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Allosteric Modulation by Single Enantiomers of a C3-Chiral 1,4-Benzodiazepine of the Gamma Aminobutyric Acid Type A Receptor Channel Expressed in *Xenopus* Oocytes

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ABSTRACT *Xenopus laevis* oocytes injected with Poly(A)$^+$-RNA isolated from neuronal tissue express membrane proteins peculiar to the origin of mRNA. The translation of gamma aminobutyric acid type A ($GABA_A$) receptors has been shown by dose/response behavior of GABA and the reversible blockade of the GABA-induced current by picrotoxin. This current was analyzed quantitatively under two electrode voltage-clamp conditions. This methodology has been applied for the first time to study the functional properties of the receptor as a function of the stereochemistry of the ligands. The (+)-S and (–)-R enantiomers of a water-soluble benzodiazepine derivative, 7-chloro-1,3-dihydro-3-hemisuccinyloxy-5-phenyl-1,4-benzodiazepin-2-one (OXHEM), obtained by preparative high performance liquid chromatographic (HPLC) resolution on chiral stationary phase, act as agonists in the in vitro modulation of the chloride channel. The (+)-S-OXHEM enantiomer was the more active. *Chirality 9:286–290, 1997.*

KEY WORDS: *Xenopus* oocytes; stereochemistry; chiral chromatography; chiral benzodiazepines; enantioselective modulation of $GABA_A$ receptor

In the central nervous system, benzodiazepine drugs exert their anxiolytic, hypnotic, and anticonvulsant effects by allosteric enhancement of the action of gamma aminobutyric acid (GABA).[1–12] In vitro receptor binding studies clearly demonstrated that the modulation of the GABA type A ($GABA_A$)-gated Cl^- channel by benzodiazepines depends on the structure of the drugs, and when they are chiral, on their stereochemistry.[6,9–12]

Recently, the modulation of the chloride ionophore by chiral benzodiazepines has been evaluated measuring the [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) binding in the presence of GABA.[12] An alternative model system for the study of neuronal channels has been developed in recent years. Oocytes of the toad *Xenopus laevis,* injected with brain mRNA, are a very useful system to express many membrane proteins such as neurotransmitter receptors and ion channels. The $GABA_A$ receptor channels expressed in *Xenopus* oocytes by this technique exhibit properties similar to those described in neurons.

In the present study, we analyzed the physiological interactions between the $GABA_A$ receptor and single (+)-S and (–)-R enantiomers of 7-chloro-1,3-dihydro-3-hemisuccinyloxy-5-phenyl-1,4-benzodiazepin-2-one (OXHEM). We measured the GABA-induced current under two electrode voltage-clamp conditions in *Xenopus* oocytes to determine differences between the pure enantiomers on the modulation of the Cl^- channels.

MATERIALS AND METHODS

Rac-OXHEM was prepared by reacting oxazepam with succinic anhydride in the presence of pyridine, according to a literature procedure.[13] The hemisuccinate derivative was extracted with chloroform from the hydrolyzed mixture and then recrystallized from ethyl acetate-hexane (20:80, v:v). OXHEM was obtained (70% yield) as crystalline powder having m.p. 152–154°C. ^{1}H-nuclear magnetic resonance (NMR) and mass spectrometric data were in accordance with the structure. Oxazepam was obtained by Soxhlet acetone extraction of commercial pharmaceuticals and characterized by NMR and mass spectrometry. Other chemicals were of reagent grade and from commercial suppliers.

Chromatographic Resolution and Spectroscopic Measurements

The high performance liquid chromatographic (HPLC) resolution of OXHEM was obtained with a Chiralcel OJ (Daicel, Tokyo, Japan 25 × 0.4 cm. i.d.) at room temperature. The mobile phase was hexane-2-propanol/acetic acid mixture (60/40/1, v/v/v), flow rate 1 ml/min.

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The chromatographic system consisted of a Jasco 887-PU HPLC pump and a Jasco Multi-340 multichannel detector (Jasco, Tokyo, Japan). The same system and chromatographic method were used for the preparative resolution of OXHEM by low loading repetitive injections, using a Rheodyne model 7125 injector (Rheodyne, Cotuti, USA with a 20 µl loop. A circular dichroism (CD)-based detection system, Jasco J700 spectropolarimeter, was also used which allows the simultaneous absorption [ultraviolet (UV)] and CD detection. The detector was equipped with a micro-HPLC cell (8 µl) and a doublet of lenses to focus the light beam in the sample compartment.

The chromatographic retention of the solutes was followed at 254 nm and reported as the capacity factor (k′), where k′ is defined as $(t_{BDZ}-t_0)/t_0$ (t_{BDZ} = retention of the BDZ enantiomer; t_0 = retention of a non-retained solute). The enantioselectivity (α), where $\alpha = k'_2/k'_1$, was also calculated (k'_2 and k'_1 are the capacity factors of the second and the first eluted enantiomers).

CD spectra were recorded with a Jasco J600 spectropolarimeter (Jasco, Tokyo, Japan) and UV spectra with a Perkin-Elmer Lambda 9 spectrophotometer (Perkin Elmer, Ubezlingen, Germany) at room temperature, with ethanol as solvent.

RNA Extraction and Expression of the Receptors

Poly(A)$^+$-RNA was isolated from rat cerebral cortex using the guanidinium thiocyanate/phenol/chloroform procedure[14] and captured on Dynabeads Oligo $(dT)_{25}$, and then washed thoroughly using a Magnetic Particle Concentrator (Dynal A.S., Oslo, Norway).

X. laevis oocytes were isolated manually from the ovary and maintained at 19°C in Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Hepes, adjusted to pH 7.4 with NaOH and supplemented with gentamicin sulfate at 0.1 mg/ml). The next day, the oocytes were injected with 50 nl of Poly(A)$^+$-RNA dissolved in water (1–2 µg/µl). Two days later, the oocytes were treated with collagenase (140 units/ml, type I A; Sigma, St. Louis, MO) for 0.5–1 h to remove the ovarian epithelial and follicular cells, and returned to the incubator (19°C).

Electrophysiological Recordings

Membrane currents were recorded 3–7 days after mRNA injection using a voltage-clamp technique with two microelectrodes. Oocytes were placed in a recording chamber (volume 0.4 ml) continuously perfused at room temperature (20–23°C) with normal frog Ringers's solution containing 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Hepes-NaOH (pH 7.4). The membrane potential was held at –80 mV. The enantiomers of OXHEM were diluted in the perfusion medium at a flow rate of 6 ml/min. Absorption and CD spectra of these solutions were recorded in order to check the concentration and enantiomeric excess of the sample. Membrane currents were recorded and stored in a computer for subsequent analysis and continuously recorded on a chart recorder during the experiments.

The peak amplitudes of GABA-induced currents were measured off-line using the "Biomedic" software package (BM 6000, Biomedica Mangoni, Pisa, Italy).

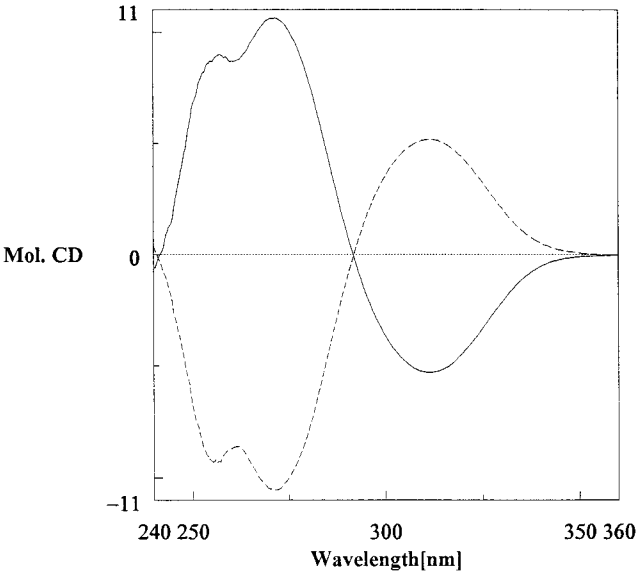

Fig. 1. CD spectra of (+)-S-OXHEM (—) and (–)-R-OXHEM (---),in 2-propanol, 1 mm cell. CD spectra carried out after 1 month are superimposable.

GABA-induced currents were recorded from the same oocyte before and after drug applications. Data are expressed as means ± standard error of the mean (S.E.M.). For statistical analysis we used a one-way analysis of variance (ANOVA) with repeated measures. We considered significant values of $P < 0.05$.

RESULTS

Chromatographic Resolution of Rac-*OXHEM and Stereochemical Characterization of the Single Enantiomers*

The enantioselective separation of OXHEM was obtained by HPLC upon a Chiralcel OJ column.

The use of the detection system measuring UV and CD signals, simultaneously, allowed us to determine the elution order of OXHEM, the enantiomer with positive CD at 254 nm being eluted first. The relationship between the sign of the CD and absolute configuration has been established.[15] Since (+)-S-OXHEM has a positive CD at 254 nm, the less retained enantiomer on Chiralcel OJ is the (+)-S-form.

The Chiralcel OJ column was also used for the preparative resolution of OXHEM. In practice, low loading repetitive injections (20 µg each injection, one injection every 20 min) allowed the collection of about 250–300 µg of each enantiomeric fraction in 20 h. The enantiomeric composition of the fractions was determined by their reinjection onto the column. The fractions gave enantiomerically pure compounds (e.e. ⩾ 99.5%) because of the high enantioselectivity in these experimental conditions (k'_1 1.3; α 2.1) and were used directly for electrophysiological tests.

CD was used to check the stereochemical stability of the samples (Fig. 1). The CD spectra of the single enantiomers of OXHEM were recorded after their HPLC preparation and immediately before their use in the electrophysiological experiments. The enantiomeric fractions obtained remained stable for 6 months.

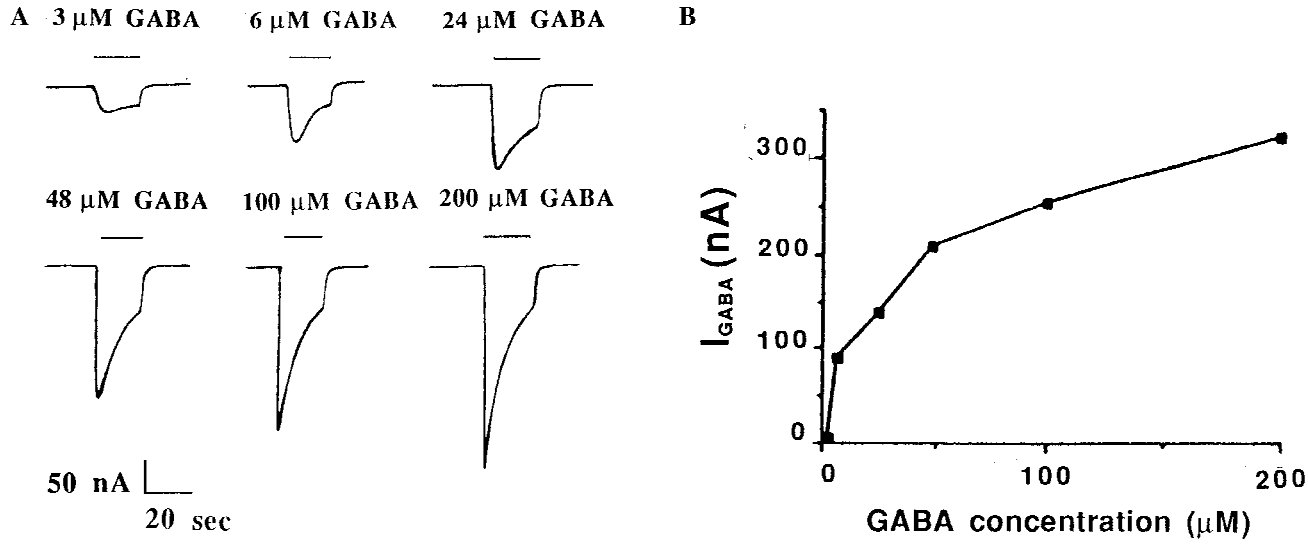

Fig. 2. Membrane currents induced by different concentrations of GABA. **A:** Sample of GABA-induced currents. Inward current is denoted by a downward deflexion of the trace. GABA was applied by bath perfusion for the duration indicated by the bars. **B:** Relation between GABA concentration and peak membrane current. The oocytes were kept at –80 mV under voltage-clamp conditions.

Functional Characterization of $GABA_A$-gated Cl^- Channels

Perfusion of mRNA-injected oocytes, clamped at –80 mV, with GABA for 20 sec produced an inward current denoted by a downward deflection of the trace. The oocytes still responded to GABA when the follicular and other enveloping cells were removed by collagenase treatment, which indicated that the GABA sensitivity resided in the oocyte membrane itself.

GABA at low concentration (1–3 μM) induced an inward steady-state current and evoked a peak current at higher concentration (6–200 μM) which decayed to a reduced steady-state level due to the time-dependent inactivation.

The peak amplitude of this current increased from 50 to 300 nA with increasing GABA concentration, as illustrated in Figure 2A. The dose-response curve for the peak membrane current, determined in five oocytes clamped at –80 mV, is presented in Figure 2B. The estimated EC_{50} of GABA for inward current was 30 μM.

The GABA-induced current was almost totally and reversibly blocked by concomitant application of a low concentration (10 μM) of picrotoxin, a $GABA_A$ receptor antagonist (Fig. 3A). Furthermore, the GABA-induced current decreased in size as the membrane was depolarized and inverted direction at about –30 mV (Fig. 3B), which corresponds to the chloride equilibrium potential in *Xenopus* oocytes.[16]

Enantioselective $GABA_A$ Agonism

After the characterization of GABA-induced inward current, the interactions between the $GABA_A$ receptors and OXHEM, resolved as single enantiomers (–)-R and (+)-S by preparative chiral HPLC, were studied. The allosteric modulation of GABA-induced current by the two enantiomers was quantitatively characterized by measuring the peak amplitude of the current induced by 4 μM of GABA, in the presence and absence of different concentrations of the OXHEM enantiomers. When the benzodiazepine was applied alone, no obvious induction of current was observed. However, concomitant application of the benzodiazepine with 4 μM of GABA potentiated the GABA-induced inward currents (Fig. 4A). At any concentration (1.6, 3.2, 4.6, 6.4, 8 μM) both (–)-R and (+)-S enantiomers enhanced GABA-induced currents. Statistical analysis of data from seven oocytes, carried out in duplicate, is shown in Table 1. The inward current induced by 4 μM GABA was increased by increasing the concentration of the benzodiazepine. The GABA response was three to four times control in the presence of 8.0 μM of (–)-R- or (+)-S-OXHEM (Fig. 4B).

It is worth mentioning that the potentiation of GABA-induced inward current by OXHEM was observed without changes in the reversal potentials of the GABA current.

DISCUSSION

To study the relationship between the stereochemistry of the benzodiazepines and the receptor functionality, the single enantiomers have to be fully characterized stereochemically. HPLC on a chiral stationary phase, coupled with a CD detector was well suited for this purpose.[17] This made possible the determination of the enantiomeric excess of each enantiomeric fraction (e.e. $\geq$ 99.5% in both cases), the absolute configuration of each fraction and their stereochemical stability. Further chiral HPLC gave the single enantiomers in sufficient amounts for electrophysiological experiments.

The GABA-induced inward current observed after injection of Poly(A)$^+$-RNA from rat confirms previous findings that the oocytes express the GABA receptor-channel complex, and that this process is caused by the injection of a specific mRNA.[18] Furthermore, the oocytes containing neuronal ion channels may be investigated under voltage-clamp conditions more easily than isolated neuronal cells. Therefore, the measures of the whole-cell currents in *Xenopus* oocytes represent a useful model to study the pharmacological effects of drugs on neuronal channels.[19]

The present results show that the GABA-induced cur-

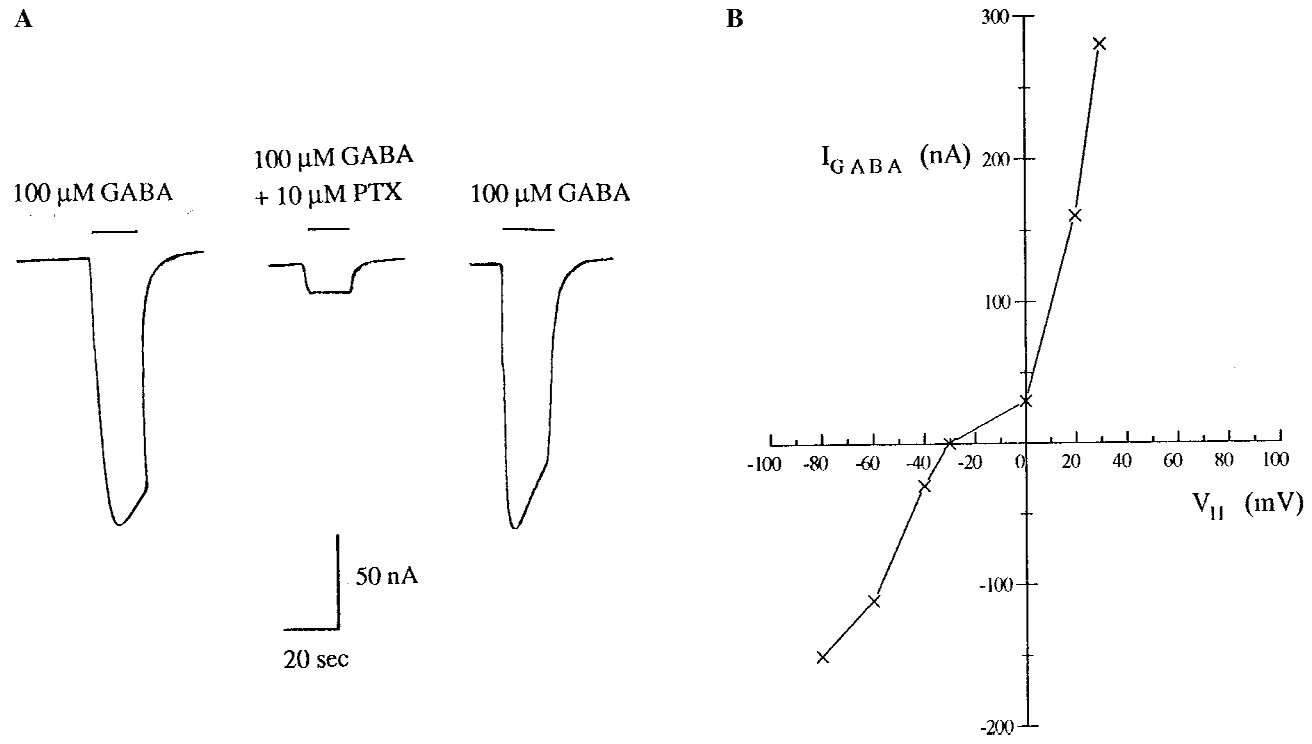

Fig. 3. Characterization of GABA-induced inward current in *Xenopus* oocytes. **A:** Reversible block of the GABA current by picrotoxin (10 μM), a selective $GABA_A$ receptor antagonist. **B:** Voltage dependence of GABA-induced current in an oocyte injected with neuronal Poly(A)$^+$-RNA. GABA (4 μM) was applied for 20 sec.

rent in the oocytes displays properties reminiscent of vertebrate central nervous system GABA currents with respect to GABA concentration dependence and sensitivity to picrotoxin.[18–23] The reversal potential of the GABA-induced current measured in our experiments is similar to that previously described in the *Xenopus* oocytes[19,23] and is close to the chloride equilibrium potential.[16,24] Therefore, it has been suggested that injection of neuronal mRNA

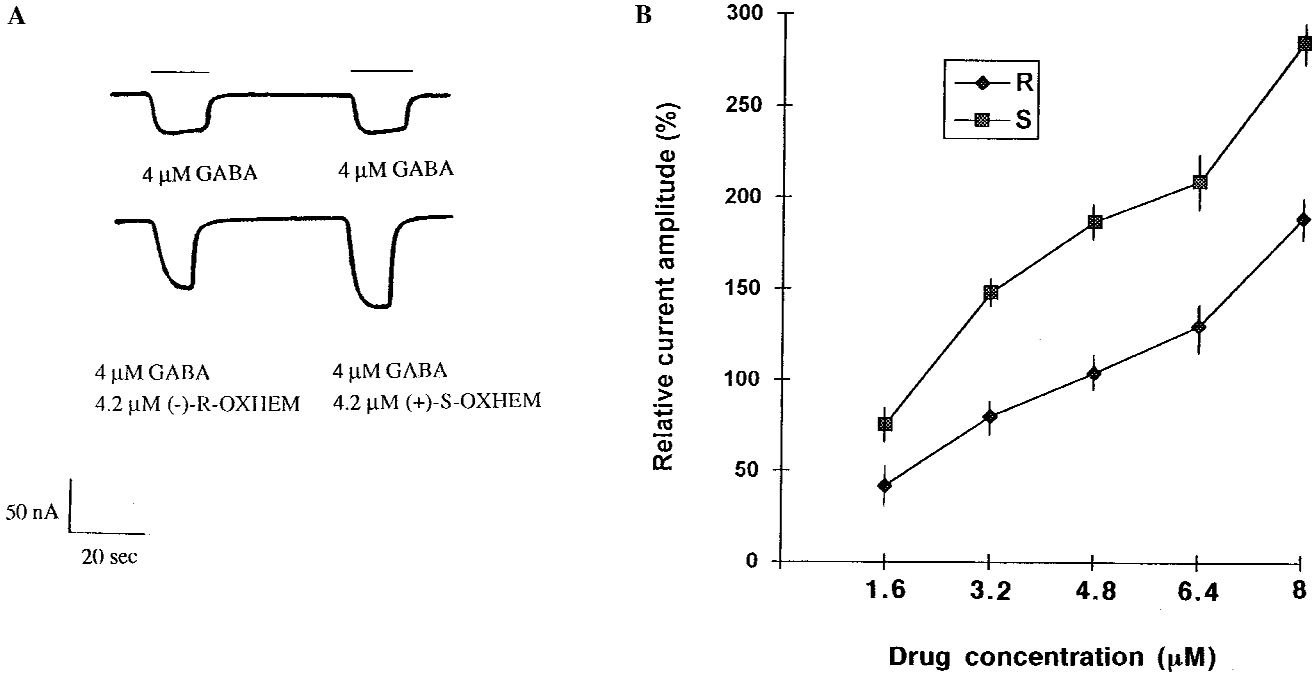

Fig. 4. A: Dose-response curves of (–)-R and (+)-S enantiomers of OXHEM on GABA-induced currents. The response induced by GABA (4 μM) alone was standardized by assigning to 0%. **B: Top trace:** Typical response to GABA 4 μM. **Lower trace:** Superfusion of the (–)-R and (+)-S enantiomers of OXHEM (4.2 μM) enhanced by the 4 μM GABA-induced current.

TABLE 1. Summary of the effects of (–)-R- and (+)-S-OXHEM on GABA-induced current[a]

	I_{GABA} (nA)										
Oocyte	GABA 4 μM	(–)-R 1.6 μM	(+)-S 1.6 μM	(–)-R 3.2 μM	(+)-S 3.2 μM	(–)-R 4.8 μM	(+)-S 4.8 μM	(–)-R 6.4 μM	(+)-S 6.4 μM	(–)-R 8 μM	(+)-S 8 μM
1	33	41	47	46	57.5	52	67.2	63	83.2	77	102
2	41	53.6	60.5	60.5	73	67	87	67	87	84	113
3	19	23.5	27.6	27	33	30.5	38.6	30.5	38.6	43.2	58.7
4	21	27	36	49.3	75.3	55	89	62.3	90.5	76	100
5	18.5	29	36	39	56.5	43	70.5	—	—	—	—
6	14	24	35	34	54.3	48.5	65	—	—	—	—
7	23	42	55	50	71	—	—	—	—	—	—
Mean	24.2	34.3	42.4	43.6	60.0	49.3	69.5	55.7	74.8	70.0	93.4
± S.E.M.	±3.5	±4.3	±4.5	±4.2	±5.6	±4.9	±7.4	±8.4	±12.1	±9.1	±11.9
Relative current amplitude (%)		41.7	75.2	80	148	104	187	130	209	189	285
		NS		$P < 0.05$		$P < 0.05$		$P < 0.05$		$P < 0.05$	

[a]For each oocyte the I_{GABA} value represents the peak amplitude of GABA current. NS = not significant.

resulted in the appearance of GABA receptors that open chloride channels when they are activated.[18]

The $GABA_A$-gated Cl^- channels expressed in *Xenopus* oocytes also appear to maintain appropriate regulatory sites to study their modulation by benzodiazepines.[23] Therefore, in the oocyte system we have investigated the effects of the single enantiomers of the OXHEM on GABA-evoked currents. Significant differences have been observed in the positive allosteric modulation of the GABA-induced currents by the two enantiomers of the benzodiazepine. (+)-S-OXHEM was more active. These electrophysiological results are in agreement with previously reported receptor binding data.[10]

In conclusion, the GABA-benzodiazepine receptor-ion channel complex expressed in *Xenopus* oocytes may represent a convenient assay system for the assessment of chiral drugs which interact with this protein complex.

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