Regulation of Neuronal and Recombinant GABA<sub>A</sub> Receptor Ion Channels by Xenovulene A, a Natural Product Isolated from Acremonium strictum


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Accepted for publication March 17, 1997

ABSTRACT

Xenovulene A (XR368) is a natural product exhibiting little structural resemblance with classical benzodiazepines yet is able to displace high-affinity ligand binding to the benzodiazepine site of the γ-aminobutyric acid (GABA<sub>A</sub>) receptor. We have characterized this compound and an associated congener (XR7009) by use of radioligand binding and electrophysiological methodologies with native neurons and the Xenopus oocyte expression system. Xenovulene A, and the more potent XR7009, inhibited [3H]flunitrazepam binding to rat forebrain with K<sub>i</sub> values of 7 and 192 nM, and 1.7 and 42 nM, respectively, each site accounting for approximately 50% of the total specific binding. In cerebellar and spinal cord membranes, these ligands identified only single binding sites. These ligands demonstrated no intrinsic agonist activity at recombinant GABA<sub>A</sub> receptors comprising α1β1γ2S subunits expressed in Xenopus oocytes, yet at 1 μM both significantly potentiated the GABA-induced response and reduced the GABA EC50 from 10.9 (control) to 5.1 (Xenovulene A) or 2.7 μM (XR7009). The rank potency order for enhancement of the 10 μM GABA response is: XR7009 (EC50, 0.02 μM) > diazepam (0.03) > Xenovulene A (0.05) > flurazepam (0.17).

It is over 30 years since the introduction of the benzodiazepines into clinical practice for the relief of anxiety. Since then, the usage of these agents has diversified into anticonvulsant, hypnotic and also muscle-relaxant applications (Woods et al., 1992). During this period of clinical application, considerable evidence has indicated that the major site of action of the benzodiazepines occurs at the GABA<sub>A</sub> receptor (Polc, 1988). Activation of this receptor accounts for the majority of inhibitory synaptic neurotransmission in the CNS and molecular cloning studies imply that the GABA<sub>A</sub> receptor probably is composed of five polypeptide subunits that participate in forming an integral ion channel (Nayeem et al., 1994; Rabow et al., 1995; Sieghart, 1995). The subunits are members of distinct “families,” designated as α, β, γ and δ, and, with the exception of the δ subunit, the families comprise multiple subunit members, i.e., α(1–6), β(1–4), γ(1–4) and δ (Sieghart, 1995).

Of particular interest to benzodiazepine pharmacology is the observation that the expression of different combinations of GABA<sub>A</sub> receptor subunits can influence the pharmacological sensitivity to benzodiazepine agonists (Wisden and Seeburg, 1992). Previously, the sensitivity of GABA<sub>A</sub> receptors to the benzodiazepines had been categorized into “type I or type II,” a feature based largely on benzodiazepine agonist selectivity. Recently, the expression of recombinant GABA<sub>A</sub> receptors has indicated that the differentiation of benzodiazepine pharmacology into type I or II, and now type III categories, depends largely on the type of α subunit present. For example, α1 subunit-containing receptors are associated with type I benzodiazepine pharmacology, whereas α2, α3 and α5 are associated with type II. The α6 subunit presently constitutes the type III category. In addition to influencing the binding and benzodiazepine pharmacology, the positive regulatory effect of the benzodiazepines on GABA<sub>A</sub> receptor function can be removed by eliminating the gamma subunit from the

Received for publication November 26, 1996

ABBREVIATIONS: GABA<sub>A</sub>, γ-aminobutyric acid receptor (type A); CNS, central nervous system; MBM, modified Barth’s medium; MEM, minimum essential medium; β-CCE, β-carboline-3-carboxylate; FLZ, flunitrazepam; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid.

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receptor complex (Pritchett et al., 1989; Wisden and Seeburg, 1992).

The recent discovery of a secondary metabolite, (2aR*, 5aR*, 7E,11E,14aS*, 14bS*, 14cS*)-2a,5a,6,9,10,13,14,14a, 14b,14c-decahydro-4 - 4 - hydroxy-5a,9,9,12-tetramethyl-1,5-dioxacycloapenta[cd]-cycloundec[fi]inden-3(2H)-one (Xenovulene A), extracted from the microorganism, Acremonium strictum, and its ability to displace high-affinity ligand binding to the benzodiazepine site on the GABA_A receptor (Ainsworth et al., 1995), was of interest, because this compound is structurally unrelated to the classical benzodiazepine ligands. We undertook the present study for two reasons: to ascertain the binding characteristics of this novel substance and a related derivative, with use of neuronal preparations noted for differences in benzodiazepine selectivity; and to analyze how these agents can functionally regulate both native neuronal and selected recombinant GABA_A receptors by binding to the allosteric “benzodiazepine site.”

A preliminary account of some of these results has been published previously in abstract form (Thomas et al., 1996; Sundaram et al., 1996).

Materials and Methods

Production of Xenovulene A (XR368). Xenovulene A was prepared from A. strictum (Accession number IMI 354451) by fermentation and down-stream processing by procedures described previously (Ainsworth et al., 1985; Blackburn et al., 1996).

Preparation of XR7009. Dibasic sodium phosphate (1.58 g, 11.13 mmol) was added to a solution of Xenovulene A (400 mg, 1.12 mmol) in anhydrous dichloromethane (20 ml). The resulting mixture was cooled to 0°C, and a solution of 3-chloroperbenzoic acid (424 mg, 1.23 mmol) in anhydrous dichloromethane (5 ml) was added dropwise with rapid stirring. After addition was complete the reaction mixture was maintained at 0°C for a further 30 min.

Further dichloromethane (100 ml) was then added and the resultant mixture filtered through celite. The filtrate was washed with sodium thiosulfate solution (5% (w/v), 3 × 25 ml), saturated sodium carbonate solution (2 × 25 ml) and finally brine (2 × 25 ml). The solution obtained was dried briefly with MgSO_4, filtered through celite and evaporated in vacuo to yield XR7009, ((2aR*, 5aR*, 7E, 11E, 14aS*, 14bS*, 14cS*)-11,12-epoxy-2a,5a,6,9,10,13,14,14a,14b,14c-decahydro-4-hydroxy-5a,9,9,12-tetramethyl-1,5-dioxacycloapenta[cd]-cycloundec[fi]inden-3(2H)-one; 275 mg, yield 66%). XR7009 had the following spectroscopic characteristics:

1H NMR (d_6-MeOH): δ 1.08 (3H, s), 1.24 (3H, s), 1.30 (3H, s), 1.36 (2H, m), 1.48 (3H, s), 1.52 (2H, m), 1.78 (1H, d, J = 12Hz), 1.92 (1H, m), 2.08 (1H, m), 2.50 (1H, m), 2.74 (2H, m), 2.96 (1H, m), 3.64 (1H, d, J = 11Hz), 3.72 (1H, m), 3.78 (1H, m), 3.96 (1H, d, J = 11Hz), 4.85 (2H, m), 5.48 (2H, m). Mass spectrum: (CI, NH_3) MH⁺ 397. Mass spectrum: (CI, CH_3COOH), Calculated for C_{29}H_{43}O_5 275.7127 (as CH_3); found 275.2164 (Δ = 0 ppm). TLC: (MeOH/CH_2Cl_2 1.80 (v/v) on silica gel), Rf = 0.2.

Radioligand binding. Rat brain membranes were prepared as described previously (Duggan and Stephenson, 1988). Membranes were isolated from adult rat forebrain, cerebellum and spinal cord, then thoroughly washed and freeze-thawed three times before incubation with 0.5 nM [3H]flunitrazepam or 5 nM [3H]Ro15–4513 in the presence and absence of competing drugs at 4°C for 1 h. Assays were performed in triplicate with a final volume of 1 ml. Samples were harvested by rapid filtration and washed in ice-cold phosphate buffered saline. Specific binding (calculated from the total and nonspecific binding) was defined as that inhibited by 10 μM diazepam and the results, expressed as mean ± S.E. mean, were analyzed by nonlinear least squares analysis using INPLOT (Ver 4.0; Graphpad Software). To assess the goodness-of-fit provided by single or two binding site models, the experimental results were compared with the theoretical fit by use of the Fisher (F) test.

Cell preparation: Xenopus oocytes. Oocytes were removed from anesthetized Xenopus laevis as described previously (Smart and Krishek, 1995) and placed in MBM containing (mM): 110, NaCl; 1, KCl; 2.4, NaHCO_3; 7.5, Tris-HCl; 0.33, Ca(NO_3)_2; 0.41, CaCl_2; 0.82, MgSO_4; 50 μg/ml gentamicin (pH 7.6). Suitable oocytes (stages IV and V) were centrifuged at 700 to 1100 × g for 10 min at 10°C to reveal the nucleus into which 10 to 20 nl of cDNA solution (1 mg/ml), encoding for murine GABA_A receptor subunits, was injected. Oocytes were then incubated at 19°C for 24 h and the MBM was replaced every 2 to 3 days. Murine cDNAs were cloned as EcoRI fragments into the mammalian expression vector pOW1, as described previously (Krishek et al., 1994).

Cultured cortical neurons. Cerebral hemispheres were removed from postnatal rats (Wistar, day 1) and cut into 3 mm cubes before incubating in 0.25% (w/v) trypsin in Hanks' calcium-magnesium free balanced salt solution for 10 min at 37°C. Trypsin was inactivated by adding serum-containing growth medium. The tissue was triturated with polished Pasteur pipettes with decreasing orifice diameters. Cells were pooled and plated on poly-L-lysine coated 35 mm dishes in fresh growth medium based on: MEM (Earle's salts); 10% (v/v) horse serum, 2 mM glutamine, 5 mg/ml glucose, 200 U/ml and 200 μg/ml penicillin G and streptomycin, respectively. The cells were incubated at 37°C in 95% air/5%CO_2. Non-neuronal cell growth was controlled after 5 days by including 10 μM cytosine arabinoside in the growth medium for 24 h. Sympathetic neurons were prepared according to the methods described by Smart (1992).

Electrophysiology: Intracellular recording. Whole-cell membrane currents and conductances were recorded from Xenopus oocytes using a two-electrode voltage clamp technique. Oocytes were superfused with an amphibian Ringer's solution containing (mM): 110, NaCl; 2, KCl; 5, HEPES; 1.8, CaCl_2 (pH 7.4), at 10 to 15 ml/min (bath volume, 0.5 ml). Voltage and current microelectrodes (1–5 MΩ) were filled with 3 M KCl and 0.6 M K_2SO_4, respectively. Currents were recorded using an Axoclamp 2A amplifier in conjunction with a Brush-Gould Ink pen recorder.

Whole-cell channel recording. Patch electrodes (1–5 MΩ) were filled with the following solution containing (mM): 140, KCl; 1, MgCl_2; 1, CaCl_2; 10, HEPES; 11, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; 2, ATP, pH 7.1. The cells were continuously superfused in the culture dish with a Krebs' solution containing (mM): 140, NaCl; 12, KCl; 1.2, MgCl_2; 2.5, CaCl_2; 10, HEPES; 11, glucose, pH 7.4. Membrane currents were recorded using an Axopatch 1C patch amplifier. Series resistance compensation of 80% was routinely achieved and membrane currents were filtered at 10 kHz (–3 dB, 6 pole Bessel filter, 36 dB/octave).

Analysis of ligand-modulated membrane conductances. The ligand-induced membrane conductance change (ΔG) was calculated by subtraction of the resting conductance. Conductances were ascertained by the application of brief hyperpolarizing voltage command steps (1 s duration, −10 mV amplitude, 0.2 Hz) which were superimposed on the holding potential (−30 to −50 mV) in the absence and presence of ligand as described previously (Krishek et al., 1994).

These data were used to construct equilibrium concentration-response relationships for GABA_A. To pool dose-conductance data from more than one oocyte, all the conductances were normalized (ΔG_{max}) to the conductance change produced by 10 μM GABA. These data were used to construct equilibrium concentration-response curves and fitted according to the following equation: ΔG/ΔG_{max} = 1/(1 + (EC_{50}/ A)^n), where ΔG and ΔG_{max} represent the conductance increases induced at a given concentration (A) and a saturating concentration of ligand, respectively. EC_{50} defines the ligand concentration producing half-maximal response, and n represents the Hill coefficient.

Compounds. For electrophysiology, stock solutions of Xenovulene A and XR7009 were made up in ethanol and diluted before
use in Ringer's solution. Final ethanol concentrations did not exceed 0.05% (v/v) and did not affect the GABA-activated responses. For radioligand binding, to avoid the precipitation of XR368 and XR7009 in the assay buffer, 10% (v/v) ethanol was also incorporated. This concentration of ethanol did not significantly affect \(^{3}H\)flunitrazepam binding which was determined as 93 ± 6 m.

Results

Structures of Xenovulene A and XR7009. Xenovulene A and XR7009 bear no apparent resemblance to the structure of classical benzodiazepines (see fig. 1). The structure of Xenovulene A comprises an 11-membered humulene ring fused to a very unusual tricyclic ring system, the structure of which has been described previously (Ainsworth et al., 1995). The humulene ring is conformationally restricted by the presence of two trans double bonds and the rigid tricycle, which contains five chiral centers present at the ring junctions. The relative stereochemistries of these chiral centers leads to an overall concave conformation for the Xenovulene A molecule. XR7009 occurs as a minor co-metabolite of Xenovulene A and was first detected on the scale-up of the fermentation of Xenovulene A. However, XR7009, which differs from the parent compound by the selective epoxidation of one face of the trisubstituted C\(_{11}-\)C\(_{12}\) double bond in the humulene ring, was routinely and more conveniently prepared as a semisynthetic derivative of Xenovulene A.

Radioligand binding studies on GABA\(_{A}\) receptors. The first indication that Xenovulene A could specifically interact with the "benzodiazepine binding site" on the mammalian GABA\(_{A}\) receptor was evident from the ability of Xe-teract with the "benzodiazepine binding site" on the mammalian GABA\(_{A}\) receptor. The humulene ring is conformationally restricted by the presence of two trans double bonds and the rigid tricycle, which contains five chiral centers present at the ring junctions. The relative stereochemistries of these chiral centers leads to an overall concave conformation for the Xenovulene A molecule. XR7009 occurs as a minor co-metabolite of Xenovulene A and was first detected on the scale-up of the fermentation of Xenovulene A. However, XR7009, which differs from the parent compound by the selective epoxidation of one face of the trisubstituted C\(_{11}-\)C\(_{12}\) double bond in the humulene ring, was routinely and more conveniently prepared as a semisynthetic derivative of Xenovulene A.

Radioligand binding studies on GABA\(_{A}\) receptors. The first indication that Xenovulene A could specifically interact with the "benzodiazepine binding site" on the mammalian GABA\(_{A}\) receptor was evident from the ability of Xenovulene A to inhibit \(^{3}H\)flunitrazepam binding to bovine brain membranes with an IC\(_{50}\) of 40 nM (Ainsworth et al., 1995). To further analyze the radioligand binding profile, three neuronal preparations containing GABA\(_{A}\) receptors exhibiting differential sensitivities to some benzodiazepines (classified as types I to III) were selected from the rat CNS. These included membranes prepared from the forebrain (types I and II), cerebellum (predominantly type I and diazepam-insensitive type III) and spinal cord (type II > type I).

In competition radioligand binding assays, Xenovulene A inhibited \(^{3}H\)flunitrazepam binding to adult rat forebrain membranes with an IC\(_{50}\) of 56 ± 9 nM (mean ± S.E.M., \(n = 5\) experiments). Analysis of the binding data revealed that a two-binding site model was required to adequately describe the displacement of \(^{3}H\)flunitrazepam by Xenovulene A (table 1). Two apparent inhibition constants (\(K_i\)) of 7 ± 2 and 192 ± 44 nM, were defined for Xenovulene A binding to designated high (\(K_{high}\)) and low (\(K_{low}\)) affinity binding sites. Each dissociation constant accounted for approximately 50% of the total number of Xenovulene A binding sites. In comparison, the binding of both the type I selective ligands, zolpidem and \(\beta\)-CCE, to forebrain membranes, were also described by a twobinding site model with equal proportions of high and low affinity sites (table 1; \(n = 3\)). In contrast, the binding of the nonselective (type I over type II) agents, diazepam, lorazepam, flurazepam and clobazepam (up to 100 \(\mu\)M, \(n = 3\)), were all described by a single binding site model (table 1). Competition assays for Xenovulene A on forebrain membranes were also analyzed in the presence of 100 \(\mu\)M GABA, which caused a 1.3-fold shift in the \(K_i\) values for Xenovulene A to a apparently higher affinity (data not shown).

Xenovulene A also inhibited the binding of \(^{3}H\)flunitrazepam to rat cerebellar and spinal cord membranes, but in contrast to the forebrain, the competition curves were adequately described by a single binding site model with apparent \(K_i\) values of 15 ± 4 (cerebellar, \(n = 5\)) and 177 ± 47 nM (spinal cord, \(n = 5\)). Competition curves for the Type I selective agents, zolpidem and \(\beta\)-CCE, and the nonselective agents (Type I > Type II), diazepam, flurazepam, lorazepam and clobazepam, on cerebellar and spinal membranes, were also fitted to single binding site models (table 1).

The binding profile of the synthetic 11,12-epoxy derivative of Xenovulene A, designated as XR7009 (see fig. 1), was also investigated. With forebrain membranes, the displacement of \(^{3}H\)flunitrazepam binding by XR7009 resulted in competition curves best described by assuming two binding sites with \(K_{high}\) of 1.7 ± 0.5 nM and \(K_{low}\) of 42 ± 8 nM (\(n = 7\); fig. 2, table 1). Thus XR7009 exhibited a 4-fold higher apparent affinity for the forebrain benzodiazepine receptors when compared with Xenovulene A. The relative proportions of high and low affinity sites for XR7009 was shifted slightly in favor of the low-affinity site (42 ± 5 and 58 ± 5%, respectively, table 1). The displacement of \(^{3}H\)flunitrazepam binding by XR7009 in cerebellar and
spinal membranes was fitted with a single binding site model yielding $K_i$ values of $6 \pm 2$ and $63 \pm 20$ nM, respectively ($n = 7$). The binding profiles for both Xenovulene A and XR7009 suggested that these agents may be Type I selective ligands. In agreement with this concept, both Xenovulene A and XR7009 did not affect the diazepam-insensitive high-affinity binding of the benzodiazepine $[^{3}H]$Ro15–4513. This compound is associated with $\alpha_6$ subunit-containing recombinant GABA$_\alpha$ receptors and does not bind with high affinity to the $\alpha_1$ subunit-containing receptors usually associated with Type I benzodiazepine pharmacology.

The apparent selectivity of Xenovulene A for the benzodiazepine receptor was confirmed by profiling Xenovulene A against a panel of 42 ligand binding assays targeting different neurotransmitter receptors and ion channel proteins. At 1 $\mu$M, Xenovulene A was found to be inactive (classified as $< 50\%$ inhibition of binding) against all of these receptors, except the central benzodiazepine receptor.

**Regulation of GABA$_\alpha$ receptors: Electrophysiological studies on native and recombinant receptors.** The investigation of the functional effects of Xenovulene A, and the derivative XR7009, was conducted initially on recombinant GABA$_\alpha$ receptors expressed in *X. laevis* oocytes. Both Xenovulene A and XR7009 (0.1–1 $\mu$M) were devoid of any intrinsic agonist activity when applied to oocytes expressing $\alpha_1\beta_3\gamma_2S$ subunit-containing GABA$_\alpha$ receptors; however, 10 $\mu$M GABA-activated responses were significantly enhanced by these agents in a reversible manner (fig. 3A). The equilibrium-concentration response curve for GABA was shifted to the left by Xenovulene A, and more so by XR7009 (1 $\mu$M), without increasing the maximum response to GABA (fig. 3B). The $EC_{50}$ for GABA was 10.9 $\pm$ 0.4 $\mu$M ($n = 7$) in control Ringer’s solution, decreasing to 5.1 $\pm$ 0.2 $\mu$M ($n = 4$) in the presence of 1 $\mu$M Xenovulene A and 2.7 $\pm$ 0.1 $\mu$M ($n = 4$) in 1 $\mu$M XR7009. This type of positive modulation at the GABA$_\alpha$ receptor is similar to that observed with benzodiazepines, but different from that expected for the barbiturates which generally increase the maximum response to GABA. Similarly, 1 $\mu$M flurazepam displaced the concentration-response curve to the left reducing the $EC_{50}$ to 3.2 $\pm$ 0.16 $\mu$M ($n = 3$) without affecting the maximum response; whereas, 50 $\mu$M pentobarbitone displaced the curve leftward, reducing the $EC_{50}$ to 1.06 $\pm$ 0.1 $\mu$M ($n = 3$), but increasing the maximum response to GABA (data not shown).

To compare the relative potencies of Xenovulene A and XR7009 with some classical benzodiazepines and pentobarbitone, the response to 10 $\mu$M GABA was measured in the presence of different concentrations of these modulators. The percentage increase in the GABA-activated membrane conductance depended on the concentration of the modulator (fig. 4). For Xenovulene A, XR7009, diazepam and flurazepam (concentration range, 0.001–2.5 $\mu$M), the maximum enhancement induced was approximately 50% of the control response induced by 10 $\mu$M GABA. In comparison, pentobarbitone (0.1–500 $\mu$M) induced a maximum enhancement of approximately 180% (fig. 4). The $EC_{50}$ values for the modulators are shown in table 1.

### Table 1

**Inhibition constants for a range of ligands displacing $[^{3}H]$flunitrazepam binding from rat brain membranes**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Forebrain</th>
<th>Cerebellum</th>
<th>Spinal Cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenovulene A</td>
<td>$7.0 \pm 2.0^a (51 \pm 4)$</td>
<td>$15 \pm 4$</td>
<td>$177 \pm 47$</td>
</tr>
<tr>
<td>XR7009</td>
<td>$1.7 \pm 0.5^a (42 \pm 5)$</td>
<td>$6.0 \pm 2$</td>
<td>$63 \pm 20$</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>$4.0 \pm 0.4^a (52 \pm 3)$</td>
<td>$9 \pm 2$</td>
<td>$97 \pm 10$</td>
</tr>
<tr>
<td>$\beta$-CCE</td>
<td>$0.23 \pm 0.05^a (57 \pm 7)$</td>
<td>$0.36 \pm 0.08$</td>
<td>$3.0 \pm 0.8$</td>
</tr>
<tr>
<td>Diazepam</td>
<td>$4.8 \pm 0.4$</td>
<td>$5.2 \pm 0.4$</td>
<td>$6.3 \pm 0.3$</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>$0.10 \pm 0.03$</td>
<td>$0.13 \pm 0.04$</td>
<td>$0.16 \pm 0.04$</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>$14 \pm 4$</td>
<td>$17 \pm 5$</td>
<td>$20 \pm 4$</td>
</tr>
<tr>
<td>Clobazepam</td>
<td>$131 \pm 9$</td>
<td>$129 \pm 2$</td>
<td>$151 \pm 9$</td>
</tr>
</tbody>
</table>

*Values are given as mean $\pm$ S.E.M.*

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**Fig. 2.** Inhibition of $[^{3}H]$flunitrazepam binding by Xenovulene A and XR7009 in rat brain membranes. Results are expressed as percentages (mean $\pm$ S.E.M. of triplicate determinations) of specific $[^{3}H]$flunitrazepam binding inhibition by Xenovulene A (X-A; A) and XR7009 (B) in rat forebrain (○), cerebellar (□) and spinal cord (▲) membranes. Curves were fitted to data by use of a nonlinear least squares method, and the goodness-of-fit to theoretical single or two-binding site models was assessed with the Fisher (F) test. Similar results were obtained for displacements by Xenovulene A and XR7009 in three to seven separate experiments, the inhibition constants for which are summarized in table 1.
Flumazenil (1–5 μM) is a zodiazepine antagonist, which inhibits the enhancements with benzodiazepines. The profiles of Xenovulene A and XR7009 were further established by the inhibition of the enhancements with the benzodiazepine antagonist, flumazenil (1–5 μM). These enhancements were monitored continuously by application of brief hyperpolarizing voltage steps (1 s, −10 mV, 0.2 Hz) superimposed on the holding potential (−30 to −50 mV). Solid lines represent the period of drug application, typically 30 s. Xenovulene A was observed to have no direct effect, yet was able to positively modulate the GABA-activated response in a reversible manner. To investigate the selectivity of Xenovulene A and XR7009 as potential agonists at the benzodiazepine recognition site (i.e., type I-III pharmacology), recombinant GABAₐ receptors were expressed in oocytes with different α subunits. Responses to GABA mediated by receptors composed of αβγδ subunits were enhanced by Xenovulene A and XR7009, resulting in displaced concentration-response curves and lower EC₅₀ values. For the control GABA curve, the EC₅₀ was 104 ± 11 μM (n = 6) for αβγδ receptors, representing a 10-fold decrease in potency when compared with the GABA EC₅₀ for αβγδ receptors. In the presence of Xenovulene A or XR7009, the EC₅₀ was reduced to 45 ± 4 μM (n = 3) and 64 ± 6 μM (n = 3), respectively. Interestingly, for αβγδ receptors, the relative potentiation of GABA-activated responses (between EC₂₀ and EC₅₀) was greater for Xenovulene A than XR7009, which is in contrast to the order obtained for αβγδ receptors, where XR7009 was the more potent moiety in terms of the displacement of the concentration-response curve (Figs. 3B and 6).

Although Xenovulene A and XR7009 both induced leftward displacements in the GABA concentration-response curves with corresponding reductions in the GABA EC₅₀ values for both α1 and α3 subunit-containing receptors, these compounds exhibited some degree of receptor subtype selectivity. The percentage enhancements of the responses to GABA (EC₂₀ concentrations) were significantly larger for α1 compared with α3 subunit-containing receptors (Table 2). Selectivity was also evident when expressing αβγδ receptors. GABA-activated responses for these αβγδ receptors were expressed in oocytes with different α subunits, resulting in a loss of activity for Xenovulene A (Fig. 5B), XR7009, flurazepam and diazepam, whereas the activity of pentobarbitone was apparently unaffected (data not shown).
insensitive receptors" were unaffected by either Xenovulene A (fig. 6) or XR7009 (not shown), but could be enhanced by 30 to 40% by the agent classified from radioligand binding studies as a partial inverse agonist, Ro15-4513 (200 nM), and previously demonstrated to interact with $\alpha_6$ subunit-containing receptors.

To establish that Xenovulene A also regulated the function of native neuronal GABA$_A$ receptors, GABA-activated responses were recorded from cultured cortical and sympathetic neurons. Whole-cell recording of GABA-activated currents revealed that Xenovulene A or XR7009 (1 mM) enhanced the current amplitudes (fig. 7). This effect could be prevented by prior incubation with flumazenil (1 mM).

The preincubation time required for Xenovulene A-induced enhancement of GABA-activated responses to attain a steady state was assessed by the rapid application of GABA to recombinant $\alpha_1\beta_1\gamma_2S$ subunit-containing GABA$_A$ receptors transiently expressed in human embryonic kidney cells. This revealed that the preincubation time required for a steady-state enhancement was approximately 30 s (fig. 8A). Moreover, by varying the preincubation times of Xenovulene A, the relationship of the enhanced GABA-activated response could be described by a single exponential function with a time constant of $5.8 \pm 0.8$ s ($n = 3$; fig. 8B).

**Discussion**

**Benzodiazepines and the GABA$_A$ receptor.** The usefulness of the benzodiazepines as therapeutic agents, particularly in treating anxiety, has been compromised by several well-
known side effects ranging from ataxia, sedation and amnesia to the chronic problems associated with benzodiazepine addiction. Two approaches have been adopted to obviate some of the side effects and to improve the therapeutic potential of anxiolytic benzodiazepines. The first has targeted the synthesis of numerous agents, derived with the purpose of improving therapeutic selectivity. Secondly, the realization of potential GABAA receptor heterogeneity, following studies with recombinant GABAA receptors, offers the prospect that novel benzodiazepines, targeting receptors comprising specific subunit constructs, may possess unique therapeutic profiles perhaps devoid of many of the deleterious side effects. In particular, agents that can distinguish Type II over Type I benzodiazepine receptors would be of considerable interest, particularly because the behavioral profile of such ligands is currently unresolved. An alternative approach to these strategies, which we have adopted, involves the isolation of novel compounds from natural sources, unrelated to the benzodiazepine class of ligands, that are identified by radioligand binding to be potential ligands for the benzodiazepine binding site(s).

**Allosteric modulation of the GABA_\alpha_ receptor by Xenovulene A: Type I vs. Type II pharmacology.** The displacement of [3H]flunitrazepam binding from rat brain membranes coupled with the electrophysiological observations of enhanced GABA-mediated currents, all indicated that the metabolite, Xenovulene A, and the synthetic derivative, XR7009, bind to the GABA_\alpha_ receptor. The ability of flumazenil to inhibit the enhancement of the GABA-induced current by XR7009, and the dependence of the potentiation by these agents on the presence of the \gamma_2 subunit in recombinant
GABA<sub>τ</sub> receptors, is in accordance with Xenovulene A and XR7009 binding to the benzodiazepine recognition site. In comparison with classical benzodiazepines (e.g., diazepam and flurazepam), XR7009 was more potent in enhancing GABA-mediated responses, whilst the parent molecule, Xenovulene A, was less potent than diazepam; however, both compounds behaved as full agonists at the benzodiazepine receptor, achieving similar maximal responses.

The degree of receptor selectivity exhibited by Xenovulene A and XR7009 was evident from the radioligand binding studies. Zolpidem, a classical Type I benzodiazepine ligand, displaced [³⁵S]flunitrazepam binding in rat forebrain membranes exhibiting both high (presumed Type I) and low (presumed Type II) affinity binding. Both Xenovulene A and XR7009 exhibited similar profiles with high and low affinity binding, which suggests approximately equal proportions of Type I and II benzodiazepine receptors. In spinal cord membranes, zolpidem, Xenovulene A and XR7009 resolved only a single, low affinity site, which indicated binding to Type II receptors. Moreover, in the cerebellum, single high affinity binding sites were also uncovered for zolpidem, Xenovulene A and XR7009. It is therefore apparent that the parent natural product, Xenovulene A, and its analog, appear to select for Type I receptors over Type II, displaying a binding profile similar to zolpidem.

The prospect of Xenovulene A and XR7009 acting as subtype-selective agents was equivocally demonstrated with recombinant GABA<sub>τ</sub> receptors. GABA-mediated responses recorded from α1 subunit-containing oocytes were enhanced to greater extents than currents recorded from α3 subunit-containing receptors. In addition, Xenovulene A and XR7009 were ineffective at enhancing responses to GABA on α6 subunit-containing receptors (Type III). Presumably the binding of Xenovulene A and XR7009 to cerebellar membranes occurs with α1 and not α6 subunit-containing receptors. This was supported by the observation of Xenovulene A displacing the binding of the partial inverse agonist, [³⁵S]Ro 15–4513, only from diazepam-sensitive sites (α1 subunit-containing receptors), whereas diazepam-insensitive sites (α6 subunit-containing receptors) were unaffected.

Comparison with other novel ligands at the benzodiazepine binding site. Apart from the benzodiazepines, there are other ligands that appear to fulfill the structural requirements necessary to interact at the allostERIC benzodiazepine binding site on the GABA<sub>τ</sub> receptor. The most notable of these is β-lumicolchicine, an inactive analog of the microtubule-disrupting agent, colchicine, which like our compounds also bears little structural resemblance to the benzodiazepines. β-Lumicolchicine enhanced musclemediated Cl<sup>-</sup> uptake in cortical microsacs and inhibited the binding of [³⁵S]flunitrazepam binding (Mihic et al., 1994). With human recombinant GABA<sub>τ</sub> receptors comprising α1β2γ2S subunits, β-lumicolchicine enhanced GABA-activated currents. An interaction at the benzodiazepine binding site was indicated by the inhibition of modulation with flumazenil and the inability of β-lumicolchicine to enhance responses mediated by α1β2 subunits (Mihic et al., 1994). Interestingly, as for Xenovulene A, β-lumicolchicine was less active on Type II GABA<sub>τ</sub> receptors (α2β2γ2S), which suggests some degree of subtype selectivity.

Structure-function relationship for Xenovulene A. The structures of the A. strictum metabolites have little resemblance to the structures of the classical benzodiazepines (fig. 1). To understand the details of the benzodiazepine pharmacophore, we attempted some preliminary structural modifications to Xenovulene A to identify those parts of the structures that are important for their functional properties at the GABA<sub>τ</sub> receptor, and to identify those which bestow subtype-selective characteristics. A range of semisynthetic derivatives were prepared and characterized. Modifications included: the removal of one or both of the humulene ring double bonds, blocking the enolized α-dicarbonyl group, complete reduction of the molecules to the corresponding diols and the selective reduction of the tricycle with retention of the humulene ring double bonds. With the exception of XR7009 (produced by epoxidation of the C<sub>1</sub>-C<sub>12</sub> double bond in the humulene ring of Xenovulene A), removal or chemically blocking any of these structural features resulted in a substantial loss of activity in radioligand displacement binding and functional electrophysiological assays. In particular, the rigidity of the humulene ring appears to be essential for activity at the GABA<sub>τ</sub>-benzodiazepine receptor. Interestingly, the diol analogs exhibited increased stability, a feature absent from the parent compounds. Despite these substantial structural modifications, we were unable to change the selectivity of the molecule such that it became a Type II preferring benzodiazepine receptor ligand. Xenovulene A and XR7009 therefore remain novel and fascinating templates for the design of new potentially subtype-selective ligands for the benzodiazepine receptor.

References


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