Molecular and Neurochemical Evaluation of the Effects of Etizolam on $GABA_A$ Receptors under Normal and Stress Conditions

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Summary

The thienobenzodiazepine derivative etizolam (CAS 40054-69-1, 6-(o-chlorophenyl)-8-ethyl-1-methyl-4H-s-triazolo-(3,4-c)thienol (1,4)diazepine) is a potent anxiolytic with a pharmacological profile similar to that of classical benzodiazepines. In order to rationalize the therapeutic use of etizolam, its pharmacodynamics properties on $GABA_A$ receptors were investigated by a comparative study with other ligands on human recombinant $GABA_A$ as well as rat brain native receptors. Etizolam inhibited in a concentration-dependent manner [^{3}H]flunitrazepam (CAS 1622-62-4) binding to rat cortical membranes, with an affinity of 4.5 nmol/l greater than that of alprazolam (CAS 28981-97-7) (7.9 nmol/l). Ethizolam enhanced GABA-induced Cl-currents in oocytes expressing human cloned $GABA_A$ receptors. With $\alpha 1\beta 2\gamma 2S$ subunit combination, etizolam produced a 73 % increase in GABA-induced currents with an EC_{50} of 92 nmol/l. At the same receptor type, alprazolam showed a higher degree of potentiation and potency (98 %, EC_{50} 56 nmol/l). At $\alpha 2\beta 2\gamma 2S$ or $\alpha 3\beta 2\gamma 2S$ subunit constructs, the effects of etizolam were similar to those of alprazolam. Flumazenil (CAS 78755-81-4) completely blocked both etizolam and alprazolam effects on GABA-induced currents. Etizolam, administered i.p., was uneffective in changing ex vivo t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]-TBPS) binding to rat cerebral cortex, whereas alprazolam and abecarnil (CAS 111841-85-1) significantly reduced this parameter. However, etizolam similarly to abecarnil and alprazolam, antagonized isoniazid-induced increase (61 %) in [^{35}S]-TBPS binding to rat cortical membranes. Further, etizolam inhibited in a dose-dependent manner basal acetylcholine release from both hippocampus and prefrontal cortex, and reversed foot-shock-induced increase of basal acetylcholine release to a control level. Altogether, these results suggest that etizolam may have a reduced intrinsic activity, at least at specific subpopulations of $GABA_A$ receptors. This property, together with the pharmacokinetic indication of a short-acting drug, may characterize etizolam as a ligand endowed with less side-effects typical of full agonits such as diazepam (CAS 439-14-5) and alprazolam. Finally, given its marked efficacy under conditions of GABAergic deficit, etizolam may represent a possible drug of choice with reduced liability to produce tolerance and dependence after long-term treatment of anxiety and stress syndromes.

Zusammenfassung

Molekulare und neurochemische Evaluierung der Wirkungen von Etizolam auf $GABA_A$-Rezeptoren unter normalen und Streßbedingungen

Das Thiobenzodiazepin-Derivat Etizolam (CAS 40054-69-1, 6-(o-Chlorphenyl)-8-ethyl-1-methyl-4H-s-triazolo-(3,4-c)thienol(1,4)diazepin) ist ein starkes Anxiolytikum mit einem pharmakologischen Profil, das dem der klassischen Benzodiazepine entspricht. Zur Bewertung des therapeutischen Nutzens von Etizolam wurden seine pharmakodynamischen Eigenschaften in Beziehung auf $GABA_A$-Rezeptoren durch eine Vergleichsstudie mit anderen Liganden untersucht, und zwar obwohl in Beziehung auf menschliche rekombinante $GABA_A$-Rezeptoren wie auf native $GABA_A$-Rezeptoren aus dem Rattenhirn. Etizolam hemmte dosisabhängig die Bindung von [^{3}H]Flunitrazepam (CAS 1622-62-4) an die Membranen der Kortexzellen von der Ratte, und zwar mit einer Affinität von 4.5 nmol/l stärker als Alprazolam (CAS 28981-97-7) (7.9 nmol/l). Etizolam verstärkte den GABA-induzierten Chlorid-IonenfluB in Oozyten, die geklonte menschliche $GABA_A$-Rezeptoren exprimierten. Bei der $\alpha 1\beta 2\gamma 22S$-Untereinheiten-Kombination aus $GABA_A$-Rezeptoren verursachte Etizolam einen Anstieg des $GABA_A$-induzierten Ionenflusses urn 73 % mit einer EC_{50} von 92 nmol/l. Am gleichen Rezeptorsubtyp bewirkte Alprazolam eine stärkere $GABA_A$-Potenzierung bei höherer Wirksamkeit (98 %, EC_{50} 56 nmol/l). Bei $\alpha 2\beta 2\gamma 22S$- oder $\alpha 3\beta 2\gamma 22S$-Untereinheitskonstrukten entsprachen die Wirkungen von Etizolam denen von Alprazolam. Flumazenil (CAS 78755-8 1-4) blockierte

sowohl die Wirkungen von Etizolam wie von Alprazolam auf $GABA_A$-induzierte Ionenströme vollständig. Bei i.p. Gabe hatte Etizolam keine Wirkung auf die Ex-vivo-Bindung von t-[^{35}S]Butylbicyclophosphorthionat ([^{35}S]-TBPS) an Kortexzellmembranen der Ratte, während Alprazolam und Abecarnil (CAS 111841-85-1) diese Bindung signifikant verminderten. Andererseits antagonisierte Etizolam in gleicher Weise wie Abecarnil und Alprazolam den durch Isoniazid verursachten Anstieg (61 %) in der Bindung von [^{35}S]-TBPS an Kortexzellen der Ratte. Etizolam hemmte weiterhin in dosisabhängiger Weise die basale Acetylcholin-Freisetzung sowohl vom Hippokampus als such vom präfrontalen Kortex und fiihrte den durch einen Elektro-Fußschock verursachten Anstieg der basalen Acetylcholin-Freisetzung wieder auf das Kontrollniveau zurück. Insgesamt sprechen diese Ergebnisse dafür, daß Etizolam eine reduzierte intrinsische Wirkung hat, jedenfalls in Beziehung auf spezifische Subpopulationen von $GABA_A$-Rezeptoren. Diese Eigenschaft, zusammen mit den pharmakokinetischen Hinweisen auf eine kurze Wirkungsdauer des Medikaments, spricht dafür, daß Etizolam ein Benzodiazepin-Ligand ist, der weniger Nebenwirkungen besitzt, als sie für volle Agonisten wie Diazepam (CAS 439-14-5) und Alprazolam bekannt sind. Schließlich konnte Etizolam in Hinblick auf seine ausgeprägte Wirksamkeit unter Bedingungen eines GABA-Mangels hier möglicherweise ein Medikament der Wahl sein, mit einem verminderten Risiko zur Entwicklung von Toleranz und Abhängigkeit bei langdauernder Behandlung von Angst- und Streßsymptomen.

Key words Anxiolytics · Benzodiazepines · CAS 40054-69-1 · Etizolam · $GABA_A$ receptors, partial agonists · Stress

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1. Introductions

Etizolam (CAS 40054-69-1, 6-(o-chlorophenyl)-S-ethyl- 1 -methyl-4H-s-triazolo-[3,4-c]thienol[1,4]-diazepine) is a prototype of a new chemical class of diazepines, the thienotriazolodiazepines, with a pharmacological profile similar to that of benzodiazepines such as diazepam (CAS 439-14-5), nitrazepam (CAS 146-22-5) and flunitrazepam (CAS 1622 62-4) [1,2]. In fact, etizolam possesses potent, clinically demonstrated, anxiolytic effects [3-5]. In addition, differently from most of the benzodiazepines, etizolam was proposed to exert antidepressant effects based on its intrinsic ability to inhibit norepinephrine turnover and uptake [6, 7].

Several studies with human volunteers have evaluated the Pharmakokinetic properties of etizolam [8], and the results suggest that this compound undergoes fairly rapid breakdown, its thiophenic ring being very susceptible to oxidation. Thus, etizolam can be classified as a short-intermediate acting anxiolytic drug [9].

As extensively reported, classical benzodiazepines as well as benzodiazepine-like ligands interact with specific recognition sites located on the γ-aminobutyric acid type A ($GABA_A$) receptor, a ligand-gated ion channel which mediates neuronal inhibition in vertebrate Central Nervous System (CNS) [10]. Thus, the anxiolytic, sedative-hypnotic, anticonvulsant, and myorelaxant properties of benzodiazepines are produced by a positive allosteric modulation of $GABA_A$ receptor function [11] . However, of fundamental importance in characterizing the pharmacological profile of a given compound are its pharmacodynamic parameters [12]. Since the various benzodiazepine receptor ligands can modulate $GABA_A$ receptors with varying degrees of potency and efficacy, these compounds can also be classified as full agonists or -partial agonists, depending on their relative intrinsic activity in potentiating the function of the Cl- channel coupled to $GABA_A$ receptors [13]. In the case of etizolam, most of this information is presently not available and a study of its neurochemical actions on the $GABA_A$ receptor is still lacking. Thus, the purpose of the present study was to evaluate, by using neurochemical and electrophysiological techniques, the effects of etizolam on native $GABA_A$ receptors from rat brain and human recombinant $GABA_A$ receptors expressed in Xenopus laevis oocytes, in comparison with those of other benzodiazepine ligands. In addition, its pharmacological profile was tested by investigating the anti-stress action evaluated on basal and foot-the shock stress-stimulated acetylcholine release in the prefrontal cortex and hippocampus of freely moving rats.

2. Materials and methods

2.1. Materials

Plasmid purification kits were obtained from Promega (Madison, WI, USA). t-[^{35}S]Butylbicyclophosphorothionate ([^{35}S]TBPS) was obtained from DuPont Biotechnology System (Boston, MA, USA). Etizolam was kindly provided by Farmadas S.p.A., Rome (Italy). All other reagents were of analytical grade and were purchased from Sigma Chemicals (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). Each drug solution was freshly prepared before each experiment. Drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted to the final concentration with buffer. The final DMSO concentration in the buffer ranged from 0.01 to 1 % and did not alter control values.

2.2. Animals

Male Sprague-Dawley CD rats (Charles River, Como, Italy), with body masses of 200 to 225 g were maintained

under a 12-h light, 12-h dark cycle at a temperature of 23 ±2°C and 65 % humidity, with water and standard laboratory food ad libitum. Animal experiments were carried out in accordance with the international guidelines, and the experimental protocols used were approved by the Animal Ethical Committee of the University of Cagliari. In experiments where different drug or stress treatments were administered, rats were randomly distributed among experimental groups. Animals were killed by decapitation, their brain rapidly removed and the cerebral cortex dissected out and used to prepare membranes for measurement of [^{3}H]flunitrazepam binding and [^{35}S]TBPS binding.

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

Cerebral cortices were homogenized in 20 vol. of ice-cold wash buffer (5 mmol/l Tris-HCl (pH 7.4),1 mmol/l EDTA) with a Teflon^{TM} pestle and glass homogenizer, and the homogenate was centrifuged at 1000 x g for 10 min. The supernatant was carefully removed and centrifuged for 20 min at 48 000 x g, and the resulting pellet washed (resuspended and centrifuged) 3 times in 20 vol. of ice-cold wash buffer, with the freeze-thaw step included between each centrifugation. The final pellet was resuspended in wash buffer and frozen at -20°C until required. On the day of the assay, the membranes were thawed and centrifuged for 20 min at 48 000 x g. The pellet was resuspended in 20 vol. of 1 mmol/l Tris-citrate (pH 7.4), 200 µl portions of which (150 to 300 µg of protein) were preincubated for 10 min at 37°C in a total volume of 1 ml containing various concentrations of drugs (dissolved in DMSO as described above) and 200 mmol/l NaCl. Then, the binding reaction was initiated by the addition of 0.5 nmol/l [^{3}H]flunitrazepam and was terminated after 60 min at 0°C by rapid filtration through a glass-fiber filter (GF/B; Whatman, Clifton, NJ, USA) in a filtration manifold (model M-24, Brandel, Gaithersburg, MD, USA). The filters were washed twice with 4-ml portions of ice-cold wash buffer and then dissolved in 3 ml of scintillation fluid (Atomlight). Filter-bound radioactivity was determined by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 5 µmol/l diazepam and represented ~10 % of total binding.

2.4. Preparation of plasmid cDNAs

CDNAs encoding human α_1,α_2,α_3, β_2,and γ_{2s} $GABA_A$ receptor subunits were purified with the Promega Wizard Miniprep DNA purification system. The cDNAs were resuspended in sterile distilled water, divided into portions, and stored at -20°C until used for injection.

2.5. Isolation of Xenopus oocytes and microinjection of cDNAs

Stage V and VI oocytes were isolated manually from a section of Xenopus ovary with the use of fine surgical forceps. The follicular cell layer was degraded by exposure for 10 min to collagenase type IA (0.5 mg/ml, Sigma). A mixture of $\alpha_1\beta_2\gamma_{2s}$, $\alpha_2\beta_2\gamma_{2s}$ or cDNAs (1.5 ng/30 nl)was microinjected into the oocyte nucleus with a 10 µl glass pipette (tip diameter 10-15 µm). Injected oocytes were maintained at 18-20°C in Modified Barth's solution (MBS) (88 mmol/l NaCl, 1 mmol/l KCl, 2.4 mmol/l $NaHCO_3$, 10 mmol/m hepes (pH 7.5), 0.82 mmol/l $MgSO_4$, 0.33 mmol/l $Ca(NO_3)_2$, 0.91 mmol/l $CaC1_2$), supplemented with 2 mmol/l sodium pyruvate, penicillin (10 U/nl), streptomycin (0.01 U/ml), gentamytin (0.05 U/ml) and 0.5 mmol/l theophylline. Oocytes were kept for up to 4 days, during which time they were transferred to fresh incubation medium each day.

2.6. Electrophysiological recording

Electrophysiological recording started 12-24 h after cDNA injection. Oocytes were placed in a small depression located on the bottom of a 100 µl rectangular recording chamber and continuously perfused with MBS at a flow rate of 2 ml/min at room temperature. Cells were impaled with two microelectrodes (0.5-3.0 MΩ) filled with filtered 3 mol/l KCl and were clamped at -70 mV with the use of an Axoclamp 2-B amplifier (Axon Instruments, Burlingame, CA, USA). Drugs were applied for 20 s, with the peak current response typically observed after 6-7 s. Between drug applications, 5-10 min were allowed to prevent current desensitization. Currents induced by the different drugs were expressed as percentage of control responses, because of variability in $GABA_A$ receptor expression. In control experiments, concentrations of up 1 % DMSO in MBS were applied to voltage-clamped oocytes and produced no appreciable change in ion currents. Each oocyte represents a single determination and oocytes from many different frog donors were used for each experiment.

2.7. Ex vivo [^{35}S]TBPS binding assay

Following drug treatment, rats were sacrificed by decapitation, their brains were rapidly removed and the cerebral cortices were dissected out. Tissues were homogenized with a Polytron PT10 (setting 5, 20 s) in 50 vol. of ice-cold 50 mmol/l Tris-citrate buffer (pH 7.4), containing 100 mmol/l NaCl. The homogenate was centrifuged at 20 000 x g for 20 min, the resulting pellet was washed (resuspended and recentrifuged) in 50 vol. of homogenization buffer without NaCl. [^{35}S]TBPS binding was determined in a final volume of 1 ml, consisting of 400 µl of membrane preparation (300 to 400 µg protein), 100 µl of 2 nmol/l [^{35}S]TBPS, 100 µ1 of 0.2 mol/l NaCl, 5 µ1 of drug or solvent, and 395 µ1 of 50 mmol/l Tris-citrate buffer. Reactions were initiated at 25°C by the addition of membranes and were terminated 90 min later by rapid filtration through glass-fiber filters. The filters were rinsed twice with 4 ml of ice-cold 50 nmol/l Tris-citrate buffer in a filtration manifold. Radioactivity was quantified by liquid scintillation spectroscopy. Nonspecific binding was defined as binding in the presence of 100 µmol/l picrotoxin and represented ~10 % of total binding. Protein concentrations were determined by the method of Lowry et al. [14] with bovine serum albumin as standard.

2.8. Measurement of acetylcholine release by microdialysis

Surgical implantation of the dialysis tube in the rat brain was performed according to the transversal microdialysis 'technique as described previously [15]. Briefly, rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.), and a dialysis tube with a wet outer diameter of 320 µm (AN 69-HF, Hospal-Dasco, Bologna, Italy) was implanted at the level of the hippocampus or the prefrontal cortex, according to the Paxinos atlas (A, -3.0 from the bregma, V, -3.0 from the dura for the hippocampus; A, +4.0 from the bregma, V, -1.5 from the dura, for the prefrontal cortex). The dialysis tube, containing a tungsten wire inside as a rigid support and not connected to the stainless steel cannula, was held directly in the micromanipulator of the stereotaxic instrument for insertion into the brain area. This technique minimizes tissue damage and reduces the glial reaction around the dialysis tube. Ringer solution (3 mmol/l KCl. 125 mmol/l NaCl, 1.3 mmol/l $CaC1_2$, 1.0 mmol/l $MgC1_2$, 23 mmol/l $NaHCO_3$, and 1.5 mmol/l potassium phosphate buffer (pH 7.3) was pumped through the dialysis probe at a constant rate of 2 µl/min. Samples (40 µ1) were collected every 20 min.

To achieve detectable amounts of acetylcholine in the dialysate, we added 0.1 µmol/l neostigmine to the Ringer's solution. Acetylcholine was measured by high-performance liquid chromatography with electrochemical detection as decribed [16]; the detection limit was 0.05 pmol per injection. Experiments were initialed 24 h after implantation of the dialysis tube. The average concentration of neurotransmitter in the last three samples before treatment was taken as 100 %, and all subsequent, posttreatment values were expressed as a percentage of basal values.

2.9. Foot-shock procedure

The foot-shock apparatus (Lafayette Instruments, Lafayette, IN, USA) consisted of a Plexiglas box with two opaque sides, measuring 28 by 22 by 27 cm. The box was connected to a scrambler-controller that delivered intermittent shocks to the stainless steel floor. An identical box was used for sham-treated animals. Rats received an acute 8-min foot-shock stress (0.2 mA for 500 ms every s) 40 min after drug or vehicle administration.

2.10. Statistical analysis

Data are presented as means ± SEM. Neurochemical and electrophyiological data were analyzed by Student's t-test. For microdialysis experiments, between-groups comparisons were performed by two-way ANOVA for repeated measures; posthoc comparisons were performed by Scheffé's test.

3. Results

3.1. High affinity interaction of etizolam at central benzodiazepine receptors

The in vitro addition of etizolam (10^{-11}-10^{-5} mol/l) produced a concentration-dependent inhibition of the specific [^{3}H]flunitrazepam binding to rat cortical membranes (Fig. 1) with an IC_{50} value of 4.5 nmol/l. Concentrations of etizolam ranging from 3 to 10 µmol/l produced a complete displacement of [^{3}H]flunitrazepam binding. Similarly to etizolam, the other benzodiazepine alprazolam (CAS 28981-97-7) (10^{-11}-10^{-5} mol/l) inhibited the specific [^{3}H]flunitrazepam binding, with an IC_{50} value of 7.9 nmol/l (Fig. 1).

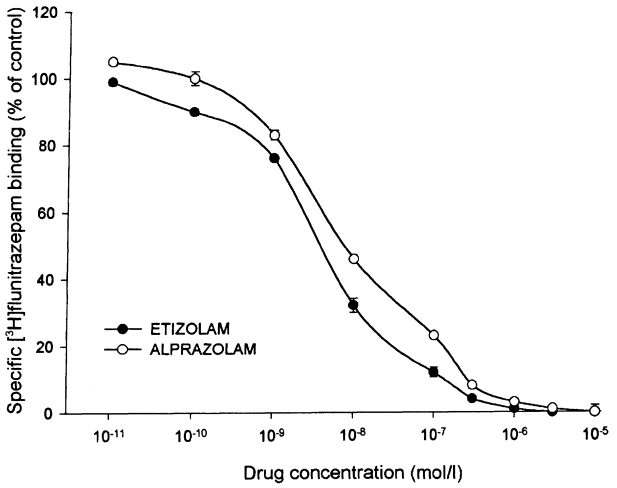

Fig. 1: Inhibition of [^{3}H]flunitrazepam binding by etizolam and alprazolam. [^{3}H]flunitrazepam binding was measured in rat cortical membranes. Data are expressed as percentage of the binding observed in the absence of test drug. Values are mean ± SEM of separate experiments.

3.2. Effects of etizolam and alprazolam on GABA-evoked Cl- currents

Etizolam (10^{-8}-10^{-5} mol/l) potentiated in a concentration-dependent manner GABA-evoked Cl- currents in Xenopus oocytes expressing different human recombinant $GABA_A$ receptor constructs (Fig. 2). In particular, etizolam increased GABA responses in receptors formed by α_1, β_2 and γ_{2s} subunits with a maximal effect of 73 % and an EC_{50} value of 92 nmol/l. With the same GABAA receptors, alprazolam produced a higher effect, with a maximal potentiation of 98 % and an EC_{50} value of 56 nmol/l (Fig. 2A). In receptors formed by α_2, β_2, and γ_{2s} subunits, etizolam showed a comparable efficacy (88 %) to that observed in α_1 containing receptors, but with an EC_{50} of 54 nmol/l (Fig. 2B). In contrast, alprazolam enhanced GABA responses with slightly lower efficacy (72 %) compared to α_1-containing receptors, and with an EC_{50} of 40 nmol/l (Fig. 2B). In $\alpha_3\beta_2\gamma_{2s}$ receptors, etizolam produced a much higher degree of potentiation of GABA responses (140 %) (EC_{50}

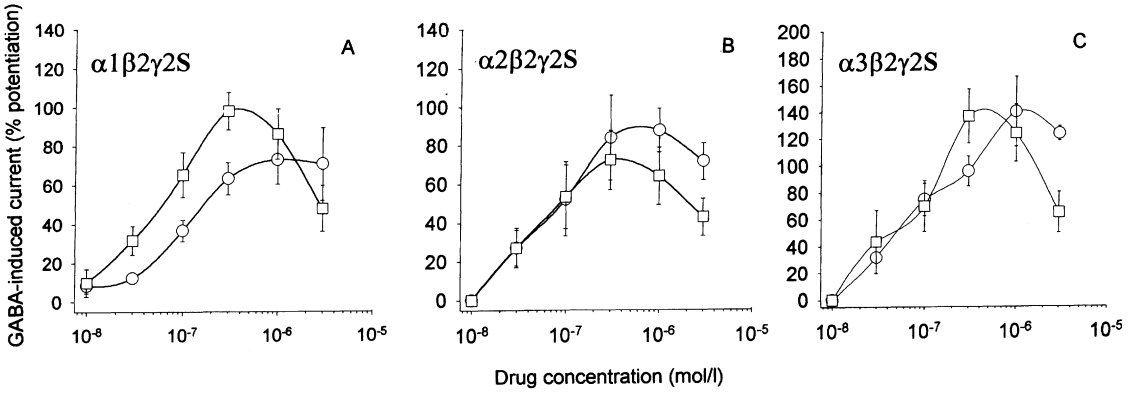

Fig. 2: Etizolam potentiates GABA-evoked Cl^- currents in oocytes expressing human $GABA_A$ receptors. The effects of etizolam (○) and alprazolam (□) were measured in different $GABA_A$ receptor constructs. Data are expressed as percent potentiation of the control response obtained with GABA EC_{20}. Values are mean ± SEM of 5 to 10 different oocytes.

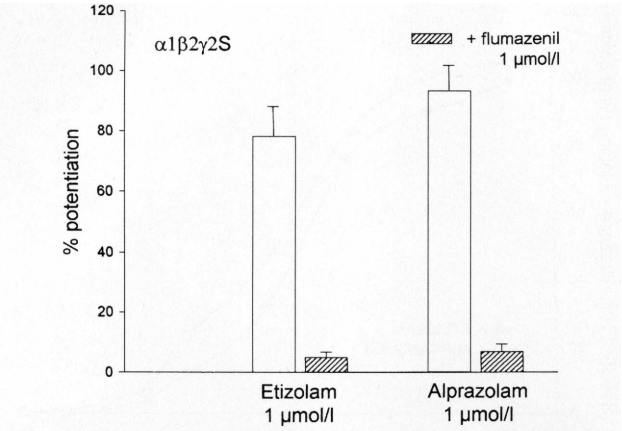

Fig. 3: Flumazenil antagonizes etizolam-induced potentiation of GABA-evoked Cl^- currents. Data were collected from oocytes expressing human α1β2γ2S $GABA_A$ receptors and are expressed as percent potentiation of the control response obtained with GABA EC_{20}. Values are mean ± SEM of 5 different oocytes.

95 nmol/l) compared to that observed with the other receptor constructs. Similarly, alprazolam showed similar efficacy (137 %) and potency (EC_{50} 116 nmol/l) (Fig. 2C). Finally, as expected, the modulatory action of etizolam, like that of alprazolam, was completely blocked by flumazenil (CAS 78755-81-4) (Fig. 3).

3.3. Effects of etizolam, alprazolam, and abecarnil on ex vivo [^{35}S]TBPS binding

In order to study the effects of etizolam at the level of the Cl- channel coupled to $GABA_A$ receptors in animals, we measured the binding ex vivo of [^{35}S]TBPS in the rat cerebral cortex. In fact, the ex vivo binding of [^{35}S]TBPS is a very sensitive index of the in vivo changes in the function of GABAergic synapses induced by the administration of several GABAergic drugs [17, 18].

The intraperitoneal administration of etizolam at the doses of 1.5, 3, 6, and 12 mg/kg did not induce any significant change on ex vivo [^{35}S]TBPS binding (Fig. 4). In contrast, administration of abecarnil (CAS 111841-85-1) (0.5 mg/kg, i.p.) or alprazolam (3 mg/kg i.p.) produced a marked reduction (20 and 25 %, respectively) of [^{35}S]TBPS binding (Fig. 4). In order to better understand the influence of etizolam on the function of $GABA_A$ receptors, its administration was repeated in rats previously treated with isoniazid, a compound that, by inhibiting the synthesis of GABA at the level of GABAergic synapses, determines a marked reduction of the inhibitory GABAergic function which in rats results, about 40-60 min following its subcutaneous injection, in two distinct effects: a) expression of typical tonic-clonic seizures and b) enhancement of [^{35}S]TBPS binding in brain [17, 19]. Thus. this animal model has proved to be useful in elucidating the pharmacological efficacy of a number of anxiolytic, hypnotic and anticonvulsant drugs [18, 20].

As shown in Fig. 5, the subcutaneous administration of isoniazid (200 mg/kg) in rats produced a 61 % enhancement in [^{35}S]TBPS binding capability to cortical membranes. This effect was inhibited by the administration of etizolam (3 mg/kg i.p.) 25 min after that of isoniazid. Similar results were obtained with abecarnil (Fig. 5) or with the benzodiazepine receptor partial agonist imidazenil (Fig. 6).

3.4. Basal acetylcholine release

Basal acetylcholine release values were monitored for ~2 h preceding the experiment, until their variation was contained within 10 %. Acetylcholine concentration in the dialysate was 3.8 ± 0.25 pmol/20 min for the hippocampus and 1.75 ± 0.3 pmol/20 min for the prefrontal cortex.

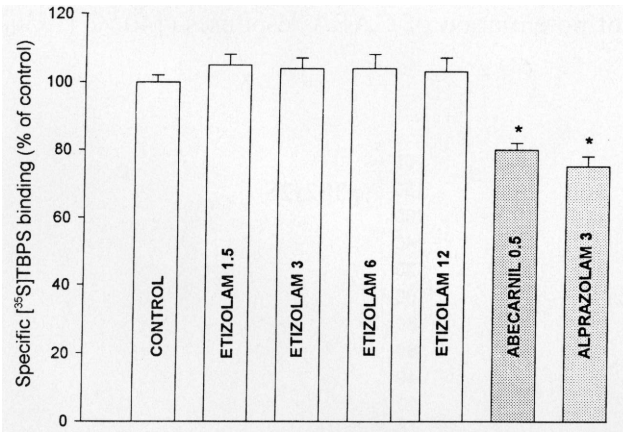

Fig. 4: Etizolam fails to modify ex vivo [^{35}S]TBPS binding. Etizolam (1.5, 3, 6 and 12 mg/kg i.p.), abecarnil (0.5 mg/kg i.p.), and alprazolam (3 mg/kg i.p.) were administered 30 min before sacrifice of rats. [^{35}S]TBPS binding was measured in unwashed cortical membranes. Data are expressed as a percentage of the binding observed in the control animals. Each value is the mean ± SEM of 3 separate experiments. * $p < 0.05$ vs. control value.

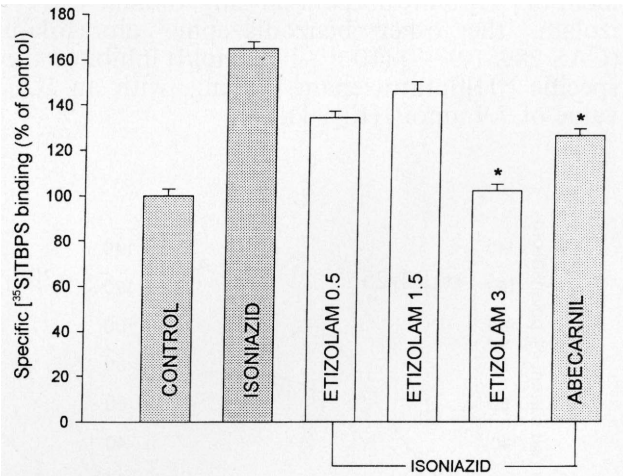

Fig. 5: Etizolam antagonizes the increase of [^{35}S]TBPS binding induced by isoniazid. Isoniazid (200 mg/kg s.c.), etizolam (0.5, 1.5, and 3 mg/kg i.p.), and abecarnil (0.5 mg/kg i.p.) were administered 45, 30, and 30 min, respectively, before sacrifice of animals. [^{35}S]TBPS binding was measured in unwashed cortical membranes. Data are expressed as percentage of the binding observed in the control animals. Each value is mean ± SEM of 3 separate experiments. * $p < 0.01$ vs. control rats treated with isoniazid.

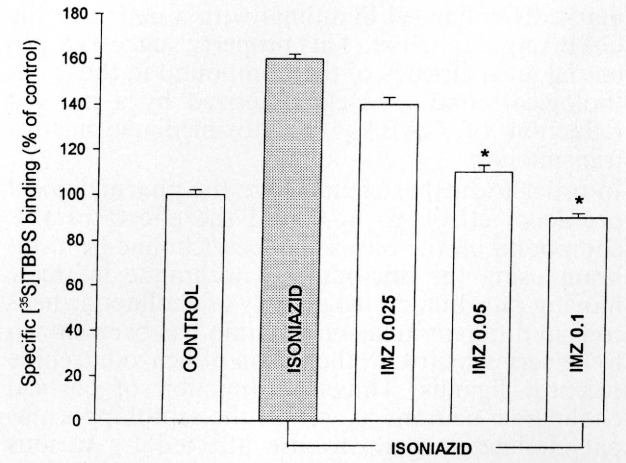

Fig. 6: Imidazenil antagonizes isoniazid-induced increase of [^{35}S]TBPS binding. Isoniazid (200 mg/kg s.c.), and imidazenil (0.025, 0.05, and 0.1 mg/kg i.p.) were administered 45 min before sacrifice of animals. [^{35}S]TBPS binding was measured in unwashed cortical membranes. Data are expressed as percentage of the binding observed in the control animals. Each value is the mean ± SEM of 3 separate experiments. * $p < 0.01$ vs. control rats treated with isoniazid.

3.4.1. Effect of etizolam on basal acetylcholine release

Etizolam (0.5-3 mg/kg i.p.) inhibited in a dose-dependent manner acetylcholine release in the rat hippocampus and prefrontal cortex (Fig. 7). In hippocampus, inhibition of basal acetylcholine release was statistically significant at the dose of 3.0 mg/kg and was maximal (45 %) after 40 min from the administration of etizolam. Values returned to control level within 80 min (Fig. 7A). In the prefrontal cortex, basal acetylcholine release was maximally inhibited by etizolam by 56 and 62 % at the doses of 1.5 and 3.0 mg/kg, respectively. This effect was reached after 60 min and acetylcholine release returned to basal values within 80 min (Fig. 7B).

3.4.2. Effect of etizolam on foot-shock stress-elicited release of acetylcholine

Foot-shock stress (0.2 mA for 500 ms) delivered continuously to rats for 8 min elicited a rapid and marked increase, 70 and 94 %, in the extracellular concentration of hippocampal and cortical acetylcholine after 20 min from stress application, respectively (Fig. 8). Values returned to baseline after further 30-50 min.

Stress delivered 40 min after etizolam (0.5-3 mg/kg i.p.) administration increased acetylcholine release by 57-130 % in the prefrontal cortex and 78-170 % in the hippocampus (Fig. 8). However, in the latter animals, the concentrations of acetylcholine were not significantly different with respect to control animals. 10 mg/kg of etizolam, however, inhibited stress-induced increase of acetylcholine release in both brain areas examined.

4. Discussion

A large body of experimental research has been devoted for the last two decades to the study of the mechanism of action of anxiolytic benzodiazepines as well as benzodiazepine-like compounds [11, 12]. Today, thanks also to cloning of the $GABA_A$ receptor subunit families, the molecular basis of the

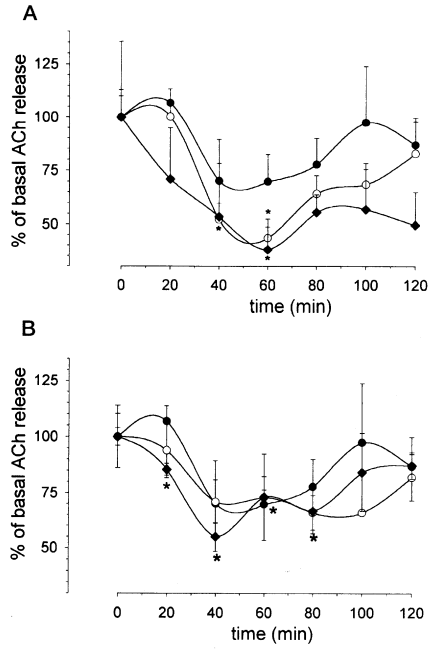

Fig. 7: Effect of etizolam on basal acetycholine release from prefrontal cortex *(A)* and hippocampus (*B*). Etizolam was administered i.p. at doses of 0.5 (●), 1.5 (○), and 3 (◆) mg/kg at time 0. Results are mean ± SEM of 6–10 rats and are expressed as percentage of basal values. Basal acetylcholine release was monitored for at least 2 h before the experiment until their variation was within 10 %. * $p < 0.05$ vs. basal values (ANOVA followed by Scheffe's test).

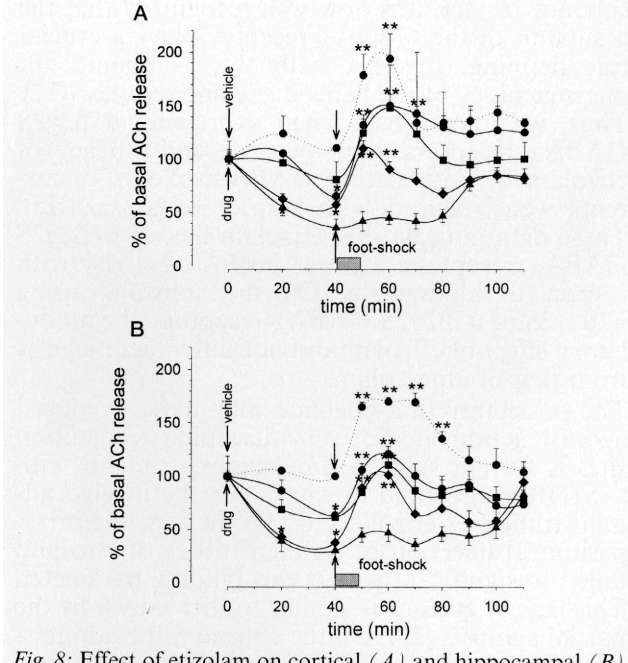

Fig. 8: Effect of etizolam on cortical *(A)* and hippocampal *(B)* acetylcholine release induced by foot-shock stress. Control animals (------) were injected with vehicle. Foot-shock was administered for a period of 8 min beginning at time 40, 40 min after administration of etizolam or vehicle. Etizolam was administered i.p. at doses of 0.5 (●), 1.5 (■), 3 (◆), and 10 (▲) mg/kg. Results are mean ± SEM of 6–10 rats and are expressed as percentage of basal values. * $p < 0.05$ vs. basal values (ANOVA followed by Scheffe's test). ** $p < 0.05$ vs. release just preceding foot-shock stress (ANOVA followed by Scheffe's test).

pharmacological effects of such class of compounds is resolved and the benzodiazepine recognition site is one of the best characterized [10, 11]. One of the major outcomes of this work indicates that, because of the high specificity and selectivity in the action of benzodiazepines and benzodiazepine-like compounds, there is an excellent correlation between their potency and efficacy as revealed in in vitro studies, and their potency and efficacy when their effects are observed in animals and humans. Thus, knowledge of the pharmacodynamic, in addition to the pharmacokinetic, properties of different benzodiazepine ligands not only is important in order to evaluate and characterize their pharmacology, but also to better understand the difference among compounds. With these premises, our study has been concerned with the neurochemical and electrophysiological characterization of the interaction of etizolam at the level of native rat brain $GABA_A$ receptors as well as cloned human $GABA_A$ receptors.

The results obtained from this research indicate that etizolam is endowed with a general neurochemical profile similar to that of other benzodiazepines and benzodiazepine-like compounds. In particular, we have shown that etizolam displaced [^{3}H]flunitrazepam binding measured in rat cortical membranes with an IC_{50} lower than that of the benzodiazepine alprazolam, suggesting that etizolam binds to the benzodiazepine site with very high affinity.

By expressing cloned human $GABA_A$ receptors in Xenopus oocytes, we have examined the action of etizolam in different $GABA_A$ receptor subunit constructs, particularly evaluating the role of the α subunit. In fact, it is now well recognized that the α subunit of the $GABA_A$ receptor plays a crucial role defining, together with the γ subunit, the pharmacology of the benzodiazepine receptor [11]. Thus, we found that, when expressing at α1β2γ2S $GABA_A$ receptors, both potency and efficacy of etizolam in potentiating GABA-evoked Cl- currents were reduced with respect to alprazolam. These data indicate that etizolam has, at a132y2S $GABA_A$ receptors, a lower intrinsic activity with respect to alprazolam. On the contrary, using α2β2γ2S or α3β2γ2S $GABA_A$ receptors, the modulatory effect of etizolam did not differ significantly from that of alprazolam.

The possibility that etizolam may have a reduced agonist action at the benzodiazepine recognition sites is further suggested by the experiments with [^{35}S]TBPS measured ex vivo after the in vivo administration of etizolam to rats. In fact, the intraperitoneal injection of different doses of etizolam failed to significantly alter this binding parameter. This lack of efficacy-is similar to that shown by the partial agonists [20]. On the contrary, the administration of alprazolam or abecarnil produced a marked inhibitiion of [^{35}S]TBPS binding to rat cortical membranes. However, when etizolam was administered to rats previously treated with the glutamic acid decarboxylase (GAD) inhibitor, isoniazid, it prevented the stimulatory action of isoniazid on ex vivo [^{35}S]TBPS binding [17]. These observations indicate that the in vivo efficacy of etizolam is markedly enhanced in animal with a deficit in the GABAergic function. This property suggests a potential great efficacy of this compound in those pathological conditions characterized by a marked reduction of $GABA_A$ receptor-mediated neurotransmission.

In order to further characterize the pharmalogical profile of etizolam, we tested the effects of this compound on the release of acetylcholine from rat brain using the microdialysis technique in freely moving rats. In fact, the activity of cholinergic neurons in different areas of rat brain has been shown to be very sensitive to the action of benzodiazepine receptor ligands. Thus, the function of cortical cholinergic neurons as well as the septohippocampal cholinergic pathway are affected by various benzodiazepine receptor ligands, including anxiolytic benzodiazepines, anxiolytic and anxiogenic β-carbolines, and the specific receptor antagonist flumazenil [21-25]. We have shown that etizolam inhibits in a dose-dependent manner acetylcholine from both prefrontal cortex and hippocampus. This result is well in accordance with the ability of benzodiazepines such as diazepam and midazolam to inhibit basal acetylcholine release from rat brain [25]. However, with respect to the effects of those benzodiazepines, etizolam resulted more potent; in fact, while diazepam and midazolam produced ~50 % inhibition of basal acetylcholine release from hippocampus at the dose of 10 mg/kg, etizolam elicited a similar release reduction at the dose of 3 mg/kg.

Foot-shock stress delivered just after etizolam failed to significantly change the release of acetylcholine with respect to the basal release in the control animals, but significantly increased acetylcholine release when compared to the level of acetylcholine release found in etizolam-treated rats just before stress administration. Based on our present neurochemical and electrophysiological data, this result is somewhat unexpected, and clearly in this respect. etizolam differs markedly from other benzodiazepines such as diazepam and midazolam as well as other anxiolytic benzodiazepine receptor ligands [25]. In fact, these drugs completely abolish the action of stress suggesting that under this effect neurons are not stimulated by stress. Since the same doses of etizolam elicit anticonflict effect in rats and anxiolytic and antistress action in humans [3-5] our results suggest that the observation that foot-shock stress did not significantly enhance acetylcholine release over control levels supports the idea that the antistress effect of etizolam is not mediated by a complete inhibition of neuronal activity. Moreover, the reduced antagonism elicited by etizolam of stress-induced increase in acetylcholine release may reflect a reduction in a tonic inhibitory input, exerted directly or indirectly by GABAergic neurons, on the prefrontal cortex and hippocampus. The lack of etizolam to completely block stress-induced increase in acetylcholine release may be the result of the reduced intrinsic activity of etizolam at particular $GABA_A$ receptor subpopulations at the level of specific pathways important in mediating the effects of foot-shock stress. This property was particularly evident at α1-

containing receptors and measuring ex vivo [^{35}S]TBPS binding in rat cortical membranes.

Altogether, these results suggest that etizolam may have a reduced intrinsic activity, at least at specific subpopulations of $GABA_A$ receptors. This property, together with the pharmacokinetic indication of a short-acting drug (lower half-life and lower drug accumulation) [8], may characterize etizolam as a ligand endowed with less side-effects typical of full agonists such as diazepam and alproazolam etc. This conclusion is consistent with the pharmacology of this compound in humans [26]. In fact, the above described properties may play a major role in the reduced liability to produce tolerance and dependence as well as the frequency of other side effects, and qualify etizolam as an ideal anxiolytic and antistress drug devoid of unwanted side-effects.

5. Literature

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