for each trace as the mean from three 512-point data segments, and it was used for setting the threshold for miniature IPSC detection. In most recordings, a ubiquitous low-amplitude synaptic "noise" contributed significantly to the overall baseline noise, preventing proper analysis of small miniature IPSC. Therefore, an amplitude threshold of six times the average RMS was used for event detection. Unless otherwise noted, data are reported as mean ± S.D.

3. Results

3.1. Effect of PCALC36 on [^{3}H]flunitrazepam binding to rat brain synaptosomes

Equilibrium binding of [^{3}H]flunitrazepam to crude synaptosomes was measured at 4 °C in the presence or absence of 1- to 100-μM PCALC36. As shown in Fig. 2, PCALC36 inhibited the binding of 0.2 nM [^{3}H]flunitrazepam in a concentration-dependent manner, with estimated IC_{50} of 13.8 ± 2.7 μM and I_{max} of 101 ± 6% as determined by nonlinear regression fitting of the data. Such effect could be due either to direct competition of PCALC36 at the benzodiazepine binding site or to an indirect effect through an allosteric site. We first considered the hypothesis of an antagonism at the GABA agonist site, which would depend on the presence of endogenous GABA in the crude synaptosome preparation. In order to test this hypothesis, we compared the effect of 10-μM PCALC36 in crude and washed preparations from rat brain. As shown in Table 1, binding of 0.2 nM [^{3}H]flunitrazepam to crude synaptosomes was only slightly stimulated by the addition of GABA but was inhibited by 30% in the presence of bicuculline, a competitive antagonist of the GABA site. In washed synaptosomes, addition of GABA increased by more than 60% the binding of [^{3}H]flunitrazepam, whereas bicuculline showed little effect. These data indicate that endogenous GABA was at nearly saturating concentrations in the crude preparation and at low, nonsaturating concentrations in the washed preparation. PCALC36 inhibited [^{3}H]flunitrazepam binding to a similar extent in both crude and washed preparations (Table 1), thus ruling out the hypothesis of an action through antagonism at the GABA site. Another classical mechanism of noncompetitive inhibition of flunitrazepam binding could be an allosteric effect at modulatory site(s) at the $GABA_A$ receptor complex. To examine the allosteric modulation hypothesis, we analysed the influence of PCALC36 on the dissociation kinetics of 0.2 nM [^{3}H]flunitrazepam in an isotopic dilution assay. Fig. 3 shows

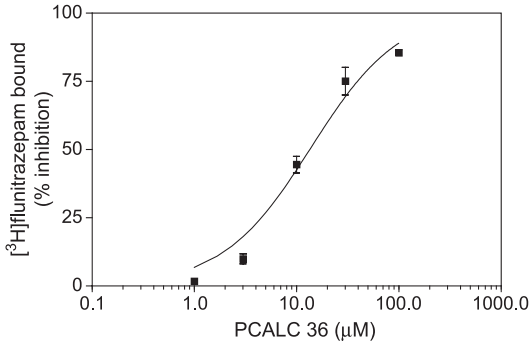

Fig. 2. Effect of PCALC36 on [^{3}H]flunitrazepam binding to crude rat brain synaptosomes. Results, expressed as percent inhibition of 0.2-nM [^{3}H]flunitrazepam binding, are means ± S.E.M. from three experiments performed in triplicate. Curves were drawn using the parameters fitted by nonlinear regression with the model of a sigmoidal dose–response curve (one binding site).

Table 1
Effect of modulators of [^{3}H]flunitrazepam binding in crude and washed preparations from rat brain

	[^{3}H]Flunitrazepam bound (%)			
	GABA 20 μM	Bicuculline 20 μM	Flunitrazepam 1 nM	PCALC36 10 μM
Crude Preparation	112.4	66.9	18.1	33.2
Washed Preparation	160.2	92.4	27.0	31.3

Synaptosomes were incubated at 4 °C for 90 min in the presence of 0.2 nM [^{3}H]flunitrazepam and Krebs solution (control) or either 20-μM GABA, 20-μM bicuculline, 1-nM unlabeled flunitrazepam, or 10-μM PCALC36. Results are expressed as % of [^{3}H]flunitrazepam bound. Data represent means of a typical experiment performed in triplicate. Similar results were obtained in two other experiments.

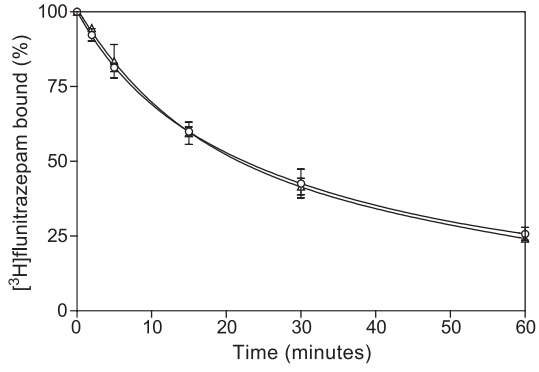

Fig. 3. Influence of PCALC36 on [^{3}H]flunitrazepam dissociation kinetics in rat brain synaptosomes. After a 90-min incubation, the dissociation of the [^{3}H]flunitrazepam-receptor complex was initiated by isotopic dilution with 300-nM unlabeled flunitrazepam alone (○, control) or with 300-nM unlabeled flunitrazepam + 30-μM PCALC36 (△). The fitted curves were obtained by nonlinear regression using the model of a biexponential decay (Chiu et al., 1981). Each point represents the mean of ± S.E.M. of triplicate determinations of specific binding in a typical experiment (repeated twice, with similar results).

that the dissociation of flunitrazepam from its binding site was not modified by the addition of a high concentration of PCALC36 because the apparent half-life was around 22 min in both cases. This result ruled out an allosteric interaction between the PCALC36 and flunitrazepam-binding sites, at least as far as dissociation kinetics is concerned. The remaining possibility of a direct interaction of PCALC36 with the benzodiazepine site was then examined. Saturation curves were obtained with [^{3}H]flunitrazepam at equilibrium, in the absence and presence of increasing concentrations of PCALC36. Fig. 4 shows that the presence of PCALC36 did not modify the affinity of flunitrazepam (Scatchard plots are nearly parallel and K_d values are very similar) but decreased its maximal binding in a concentration-dependent manner (Table 2). Analysis of the time course of flunitrazepam binding (Fig. 5) indicated that PCALC36 slightly decreased the association rate constant (k_{+1}) of flunitrazepam (from 0.110 to 0.058 nM/min). This very small decrease of k_{+1} (less than two times) associated with the lack of effect on k_{-1} is compatible with the lack of a significant effect of 30-μM PCALC36 on the K_d (k_{-1}/k_{+1}) of flunitrazepam; in fact, a small increase of K_d (around 1.5 times) was measured, but this was not statistically significant (Table 2).

Considering the possibility that irreversible binding of PCALC36 to the benzodiazepine binding sites underlay the decrease of B_{max} for flunitrazepam (i.e., by irreversible competition), we carried out experiments to investigate the stability of the PCALC36–$GABA_A$ receptor complex. In this reversibility assay, we compared the binding of [^{3}H]flunitrazepam to crude synaptosomes pretreated with 30-μM PCALC36 or with buffered Krebs solution (control) after extensive washing to promote the dissociation of bound PCALC36. Analysis of the Scatchard plots indicated that the number of [^{3}H]flunitrazepam binding sites (B_{max}) after the washout of PCALC36 (5417 ± 216 fmol/mg) was not significantly smaller than in the untreated control (5712 ± 181 fmol/mg) and that there was no alteration of the K_d (4.05 ± 0.24 and 3.85 ± 0.18 nM for pretreated and control, respectively; Fig. 6). This suggests that the binding of PCALC36 to its site on the $GABA_A$ receptor is reversible.

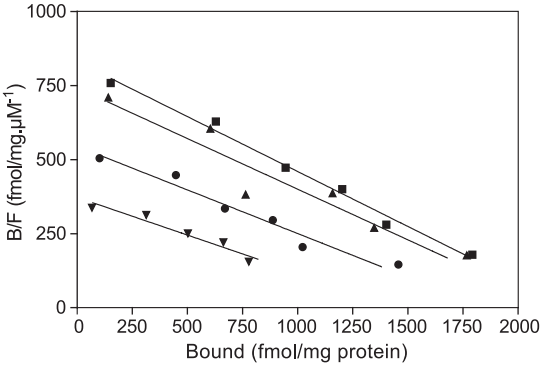

Fig. 4. Scatchard plot for the specific binding of [^{3}H]flunitrazepam to rat brain crude synaptosomes in the presence of PCALC36. About 150–200 μg protein was incubated at 4 °C for 90 min in the absence (■) or presence of 3 μM (▲), 10 μM (●), or 30 μM (▼) PCALC36. The fitted curves were obtained by nonlinear regression using the model of a single class of binding sites. Data are means from three experiments performed in triplicate.

Table 2
Values of K_d and B_{max} for [^{3}H]flunitrazepam binding measured in the presence and absence of PCALC36

	K_d (nM)	B_{max} (fmol/mg)
Control	2.70 ± 0.14	2239 ± 70.0
PCALC36 3 μM	2.92 ± 0.50	2169 ± 226
PCALC36 10 μM	3.39 ± 0.36	1849 ± 123
PCALC36 30 μM	3.93 ± 0.56	1463[a] ± 147

K_d and B_{max} values were calculated by nonlinear regression from the data shown in Fig. 4. The calculated standard error represents here the precision of the parameter estimated by fitting (goodness of fit for this parameter) of the mean curve and not a classical standard error indicating the interassay variability.

[a] $P < 0.05$.

As a first attempt to investigate the intrinsic efficacy of PCALC36, we performed a classical in vitro test, the so-called GABA shift assay (Braestrup et al., 1982, 1984), where the IC_{50} of the ligand for inhibition of [^{3}H]flunitrazepam binding is measured in the absence and presence of GABA. Fig. 7A shows that the addition of 20-μM GABA to the incubation medium decreased the apparent affinity of PCALC36 in washed synaptosomes, as observed by a shift to the right of the inhibition curve (IC_{50} ratio = 0.68). This effect of GABA was similar to that of methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM; IC_{50} ratio = 0.72; Fig. 7B), a classical full inverse agonist at the benzodiazepine binding site, and opposite to the one measured with the agonist flunitrazepam (IC_{50} ratio = 1.47; Fig. 7C).

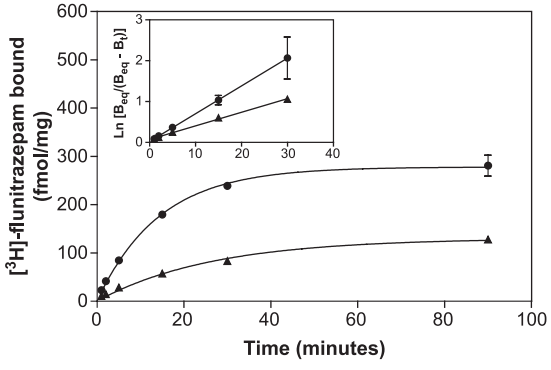

Fig. 5. Effect of PCALC36 on [^{3}H]flunitrazepam association kinetics in rat brain synaptosomes. Proteins (200 μg) were incubated at 4 °C for 90 min in the absence (●) or presence of 30-μM PCALC36 (▲). The fitted curves were obtained by nonlinear regression using the model of one-phase exponential association. Data are means ± S.E.M from two experiments (each performed in triplicate). Inset: plot of Ln [B_{eq}/($B_{eq} - B_t$)] versus time where B_t is the amount of specifically bound ligand at time t, B_{eq} is the bound ligand at the steady state, k_{-1} is the dissociation rate constant (see Fig. 4) and [L] is the (free) ligand concentration. The slopes of the lines (k_{obs} = 0.068 min^{-1} and 0.046 min^{-1}) were calculated by linear regression and used for calculation of k_{+1} (see Material and methods).

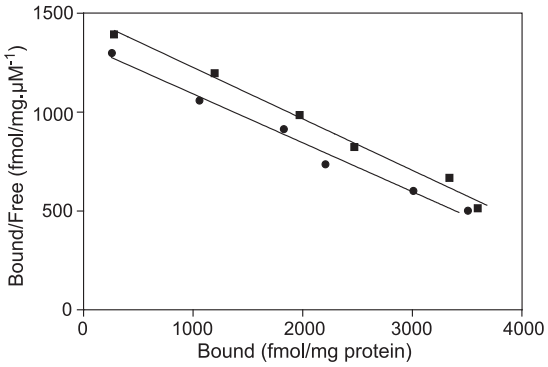

Fig. 6. Reversibility of PCALC36 inhibition of flunitrazepam binding. Scatchard plot for specific binding of [^{3}H]flunitrazepam in crude synaptosomes pretreated either with 30-μM PCALC36 (●) or Krebs Tris–HCl buffer (■, control) and extensively washed (see Materials and methods). The fitted curves were obtained by nonlinear regression using the model of a single class of binding sites. Each point represents the mean of three experiments performed in triplicate.

3.2. Electrophysiology

All hippocampal neurones sampled in this study expressed $GABA_A$ receptors, as inferred from the presence of large spontaneous IPSC that reversed the direction at the Cl^- equilibrium potential and could be blocked by 10-μM bicuculline. The application of 3-μM GABA consistently evoked currents greater than 1 nA (data not shown). However, we could not detect a significant effect of midazolam (0.1–1 μM) on currents evoked by 1- to 10-μM GABA. When macroscopic synaptic activity was blocked by tetrodotoxin, most neurones (88 out of 90) also exhibited a tonic GABAergic current (I_T), as revealed by fast baseline shifts and reduction of current fluctuations in response to pulses of 10-μM bicuculline (Fig. 8). The tonic current was insensitive to the perfusion rate, suggesting that it was not due to accumulation of endogenous GABA in the bulk solution, as previously described in cerebellar (Leão et al., 2000) and hippocampal neurones (Bai et al., 2001). Compound PCALC36 (60 μM) applied alone seemed to have a small inhibitory effect, reducing the gross fluctuations of the control I_T in 10 out of 11 tested neurones (Fig. 8). In most neurones, there was no clear baseline shift upon application of PCALC36, and the macroscopic effect seemed to involve inhibition of miniature IPSC. In contrast, midazolam (0.1 μM) potentiated I_T in two thirds of the tested neurones, with an average change to 211 ± 33% of control (n = 17). In responding neurones, when 60-μM PCALC36 was added in the presence of midazolam, I_T returned to 101.5 ± 28.0% (n = 6) of control levels, showing a complete inhibition of midazolam-induced potentiation (Fig. 8). The currents decayed to an apparent plateau in 25.2 ± 11.3 s when perfusion of PCALC36 was initiated, and they returned to the previous level in 12.7 ± 5.4 s when it ended, showing that the inhibition could be quickly reversed. In these six cells, the effects of PCALC36 (60 μM) alone on miniature IPSC were further investigated. In addition to clearly identified IPSC, the traces showed a high frequency of low-amplitude current transients that seemed to be of synaptic origin and were blocked by bicuculline. To minimise contamination with synaptic "noise", only miniature IPSC larger than six times the average RMS current fluctuations were analysed in 60- to 100-s data segments recorded

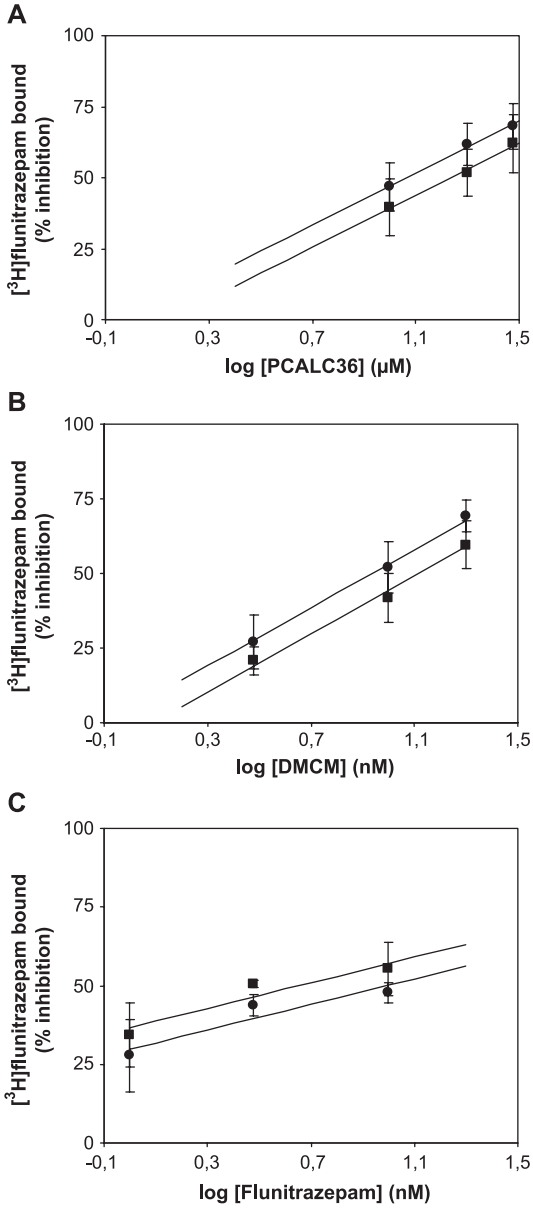

Fig. 7. Inhibitory effect of PCALC36, DMCM, and flunitrazepam on [^{3}H]flunitrazepam binding to rat brain synaptosomes in the presence and absence of GABA (GABA shift). Around 200 μg washed protein was incubated at 4 °C for 90 min in the absence (●) or presence of 20-μM GABA (■) and increasing concentrations of PCALC36 (A), DMCM (B), or flunitrazepam (C). Data are expressed as % inhibition of 0.2 nM [^{3}H]flunitrazepam (specific) binding. Each point represents the mean ± S.E.M. of three experiments performed in triplicate, except for control experiments (B and C) where n = 2.

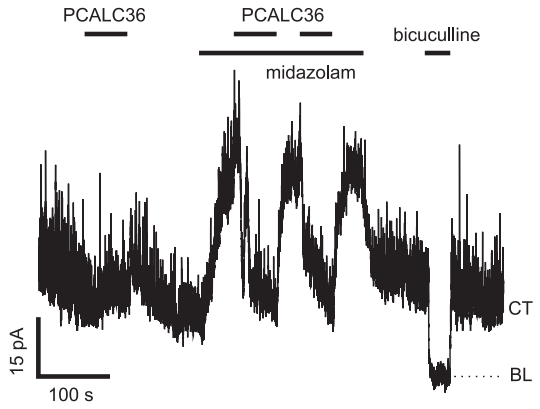

Fig. 8. Effects of PCALC36 on GABAergic tonic currents. Sample recording from a hippocampal neurone kept for 40 days in culture, clamped at 0 mV. Upward (spiky) deflections are miniature inhibitory synaptic currents, which are not resolved at the time scale of this figure. Periods of drug application are shown by the horizontal bars on top. PCALC36 60 μM alone seemed to reduce current fluctuations, while 0.1 μM midazolam more than doubled the control current level (CT), which was measured relative to the baseline (BL) seen during the application of 10 μM bicuculline (right). Two pulses of 60-μM PCALC36 transiently and reproducibly abolished the effect of midazolam.

before the application of PCALC36 (control), during its application, and after its removal (washout).

Data obtained in the three conditions were analysed by repeated-measures ANOVA. The mean control amplitude ranged from 12.9 to 25.3 pA ($n = 39$ to 308 events). In the presence of PCALC36, the mean amplitude was reduced in all six cells ($P < 0.05$) with an average reduction of $20.5 \pm 10.2\%$. This effect was not significantly reversed after 1 min of washout. Together with the reduction in amplitude, there was a reduction of $62.2 \pm 15.8\%$ ($P < 0.05$) in the frequency of detected events that was partially reversed in four of the six cells within 1 min of the removal of PCALC36.

4. Discussion

The search for new ligands of the central benzodiazepine receptor with more selective patterns of effects than the classical 1,4-benzodiazepines has been stimulated by recent evidences that specific $GABA_A$ receptor subtypes discriminate between benzodiazepine site ligands (Rudolph et al., 1999; Möhler et al., 2001). Among the many, structurally diverse compounds that bind with high affinity to the benzodiazepine binding site (Braestrup et al., 1984; Gardner et al., 1993; Chebib and Johnston, 2000), we draw a special attention to flavonoids, either naturally occurring or synthetic derivatives (Hui et al., 2002; Medina et al., 1990; Viola et al., 1994; Wolfman et al., 1998; Salgueiro et al., 1997; Viola et al., 2000; Kahnberg et al., 2002; Nielsen et al., 1988). Here we described the molecular mechanism of the action of a newly synthesized coumestan (2-methoxy-3,8,9-trihydroxy coumestan—PCALC36) on the $GABA_A$ receptor complex. PCALC36 has moderate to low affinity for inhibiting flunitrazepam binding to the central benzodiazepine receptor (IC_{50} around 10 μM) when compared to classical 1,4-benzodiazepines (nanomolar range) and some flavonoids (Marder et al., 2001). To the best of our knowledge, this is the first report to state that coumestans, chemically classified as isoflavonoids, interact with the central benzodiazepine receptor. This effect was not due to antagonism at the GABA site, where GABA positively modulates the affinity of benzodiazepine ligands to their site on the $GABA_A$ receptor complex. This hypothesis has been ruled out by the finding that PCALC36's effect was not abolished in a washed preparation depleted of endogenous GABA (Table 1). A priori, an allosteric effect on benzodiazepine binding could also be ruled out because PCALC36 did not increase the dissociation rate of flunitrazepam (Fig. 3). It is worth noting that the protocol used in our dissociation assay has already been used successfully for demonstrating the (negative) allosteric modulation of pentobarbital at the picrotoxin/cage convulsant site (Trifiletti et al., 1984). Theoretically, an allosteric effect should not be definitively excluded because we detected a small decrease of the association rate of flunitrazepam binding in the presence of PCALC36 (Fig. 5). A more direct effect of PCALC36 on the benzodiazepine-binding site is suggested by the saturation curves of flunitrazepam performed in the presence of increasing concentrations of PCALC36 (Fig. 4). However, the decrease of B_{max} with no alteration of the K_d of flunitrazepam ruled out the classical hypothesis of a reversible competition for the same sites. The simple alternative mechanism would be that PCALC36 binds to the benzodiazepine-binding site, but in an irreversible manner. However, this straightforward explanation has not been supported by the results of the reversibility assay described in Fig. 6. There, a simple washing procedure was enough to dissociate completely PCALC36 from its binding site. Inasmuch as a different tissue (hippocampal) was used for current recordings, the fast reversal of the inhibition of I_T when PCALC36 was removed, particularly in the presence of midazolam, is also strong evidence of reversible binding. A convincing explanation for the apparent contradictory results is not easy, but two hypotheses could be raised. First, PCALC36 could bind to an allosteric site but could only decrease the association rate of flunitrazepam binding so that no effect would be observed in our dissociation assay. However, in this case, we should have detected a large decrease of the association rate constant for flunitrazepam and a significant decrease of its affinity, but this was not apparent in the Scatchard plots presented in Fig. 4. Alternatively, binding of PCALC36 could be too stable at 4 °C to allow true competition with flunitrazepam, but not irreversible, so that dissociation could occur during the washing procedure of the reversibility assay. Furthermore, in the hippocampal neurons,

only the effect on I_T was quickly reversed by washout, while the effect on synaptic currents was only slowly and incompletely reversed after minutes of washout. This is consistent with the concept that I_T is mediated by a pharmacologically distinct and probably small subset of $GABA_A$ receptors (Bai et al., 2001; Yeung et al., 2003). The relatively fast unbinding kinetics of PCALC36 to these receptors may have been missed in the radioligand experiments if they comprise only a small fraction of the binding sites in the whole brain preparation. It is also possible that the synaptic hippocampal receptors sensitive to PCALC36 belong to the larger fraction that showed features of pseudo-irreversible binding at 4 °C. Unfortunately, the small magnitude of I_T and the high frequency of spontaneous miniature IPSC precluded the analysis of the concentration–inhibition relationships in the patch-clamp experiments, which would have allowed further testing of the competition hypothesis. This issue requires further experiments and may be better addressed in a different preparation showing large benzodiazepine-sensitive responses to applied GABA.

Regarding the intrinsic efficacy of PCALC36, the binding assay indicated that it could act as an inverse agonist because a GABA shift to the right was observed (IC_{50} ratio < 1). The shifts described in the literature are always relatively small (from about 0.5 to 2, for full inverse and "direct" agonists, respectively), and they are more easily quantified when an antagonist is chosen as ligand. In the present case, we used an agonist, following the lead of the pioneers of this technique (Braestrup et al., 1982) as well as others, more recently (e.g., Kawasaki et al., 1996). Positive controls for an agonist (flunitrazepam) and an inverse agonist (DMCM) were included in order to show that the present conditions were able to discriminate between these two kinds of drugs and in order to facilitate the comparison with PCALC36. The predictive value of the GABA shift has recently been further confirmed by Visser et al. (2003), who reported a highly significant linear correlation between the in vivo intrinsic efficacy and the log GABA shift. In our hands too, the prediction of the GABA shift assay was consistent with the inhibitory effect seen in the electrophysiological experiments. PCALC36 produced little change in the GABAergic I_T, but it caused a 20% reduction in miniature IPSC amplitude and completely antagonised the full (direct) agonist midazolam. These effects suggest an inverse agonism, possibly with low intrinsic efficacy. Unfortunately, full efficacy standards for comparison are lacking because few studies have addressed the effects of benzodiazepine-site inverse agonists on miniature IPSC. Two studies showed that the partial inverse agonist RO 15-4513 (which is a full positive agonist at $GABA_A$ receptors containing the α4 subunit) accelerates the decay but has no effect on the amplitude of the miniature IPSC (Cagetti et al., 2003; Hsu et al., 2003). To the best of our knowledge, the effect of the classical BZP inverse agonist DMCM on these currents has not been described so far. Thus, the conclusion that PCALC36 has low efficacy is conservative, based on the lack of a clear inhibition of the tonic currents.

PCALC36 was recently shown to inhibit Na^+/K^+-ATPase (Poças et al., 2003), raising the possibility that part of the effect on synaptic currents was presynaptic in origin. Intracellular sodium accumulation promoted by a Na^+/K^+-ATPase inhibitor would lead to a reversal of the Na^+/Ca^{2+} exchanger, increasing intracellular $[Ca^{2+}]$, and then enhancing neurotransmitter release. Similarly, the Na^+-dependent GABA transporter could operate in reverse mode, leading to GABA release instead of GABA uptake. In both cases, an increase in the frequency of miniature IPSC or in I_T would be expected, contrary to what was observed in the presence of PCALC36. Furthermore, ouabain causes no significant change in the frequency or the amplitude of miniature IPSC from rat neurons in ~ 5-min applications (Doi et al., 2002).

Our data indicate that PCALC36 acts like an inverse agonist at the benzodiazepine site of the $GABA_A$ receptor complex. Although inverse agonists are capable of producing convulsions and anxiety, recent reports indicate that the effects of such drugs in cognitive function could be clinically useful (Eid and Rose, 1999; Maubach, 2003). Inverse agonists might enhance cognition because of their ability to selectively disinhibit cholinergic neurones in the basal forebrain (Sarter and Bruno, 1997). This disinhibition would be particularly important in Alzheimer's disease, where failure of memory has been attributed to the degeneration and functional impairment of the basal cholinergic system (Whitehouse, 1998). It appears that the beneficial effects of inverse agonists at the benzodiazepine receptor could be generalised to the aged nervous system, based on the finding that RO 15-3505 improved the working memory performance of memory-impaired aged mice (Forster et al., 1995).

Much interest is now directed to the development of selective inverse agonists. A newly described inverse agonist selective for α_5 $GABA_A$ receptors improves performance in a hippocampal-dependent memory task, while being devoid of proconvulsant activity (Chambers et al., 2003). Because the α_5 receptors are expressed in cultured hippocampal neurons and may in part be extrasynaptic (Christie and De Blas, 2002; Brunig et al., 2002), it is tempting to speculate that they contributed to I_T in our experiments, thus being a possible target of PCALC36.

In addition to their proposed use as cognition enhancers, inverse agonists could be useful for the (partial) reversal of intoxication with central nervous system depressants, like alcohol (Korpi et al., 1997). Indeed, RO 15-4513, a partial inverse agonist of the benzodiazepine receptor, has been shown to have some benefits as an alcohol antidote, and alcohol consumption in rodents has been shown to decrease after use of negative modulators (Korpi et al., 1997). The introduction of PCALC36 may inspire structure–activity relationship studies based on this new structural pattern aimed at developing drug candidates for the treatment of cognition impairment and/or ethanol intoxication.

Acknowledgements

This work was supported by Pronex (Programa Nacional de Núcleos de Excelência), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Faperj (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro), and CNPq (Conselho Nacional de Ciência e Tecnologia). We thank Roche Indústrias Químicas for the kind gift of flunitrazepam and midazolam.

References

Bai, D., Zhu, G., Pennefather, P., Jackson, M.F., MacDonald, J.F., Orser, B.A., 2001. Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric acid$_A$ receptors in hippocampal neurons. Mol. Pharmacol. 59, 814–824.

Bennett Jr., J.P. 1978. Methods in binding studies. In: Yamamura, H.I., Enna, S.J., Kuhar, M.I. (Eds.), Neurotransmitter Receptor Binding. Raven Press, New York, pp. 41–55.

Braestrup, C., Schmiechen, R., Neef, G., Nielsen, M., Petersen, E.N., 1982. Interaction of convulsive ligands with benzodiazepine receptors. Science 216, 1241–1243.

Braestrup, C., Honoré, T., Nielsen, M., Petersen, E.N., Jensen, L.H., 1984. Ligands for benzodiazepine receptors with positive and negative efficacy. Biochem. Pharmacol. 33, 859–862.

Brickley, S.G., Cull-Candy, S.G., Farrant, M., 1996. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABA$_A$ receptors. J. Physiol. 497 (Pt 3), 753–759.

Brunig, I., Scotti, E., Sidler, C., Fritschy, J.M., 2002. Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J. Comp. Neurol. 443, 43–55.

Cagetti, E., Liang, J., Spigelman, I., Olsen, R.W., 2003. Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABA$_A$ receptors. Mol. Pharmacol. 63, 53–64.

Castro, N.G., de Mello, M.C.F., de Mello, F.G., Aracava, Y., 1999. Direct inhibition of the *N*-methyl-D-aspartate receptor channel by dopamine and (+)-SKF38393. Br. J. Pharmacol. 126, 1847–1855.

Chambers, M.S., Atack, J.R., Broughton, H.B., Collinson, N., Cook, S., Dawson, G.R., Hobbs, S.C., Marshall, G., Maubach, K.A., Pillai, G.V., Reeve, A.J., MacLeod, A.M., 2003. Identification of a novel, selective GABA(A) alpha5 receptor inverse agonist which enhances cognition. J. Med. Chem. 46, 2227–2240.

Chebib, M., Johnston, G.A., 2000. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. J. Med. Chem. 43, 1427–1447.

Chiu, T.H., Dryden, D.M., Rosenberg, H.C., 1981. Kinetics of [^{3}H]flunitrazepam binding to membrane-bound benzodiazepine receptors. Mol. Pharmacol. 21, 57–65.

Christie, S.B., De Blas, A.L., 2002. α5 Subunit-containing GABA$_A$ receptors form clusters at GABAergic synapses in hippocampal cultures. NeuroReport 13, 2355–2358.

da Silva, A.J., Melo, P.A., Silva, N.M., Brito, F.V., Buarque, C.D., de Souza, V.P., Rodrigues, V.P., Poças, E.S., Noël, F., Albuquerque, E.X., Costa, P.R., 2001. Synthesis and preliminary pharmacological evaluation of coumestans with different patterns of oxygenation. Bioorg. Med. Chem. Lett. 11, 283–286.

da Silva, A.J., Buarque, C.D., Brito, F.V., Aurelian, L., Macedo, L.F., Malkas, L.H., Hickey, R.J., Lopes, D.V., Noël, F., Murakami, Y.L., Silva, N.M., Melo, P.A., Caruso, R.R., Castro, N.G., Costa, P.R., 2002. Synthesis and preliminary pharmacological evaluation of new (+/-) 1,4-naphthoquinones structurally related to lapachol. Bioorg. Med. Chem. 10, 2731–2738.

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.

Doi, A., Kakazu, Y., Akaike, N., 2002. Na^+/Ca^{2+} exchanger in GABAergic presynaptic boutons of rat central neurons. J. Neurophysiol. 87, 1694–1702.

Eid, C.N.J., Rose, G.M., 1999. Cognition enhancement strategies by ion channel modulation of neurotransmission. Curr. Pharm. Des. 5, 345–361.

Forster, M.J., Prather, P.L., Patel, S.R., Lal, H., 1995. The benzodiazepine receptor inverse agonist RO 15-3505 reverses recent memory deficits in aged mice. Pharmacol. Biochem. Behav. 51, 557–560.

Gardner, C.R., Tully, W.R., Hedgecock, C.J., 1993. The rapidly expanding range of neuronal benzodiazepine receptor ligands. Prog. Neurobiol. 40, 1–61.

Gordon, J.A., 2002. Anxiolytic drug targets: beyond the usual suspects. J. Clin. Invest. 110, 915–917.

Goslin, K., Banker, G., 1991. Rat hippocampal neurons in low-density culture. In: Banker, G., Goslin, K. (Eds.), Culturing Nerve Cells. MIT Press, Cambridge, p. 251.

Hsu, F.C., Waldeck, R., Faber, D.S., Smith, S.S., 2003. Neurosteroid effects on GABAergic synaptic plasticity in hippocampus. J. Neurophysiol. 89, 1929–1940.

Huang, Q., He, X., Ma, C., Liu, R., Yu, S., Dayer, C.A., Wenger, G.R., McKernan, R., Cook, J.M., 2000. Pharmacophore/receptor models for GABA$_A$/BzR subtypes (α1β3γ2, α5β3γ2, and α6β3γ2) via a comprehensive ligand-mapping approach. J. Med. Chem. 43, 71–95.

Hui, K.M., Huen, M.S., Wang, H.Y., Zheng, H., Sigel, E., Baur, R., Ren, H., Li, Z.W., Wong, J.T., Xue, H., 2002. Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from *Scutellaria baicalensis* Georgi. Biochem. Pharmacol. 64, 1415–1424.

Kahnberg, P., Lager, E., Rosenberg, C., Schougaard, J., Camet, L., Sterner, O., Ostergaard, N.E., Nielsen, M., Liljefors, T., 2002. 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.

Kaneda, M., Farrant, M., Cull-Candy, S.G., 1995. Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. J. Physiol. 485 (Pt 2), 419–435.

Kawasaki, K., Eigyo, M., Ikeda, M., Kihara, T., Koike, K., Matsushita, A., Murata, S., Shiomi, T., Takada, S., Yasui, M., 1996. A novel benzodiazepine inverse agonist, S-8510, as a cognitive enhancer. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 20, 1413–1425.

Korpi, E.R., Mattila, M.J., Wisden, W., Lüddens, H., 1997. GABA$_A$ receptor subtypes: clinical efficacy and selectivity of benzodiazepine site ligands. Ann. Med. 29, 275–282.

Korpi, E.R., Grunder, G., Lüddens, H., 2002. Drug interactions at GABA$_A$ receptors. Prog. Neurobiol. 67, 113–159.

Leão, R.M., Mellor, J.R., Randall, A.D., 2000. Tonic benzodiazepine-sensitive GABAergic inhibition in cultured rodent cerebellar granule cells. Neuropharmacology 39, 990–1003.

Marder, M., Estiú, G., Blanch, L.B., Viola, H., Wasowski, C., Medina, J.H., Paladini, A.C., 2001. Molecular modeling and QSAR analysis of the interaction of flavone derivatives with the benzodiazepine binding site of the GABA$_A$ receptor complex. Bioorg. Med. Chem. 9, 323–335.

Maubach, K., 2003. GABA$_A$ receptor subtype selective cognition enhancers. Curr. Drug Target CNS Neurol. Disord. 2, 233–239.

Medina, J.H., Paladini, A.C., Wolfman, C., Levi de Stein, M., Calvo, D., Diaz, L.E., Peña, C., 1990. Chrysin (5,7-di-OH-flavone), a naturally-occurring ligand for benzodiazepine receptors, with anticonvulsant properties. Biochem. Pharmacol. 40, 2227–2231.

Möhler, H., Crestani, F., Rudolph, U., 2001. GABA$_A$ receptor subtypes: a new pharmacology. Curr. Opin. Pharmacol. 1, 22–25.

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

Nielsen, M., Frokjaer, S., Braestrup, C., 1988. High affinity of the naturally-occurring biflavonoid, amentoflavon, to brain benzodiazepine receptors in vitro. Biochem. Pharmacol. 37, 3285–3287.

Poças, E.S.C., Costa, P.R.R., Silva, A.J.M., Noël, F., 2003. 2-Methoxy-3,8,9-Trihydroxy Coumestan: a new synthetic inhibitor of Na^+,K^+-ATPase with an original mechanism of action. Biochem. Pharmacol. 66, 2169–2176.

Rossi, D.J., Hamann, M., Attwell, D., 2003. Multiple modes of GABAergic inhibition of rat cerebellar granule cells. J. Physiol. 548, 97–110.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.R., Martin, J.R., Bluethmann, H., Mohler, H., 1999. Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. Nature 401, 796–800.

Salgueiro, J.B., Ardenghi, P., Dias, M., Ferreira, M.B., Izquierdo, I., Medina, J.H., 1997. Anxiolytic natural and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory tasks in rats. Pharmacol. Biochem. Behav. 58, 887–891.

Sarter, M., Bruno, J.P., 1997. Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. Brain Res. Brain Res. Rev. 23, 28–46.

Silva, C.L., Cruz, H.N., Violante, F.A., Cordeiro, R.S., Martins, M.A., Noel, F., 1996. Formation of a highly stable complex between BN 50730 [tetrahydro-4,7,8,10 methyl-1(chloro-2 phenyl)-6 (methoxy-4 phenyl-carbamoyl)-9 pyrido [4′,3′ -4,5] thieno [3,2-f] triazolo-1,2,4 [4,3-a] diazepine-1,4] and the platelet-activating factor receptor in rabbit platelet membranes. Biochem. Pharmacol. 51, 193–196.

Stell, B.M., Mody, I., 2002. Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons. J. Neurosci. 22, RC223.

Trifiletti, R.R., Snowman, A.M., Snyder, S.H., 1984. Barbiturate recognition site on the GABA/benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site. Eur. J. Pharmacol. 106, 441–447.

Valeyev, A.Y., Cruciani, R.A., Lange, G.D., Smallwood, V.S., Barker, J.L., 1993. Cl^- channels are randomly activated by continuous GABA secretion in cultured embryonic rat hippocampal neurons. Neurosci. Lett. 155, 199–203.

Viola, H., Wolfman, C., Levi de Stein, M., Wasowski, C., Peña, C., Medina, J.H., Paladini, A.C., 1994. Isolation of pharmacologically active benzodiazepine receptor ligands from *Tilia tomentosa* (Tiliaceae). J. Ethnopharmacol. 44, 47–53.

Viola, H., Marder, M., Wasowski, C., Giorgi, O., Paladini, A.C., Medina, J.H., 2000. 6,3′ -dibromoflavone and 6-nitro-3′ -bromoflavone: new additions to the 6,3′ -disubstituted flavone family of high-affinity ligands of the brain benzodiazepine binding site with agonistic properties. Biochem. Biophys. Res. Commun. 273, 694–698.

Visser, S.A.G., Wolters, F.L.C., Gubbens-Stibbe, J.M., Tukker, E., Van Der Graaf, P.H., Peletier, L.A., Danhof, M., 2003. Mechanism-based pharmacokinetic/pharmacodynamic modeling of the electroencephalogram effects of $GABA_A$ receptor modulators: in vitro–in vivo correlations. J. Pharmacol. Exp. Ther. 308, 88–101.

Wall, M.J., Usowicz, M.M., 1997. Development of action potential-dependent and independent spontaneous $GABA_A$ receptor-mediated currents in granule cells of postnatal rat cerebellum. Eur. J. Neurosci. 9, 533–548.

Whitehouse, P.J., 1998. The cholinergic deficit in Alzheimer's disease. J. Clin. Psychiatry 59 (Suppl. 13), 19–22.

Wolfman, C., Viola, H., Marder, M., Ardenghi, P., Wasowski, C., Schröder,N.,Izquierdo,I.,Rúveda,E.,Paladini,A.,Medina,J.H.,1998.1998. 1998. Pharmacological characterization of 6-bromo-3′ -nitroflavone, a synthetic flavonoid with high affinity for the benzodiazepine receptors. Pharmacol. Biochem. Behav. 61, 239–246.

Yeung, J.Y., Canning, K.J., Zhu, G., Pennefather, P., MacDonald, J.F., Orser, B.A., 2003. Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. Mol. Pharmacol. 63, 2–8.