

## Characterization of $\alpha$ -casozepine, a tryptic peptide from bovine $\alpha_{s1}$ -casein with benzodiazepine-like activity

Laurent Miclo\*, Emmanuel Perrin\*, Alain Driou\*, Vassilios Papadopoulos<sup>†</sup>, Noureddine Boujrad<sup>†,‡</sup>, Régis Vanderesse<sup>‡</sup>, Jean-François Boudier<sup>§</sup>, Didier Desor<sup>||</sup>, Guy Linden\*, and Jean-Luc Gaillard\*

\*Laboratoire des Biosciences de l'Aliment UA 885 INRA, Faculté des Sciences, Université Henri Poincaré-Nancy 1, Vandoeuvre-lès-Nancy, France; <sup>†</sup>Departments of Cell Biology, Pharmacology and Neuroscience, Georgetown University Medical Center, Washington, DC; <sup>‡</sup>Laboratoire de Chimie Physique Macromoléculaire UMR 7568 CNRS, ENSIC, INPL, Nancy, France; <sup>§</sup>Ingredia, Arras, France; <sup>||</sup>Laboratoire des Aspects Fonctionnels et du Développement des Comportements URA 1293 CNRS, Faculté des Sciences, Université Henri Poincaré-Nancy 1, Vandoeuvre-lès-Nancy, France; and <sup>#</sup>Endocrinologie Moléculaire de la Reproduction UMR 6026 CNRS Interactions Cellulaires et Moléculaires, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes Cedex

Corresponding author: Laurent Miclo, LBSA, Faculté des Sciences, Université Henri Poincaré, B.P. 239, 54506 Vandoeuvre-lès-Nancy, France. E-mail: Laurent.Miclo@sbiol.uhp-nancy.fr

### ABSTRACT

Caseins are a known source of biologically active peptides. In this study, we have shown evidence of a novel anxiolytic activity in a tryptic hydrolysate of bovine  $\alpha_{s1}$ -casein. Injection of 3 mg/kg of this hydrolysate significantly reduced the epileptic symptoms caused by pentylentetrazole in rats. Anxiety reduction was also observed when the hydrolysate was tested in the elevated plus-maze and in the conditioned defensive burying rat models. Peptides isolated from the hydrolysate were examined for their affinity for the  $\gamma$ -amino-butyric acid (GABA) type A receptor. Only one peptide, named  $\alpha$ -casozepine, corresponding to the 91–100 fragment from bovine  $\alpha_{s1}$ -casein, expressed affinity for GABA<sub>A</sub> receptor. *In vitro*, the peptide had 10,000 less affinity for the benzodiazepine site of the GABA<sub>A</sub> than did diazepam. However, in the conditioned defensive burying paradigm it was 10-fold more efficient than diazepam. The difference observed between the *in vitro* and *in vivo* activity of  $\alpha$ -casozepine could not be explained by an action via the peripheral-type benzodiazepine receptor;  $\alpha$ -casozepine had no affinity for this receptor. The  $\alpha$ -casozepine amino acid sequence could be related to the carboxy-terminal sequence of the polypeptide diazepam binding inhibitor, an endogenous ligand of the central GABA<sub>A</sub> and peripheral-type benzodiazepine receptors.

Keywords: milk • casein peptide • anxiolysis • anticonvulsant • diazepam binding inhibitor

**M**any physiological benefits are attributed to milk. A gastroprotective action in an *in vivo* rat stress-induced ulcer model of numerous dairy foods, including cream, whole milk, and skim milk, has been reported (1). In folk wisdom, milk intake would improve sleep

or provide a calming effect. More than 60 years ago, it was reported that adults showed a higher tendency toward uninterrupted sleep after consuming a meal of cornflakes and milk (2). Brezinova and Oswald (3), using electroencephalography, have found that sleep of old people is significantly longer and less broken after cereal–milk intake at bedtime and that the action of milk is more effective with serial administration. Those results would be associated with a conditioned response to milk that persists from infancy.

Milk proteins are the only proteins synthesized by mammals in order to feed their young. The effects of caseins in addition to being nitrogen providers for the newborn are considered, because many works have shown in the past 15 years that their enzymatic hydrolysis produces peptides with various biological activities (4). The peptides found are opioid and opioid-antagonist peptides, angiotensin-converting-enzyme inhibitors, immunostimulating peptides, platelet-aggregation inhibitors, phosphopeptides carriers of minerals ( $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ ), mitogenic peptides, antibacterial peptides, and protease inhibitors. Their physiological role is often putative as most of these properties have been discovered by *in vitro* experiments. However, opioid peptides have been detected *in vivo* in duodenum of mini-pigs after bovine milk intake (5), in human adult gut after milk ingestion (6), in serum of newborn calves after first milk intake (7), and in plasma of pregnant or breast-feeding women (8). *In vivo* activities have been evidenced for angiotensin-converting-enzyme peptidic inhibitors, because per os ingestion of fermented milk containing these peptides reduces blood pressure in rats suffering from genetic hypertension (9). Nevertheless, the physiological purposes of the biological active peptides from milk caseins are not yet elucidated (10).

These considerations have led us to try to determine whether the popular sedative and calming properties of milk could be carried by a peptide. On the one hand, the compounds reducing the function of the GABA-coupled chloride channel, as beta-carboline derivatives, produce pharmacological effects such as anxiety and convulsions (11, 12). On the other hand, benzodiazepines (BDZs) or barbiturates, which enhance the GABAergic transmission, are anxiolytic and anticonvulsant. So, the GABA<sub>A</sub> receptor complex plays a major role in the pharmacology, neurochemistry and physiopathology of stress and anxiety (13, 14). A deficiency of GABAergic transmission is also linked to epilepsy, because the inhibition of the expression of the GABA<sub>A</sub> receptor  $\gamma 2$  subunit by antisense oligonucleotide causes electrographic seizures (15). A second BDZ-binding site with a predominantly mitochondrial localization has been identified and named “peripheral-type benzodiazepine binding site” (PBR), although it is present in all tissues, including the central nervous system.

PBRs are implicated in steroidogenesis (16). Drugs that bind to PBR in the brain may regulate neurosteroid production by glial cells, which in turn can act on the neuronal GABA<sub>A</sub> receptor and modulate neuronal activity and brain function (17, 18). Some BDZs as 4'-chlorodiazepam (Ro5-4864) bind with high affinity to the PBR and isoquinolines, which are not BDZs, have a great selectivity for this receptor.

In the present study, we show that tryptic hydrolysate of bovine  $\alpha_{s1}$ -casein have *in vivo* BDZ-like activity. We identified the active peptide by *in vitro* screening by using the  $\gamma$ -amino butyric acid type A (GABA<sub>A</sub>) receptor binding assay and was checked for *in vivo* activity.

## MATERIALS AND METHODS

## **Bovine $\alpha_{s1}$ -casein preparation**

Raw bovine milk from Holstein cows was skimmed by centrifugation (2,000 g, 20 min, 30°C). Caseins were precipitated by adjusting milk to pH 4.6 with 1 M HCl. After centrifugation (1,500 g, 20 min, room temperature), the precipitate was washed twice with distilled water and solubilized at pH 7.0 with 1 M NaOH. The precipitation at pH 4.6, washing steps and resolubilization in water at neutral pH were repeated twice. Sodium caseinate was lyophilized after the last solubilization at pH 7.5 with 1 M NaOH.  $\alpha_{s1}$ -Casein was prepared from sodium caseinate by batch fractionation adapted from that described in ref. 19. A quantity of 20 g of dry diethylaminoethyl (DEAE)-cellulose DE23 (Whatman, Maidstone, UK) was equilibrated in 150 ml of a 0.02 M sodium acetate buffer, pH 6.6, with 3.3 M urea, 0.035 M EDTA, and 0.1% (v/v) 2-mercaptoethanol and was mixed with 5 g of sodium caseinate solubilized in 100 ml of the same buffer. DEAE-cellulose and sodium caseinate were mixed gently for 15 min and filtered through Whatman n°41 filter paper. The DEAE-cellulose cake was dispersed in 250 ml of the same buffer, and the procedure was repeated twice. Elution of caseins was performed in two steps: first with 35 mM CaCl<sub>2</sub> and then with 70 mM CaCl<sub>2</sub> dissolved in 0.02 M sodium acetate buffer with 3.3 M urea. Each elution step was repeated twice. Filtrates were dialyzed and lyophilized.

## **Tryptic hydrolysis of $\alpha_{s1}$ -casein**

$\alpha_{s1}$ -Casein was dissolved in 50 mM ammonium formate buffer pH 8.5 to a final concentration of 0.2% (w/v). Seven N- $\alpha$ -benzoyl-arginine-ethyl-ester units of immobilized trypsin from bovine pancreas (EC 3.4.21.4) (Sigma, St. Louis, MO) were added to the solution, and hydrolysis was performed during 1 h at 37°C with gentle stirring. Hydrolysis was stopped by centrifugation (1,700 g, 5 min, 4°C). The supernatant was evaporated under vacuum to remove volatile buffer and  $\alpha_{s1}$ -casein total hydrolysate was redissolved in distilled water. The evaporating procedure was repeated five times.  $\alpha_{s1}$ -Casein hydrolysate was then lyophilized.

## **Purification of $\alpha_{s1}$ -casein tryptic peptides**

$\alpha_{s1}$ -Casein peptides were resolved by reversed-phase chromatography by using a Hitachi-Merck system with an L6200 ternary pumping system, a Model 655A-40 automated injection and a sampling system (Merck, Darmstadt, Germany) coupled with a Waters Model 996 photodiode array detector controlled by a computer. Casein hydrolysate was run on a LichroCart C<sub>18</sub> column (250 × 4 mm i.d., 5 μm particle size, 10 nm porosity) (Merck). Peptides were eluted with a gradient from 5% to 40% of acetonitrile (Rathburn, Walkerburn, UK) in Ultra-High Quality water containing 0.1% (v/v) trifluoroacetic acid (TFA) (sequencing grade, Sigma) for 70 min at a flow rate of 1 ml/min. Peptides were collected manually. After lyophilization, these partially purified peptides were re-run in isocratic conditions. Peptidic fractions were lyophilized.

## **Amino acid analysis**

Purified peptides were hydrolyzed in 6 M HCl with 0.5% (w/v) phenol and 0.1% (v/v) 2-mercaptoethanol under vacuum at 110 ± 2°C during 24 h. The amino acid compositions were

performed on a cation exchanger resin BTC 2410 with a Biotronik LC3000 analyzer (Munich, Germany) by using a ninhydrin derivatization of amino acids.

### **Peptide sequence**

The sequence was determined in an automatic Edman degradation microsequencer Model 476A (Perkin Elmer Applied Biosystems Division, Foster City, CA).

### **Mass analysis**

Mass of  $\alpha_{s1}$ -casein peptides was checked by fast atom bombardment-MS (FAB-MS). FAB-mass spectra were acquired on a VG ZAB-HF double-focusing mass spectrometer (VG Instruments, Manchester, UK). The matrix used was thioglycerol, and samples were dissolved in methanol for loading onto the target. Ionization was achieved with a fast atom gun operated at 10 kV. For peptides in which mass was superior to 3,000 Da, we performed analysis by electrospray ionization-MS (ESI-MS) on a Bio-Q quadrupole mass spectrometer (VG Instruments) equipped with an electrospray ion source operating in the positive-ion mode. Peptidic fraction was mixed with 50% acetonitrile and 1% formic acid in water before positive ion electrospray. Scanning was from  $m/z$  500 to 1500. Bovine  $\alpha_{s1}$ -casein was analyzed by ESI-MS in the negative-ion mode.

### **Peptide synthesis**

The peptide was synthesized on a Dupont Coupler 250 peptide synthesizer (DuPont Co., Wilmington, DE) from  $N\alpha$ -tert-butoxycarbonyl(Boc)-mesitylene-2-sulfonyl-arginine-4-(hydroxymethyl)-phenylacetamidomethyl-polystyrene resin (0.56 mmol/g substitution, Neosystem, Strasbourg, France), by using Boc solid-phase peptide-synthesis protocols. The peptide chain was assembled starting from 0.5 mmol mesitylene-2-sulfonyl-arginine linked to the resin by 2 h coupling of the Boc-Xaa (1.5 mmol), with the side chains of glutamic and tyrosine residues protected, respectively, by benzyl ester and 2,6-dichlorobenzyl ester in the presence of 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium tetrafluoroborate (1.5 mmol), and diisopropylethylamine (4.5 mmol) in dimethylformamide/dichloromethane (1:3). The completion of each coupling step was monitored by Kaiser ninhydrin and 2,4,6-trinitrobenzenesulfonic acid tests. Deprotection of the Boc group was achieved by 40% (v/v) TFA in dichloromethane. A standard cleavage with 18% (v/v) trimethylsilyl trifluoromethane sulfonate, 65% (v/v) TFA, 11% (v/v) thioanisole, and 6% (v/v) 1, 2-ethanedithiol afforded the crude peptide. Desalting was achieved on Sephadex G-25 with n-butanol/pyridine/acetic acid/water (15:10:3:12) as eluent. Peptide was purified by HPLC on a LichroCart C<sub>18</sub> column as described above.

### **GABA<sub>A</sub> receptor binding assay**

Binding of tryptic  $\alpha_{s1}$ -casein hydrolysate and purified peptides to GABA<sub>A</sub> receptors was studied by the drug discovery system kit (Nenquest™, NEN Life Sciences, Boston, MA) consisting of bovine cerebral cortex membranes in sodium phosphate buffer pH 7.7, [methyl-<sup>3</sup>H]flunitrazepam (specific activity 83 Ci/mmol), and 2.5  $\mu$ M flunitrazepam in 0.05 M Tris-HCl buffer pH 7.7 (binding buffer). For the binding assay, 400  $\mu$ l of the membrane solution was incubated at 4°C for 60 min with 50  $\mu$ l [methyl-<sup>3</sup>H]flunitrazepam (0.48 nM final concentration) and either 50  $\mu$ l of

binding buffer to determine total binding or 50  $\mu$ l of binding buffer containing nonradioactive flunitrazepam (0.25  $\mu$ M final concentration) to determine nonspecific binding, or 50  $\mu$ l of binding buffer containing various concentration of unlabeled flunitrazepam (final concentration ranging from 12.5 nM to 0.39 nM) to establish a standard curve, or peptides or peptidic fractions to be tested in 50  $\mu$ l of binding buffer (final concentration ranging from  $10^{-4}$  to  $10^{-7}$  M). Ice-cold binding buffer (3 ml) was added, and rapid filtration through Whatman GF/B filter under vacuum stopped the incubation. Filters were washed twice with 3 ml of the same ice-cold buffer and put in counting vials with 10 ml of scintillation fluid (Ultima Gold, Packard, Meriden, CT). After one night at room temperature, the radioactivity was measured during 10 min on a Betamatic V counter (Kontron Instrument, Milton Keynes, UK s). Nonspecific binding value was subtracted. IC<sub>50</sub> and Hill number were obtained from Scatchard plot representation.

### **PBR radioligand binding assays**

The assays were performed on MA-10 mouse Leydig cell mitochondrial preparations. MA-10 cells contain high levels of PBR localized primarily on the mitochondria (17). MA-10 cells were scraped from 150 mm culture dishes into PBS and centrifuged at 500 g for 15 min. Mitochondria were prepared by differential centrifugation as previously described (20) and resuspended in PBS at 1  $\mu$ g to 10  $\mu$ g of protein per sample. [N-methyl-<sup>3</sup>H]Ro5-4864 (specific activity 86 Ci/mmol) and [N-methyl-<sup>3</sup>H]PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide; specific activity 75 Ci/mmol), were obtained from NEN. PK 11195 and Ro5-4864 were obtained from Research Biochemicals Incorporated. [<sup>3</sup>H]PK 11195 or [<sup>3</sup>H]Ro5-4864 binding studies were performed at 4°C, in a final incubation volume of 0.3 ml, by using radioligands in the concentration range of 0.03–20 nM and 200-fold excess of unlabelled ligand, as previously described (17). Competition studies were carried out with a concentration of radioligand from 1 nM to 5 nM, and increasing amounts of the peptide. After 90 min incubation, assays were stopped by filtration through Whatman GF/B filters equilibrated in 0.1% polyethyleneimine and washed with 20 ml ice-cold PBS. Radioactivity trapped on the filters was determined by liquid scintillation counting. The dissociation constant (K<sub>d</sub>) and the number of binding sites (B<sub>max</sub>) were determined by Scatchard plot analysis of the saturation isotherms generated by using the LIGAND program (KELL, v.4.0, Biosoft, Inc., Ferguson, MO) (21). The IC<sub>50</sub> from competition studies were also calculated by using the LIGAND program.

### **Sequence alignment**

Alignment of diazepam binding inhibitor (DBI) from various species with  $\alpha_{s1}$ -CN-(f91-100) was performed according to CLUSTALW method (22).

### **Subjects**

A group of 60 non-blood-related Wistar rats, weighing 180 to 230 g at the beginning of the elevated plus-maze experiment; 27 rats weighing 230 to 260 g at the beginning of the anticonvulsant experiment; and 48 rats weighing 280 to 300 g at the beginning of the conditioned defensive burying experiment were obtained from Iffa Credo (Saint-Germain sur l'Arbresle, France). Animals were adjusted to the laboratory environment 3 weeks before the beginning of the experiment. On receipt, the rats were individually identified and housed by groups of four to avoid the isolation stress in an air-conditioned room maintained at a constant temperature ( $22 \pm$

2°C) with 12 h inverted day/night cycle (light 8:00 p.m. to 8:00 a.m.). Tap water and standard diet (Extralabo, Pietrement, Provins, France) were available ad libitum. During the acclimatization period of two weeks, rats were weighed three times by week to avoid manipulation stress. Experiments were performed during the obscure phase of the cycle. All animal studies were conducted in accordance with the principles and procedures defined by the French Ministère de l'Agriculture et de la Pêche under agreement number A54540.

### **Elevated plus-maze experiment**

Rats were randomly divided into three groups. The first group received an i.p. injection (2 ml/kg) of an aqueous solution of 0.5% (w/v) gelatin and 5% (w/v) mannitol. The second group received an i.p. injection of diazepam (2 mg/kg) (Hoffman-La Roche, Basel, Switzerland) suspended in the gelatin–mannitol solution. The last group received an i.p. injection of  $\alpha_{s1}$ -casein hydrolysate (3 mg/kg) solubilized in the gelatin–mannitol solution. Rats were tested in the elevated plus-maze 30 min after the injection. They were removed from their cages and, according to Pellow et al. (23), placed in a box (30×30×30 cm) 5 min before the beginning of the experiment. Pellow et al. (23) describe the plus-maze in detail. The maze consisted of a cross with two opposite open arms and two opposite enclosed arms with 40 cm high walls. The apparatus was elevated at 50 cm from the floor. Rats were placed individually in the center of the maze, which was cleaned after each rat in order to avoid persistent smells, and their behavior tape-recorded for 5 min. The videotape observer was unaware of the experimental conditions of the animals. The number of entries into open and enclosed arms and time spent on open arms were scored. The percentage of entries in open arms was also calculated. The number of entries into closed arms provides a measure of general activity. Data were analyzed using ANOVA (24).

### **Inhibition of pentylenetetrazole-induced seizures**

On day 1, each of the 27 rats received an i.p. injection of 25% (v/v) dimethyl isosorbide ether (DMI) (Sigma) solution in water 30 min before receiving an i.p. injection of pentylenetetrazole (PTZ) (Sigma) (60 mg/kg) in saline (control n°1). Only the 17 rats that had developed seizures were then used for the control and treatment experiments. Such a procedure decreases the variance because response to PTZ differs among rats. For each experiment, an individual rat was observed during the 45 min following the PTZ injection. The parameters scored: crisis severity according to Racine scale (25) (“0” means absence of crisis after PTZ administration, “5” means complete crisis with balance losing); crisis latency (time between PTZ injection and the first visible sign of crisis); and crisis duration (time between the first and the last visible sign of crisis). On day 4, the rats received an i.p. injection of  $\alpha_{s1}$ -casein tryptic hydrolysate (1 mg/kg) dissolved in 25% (v/v) DMI in water 30 min before the i.p. injection of PTZ (60 mg/kg) (assay n°1). On day 6, rats received an i.p. injection of 25% (v/v) DMI in water 30 min before the i.p. injection of PTZ (60 mg/kg) (control n°2). On day 10, the same experiment as day 4 was performed with an increased  $\alpha_{s1}$ -casein tryptic hydrolysate dose (3 mg/kg) (assay n°2). Finally, on day 12, a third control was done (control n°3) under the same conditions as on days 1 and 6. Data were corrected by using the transformation  $x \rightarrow \log(1+x)$ . Analyses were performed by using repeated measures ANOVA procedure corrected by Greenhouse–Geisser epsilon (26). Multiple comparisons were performed by using Fischer’s PLSD test. Assay n°1 was compared with control n°1 and assay n°2 to control n°2.

## Conditioned defensive burying experiment

The conditioned defensive burying test is based on Pinel and Treit's procedure (27). On each of the 2 days before the test, the rats were placed individually in the test chamber without the shock-probe for 20 min. The conditioned defensive burying test was performed during the obscure phase of the cycle. The floor of the test chamber was covered with 5 cm of bedding material made of wood sawdust. On the center of one wall 2 cm above the level of the bedding material was a small hole through which the shock-probe was inserted. The rat was placed in the test chamber on the side opposite to the shock-probe. A single 2 mA shock was manually delivered when the rat touched the shock-probe with its forepaws for the first time. Once the rat received the shock, it recognized the shock-probe as the aversive stimulus. Burying behavior consists in a series of rapid and alternating movements of animal forepaws, moving and pushing a pile of bedding material over this aversive stimulus. The behavior of the rat was recorded for 5 min after it received the shock.

The 48 rats were put randomly into four groups. The first group received an i.p. injection (2 ml/kg) of a 0.9% (w/v) NaCl solution, the second group received an i.p. injection of diazepam (1 mg/kg) suspended in 0.9% NaCl solution, the third group received an i.p. injection of  $\alpha_{s1}$ -casein hydrolysate (3 mg/kg) solubilized in 0.9% NaCl solution, and the last group received an i.p. injection of purified  $\alpha_{s1}$ -CN-(f91-100) (0.4 mg/kg) solubilized in 0.9% NaCl solution. Compounds were injected 30 min before the test. An observer, who analyzed the results, was unaware of the experimental conditions of the animal. Duration of probe burying, number of head stretchings towards the probe, number of approaches towards the probe, and number of retreats away from the probe were scored. The percentage of approaches toward the probe followed by retreats was calculated (i.e., the ratio of the retreats to the approaches). An approach was scored when the animal approached the shock-probe at less than 2 cm or touched it. A retreat was scored when an animal, close to the shock-probe, ran away speedily at the opposite end of the probe. A subject that was not shocked after 5 min was discarded from the study. We analyzed all data by using ANOVA (24).

## RESULTS

### Preparation of $\alpha_{s1}$ -casein tryptic hydrolysate

The purity of  $\alpha_{s1}$ -casein prepared by batch chromatography with  $\text{CaCl}_2$  step gradient elution was estimated to be 96% by PAGE with 4 M urea. Analysis of  $\alpha_{s1}$ -casein by ESI-MS shows most intense mass anion at  $m/z$  23617. The B variant of bovine  $\alpha_{s1}$ -casein-8P, which is the major variant in Holstein cows, has a calculated  $M_r$  of 23615. Mass anion  $m/z$  23696 was also observed. The difference  $m/z$  79 observed between the two mass anions corresponds to the molecular mass of the phosphate group of a phosphoserine residue. Thus, the  $\alpha_{s1}$ -casein fraction contained  $\alpha_{s1}$ -casein-8P and  $\alpha_{s1}$ -casein-9P, of which the sequence contained an additional phosphorylated serine residue. The main  $\alpha_{s1}$ -casein tryptic peptides ([Fig. 1](#)) were characterized and identified by retention time and spectral analysis in reversed-phase HPLC (28), amino acid analysis and FAB-MS. In our conditions, we found nonspecific tryptic cleavage, whereas some bonds involving lysine or arginine residue were found totally or partially resistant to tryptic hydrolysis. The  $\text{Arg}^1\text{-Pro}^2$  bond, which has been already described by Kaminogawa et al. (29),

and the Lys<sup>36</sup>-Val<sup>37</sup> bond were totally resistant to trypsin in our experimental conditions. The Lys<sup>105</sup>-Val<sup>106</sup>, Lys<sup>102</sup>-Lys<sup>103</sup>, Lys<sup>83</sup>-Glu<sup>84</sup>, Lys<sup>132</sup>-Glu<sup>133</sup> bonds were only partially cleaved by trypsin. After hydrolysis, the volatile salts were removed by evaporation under partial vacuum, and the lyophilized hydrolysate contained only 18 ± 5% (w/w) of ammonium formate.

### **α<sub>s1</sub>-Casein tryptic hydrolysate anticonvulsant activity**

Anticonvulsant activity of the hydrolysate was assessed *in vivo* with rats. A value of 2,700 s (observation duration) was arbitrarily assigned to crisis latency when an animal did not develop an epileptic crisis. [Figure 2](#) shows a sensitization of animals to PTZ with increasing experiences. The latency of crisis significantly decreased from 156 ± 10 s (control n°1) to 116 ± 10 s (control n°3) ( $P < 0.01$ ). Difference was also significant between controls n°1 and n°2 (125 ± 12 s) ( $P < 0.02$ ). The same phenomenon was observed with the clonus duration because the mean value increased nonsignificantly about 4 min between control n°1 (562 ± 122 s) and control n°3 (801 ± 169 s). No modification in crisis severity was observed in the course of the three control experiments, because animals were selected according to their great sensitivity to PTZ. Intraperitoneal injection of 1 mg/kg of α<sub>s1</sub>-casein tryptic hydrolysate significantly reduced the severity of the crisis ( $P < 0.02$ ). Compared with the first control, the mean value of the severity decreased from 3.7 ± 0.2 on Racine scale to 2.8 ± 0.4. With this dose, the clonus duration did not differ significantly from the control and the increase of crisis latency was not significant. The i.p. injection of 3 mg/kg of α<sub>s1</sub>-casein tryptic hydrolysate led to a very significant reduction of PTZ action. Compared with control n°2, the mean value of crisis severity decreased from 3.6 ± 0.4 to 2.1 ± 0.5 ( $P < 0.002$ ) ([Fig. 2A](#)). The mean value of crisis latency significantly increased ( $P < 0.005$ ) from 125 ± 12 s to 1047 ± 314 s and the mean value of clonus duration significantly decreased from 728 ± 174 s to 404 ± 123 s ( $P < 0.005$ ). Because of the sensitization of animals during experiments, α<sub>s1</sub>-casein tryptic hydrolysate would probably have a more intense effect. These results show a significant antagonist effect of α<sub>s1</sub>-casein tryptic hydrolysate on PTZ action in Wistar rats.

### **α<sub>s1</sub>-Casein tryptic hydrolysate anxiolytic activity**

The elevated plus-maze test was used to evaluate the anxiolytic effect of the α<sub>s1</sub>-casein hydrolysate ([Fig. 3](#)). The general activity of the rats on the plus-maze was not modified by saline, diazepam, or α<sub>s1</sub>-casein tryptic hydrolysate i.p. injection because the number of closed-arm entries did not differ significantly among the three groups. The percentage of time spent in the open arms differed significantly in the three groups ( $P < 0.02$ ). The diazepam-treated rats spent significantly more time in the open arms than did the control rats. For the α<sub>s1</sub>-casein tryptic hydrolysate, this parameter was not raised significantly. The percentage of time spent in the open arm is generally less straight than the percentage of entries in the open arms to measure anxiety (30). Diazepam and α<sub>s1</sub>-casein tryptic hydrolysate significantly ( $P < 0.02$ ) enhanced the percentage of entries into the open arms (15.1 ± 3.0% of entries for the saline control group vs. 33.8 ± 5.9% and 29.3 ± 5.3% for the diazepam and α<sub>s1</sub>-casein tryptic hydrolysate-treated groups, respectively). An experiment of conditioned defensive burying was carried out on male Wistar rats to confirm the anxiolytic activity of the α<sub>s1</sub>-casein tryptic hydrolysate. Similarly to diazepam (1 mg/kg i.p.), the α<sub>s1</sub>-casein tryptic hydrolysate, administered at 3 mg/kg by i.p., significantly decreased the duration of probe burying compared with saline, from 81.4 ± 19.7 s to 24.2 ± 10.8

s ( $P < 0.005$ ) (Fig. 4A), the number of head stretchings towards the probe ( $P < 0.01$ ) (data not shown), and the percentage of approaches toward the probe followed by retreats ( $P < 0.01$ ) (Fig. 4B).

### **Affinity of $\alpha_{s1}$ -casein tryptic hydrolysate and $\alpha$ -casozepine for GABA<sub>A</sub> receptor**

Affinity for the BDZ site of GABA<sub>A</sub> receptor was tested because the tryptic hydrolysate produced the same effects as did drugs acting at the BDZ receptor (anticonvulsant activity, increase of the entries in the open arms without general activity change in the elevated plus-maze test, and modification of the conditioned defensive burying behavior). Anxiolytics that act through the 5-hydroxytryptamine receptors do not necessarily give positive results in the elevated plus-maze test (buspiron acts as an anxiogenic drug in this test (31). Displacement studies of [methyl-<sup>3</sup>H]flunitrazepam showed that  $\alpha_{s1}$ -casein tryptic hydrolysate interacted with the BDZ site of GABA<sub>A</sub> receptor with an IC<sub>50</sub> of 72  $\mu$ M and a Hill number of 0.8. The peptides of the  $\alpha_{s1}$ -casein tryptic hydrolysate were prepurified by reversed-phase HPLC on C<sub>18</sub> column in gradient conditions and lyophilized twice to remove TFA. The purified peptides were rechromatographed on a C<sub>4</sub> column in isocratic conditions, and the same lyophilisation procedure was carried out. All the peptides were recovered and tested in competition with [methyl-<sup>3</sup>H]flunitrazepam. Only one fraction competed with flunitrazepam for the BDZ site of the GABA<sub>A</sub> receptor. This fraction contained only one peptide, of which the sequence was found to be Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg and the molecular mass, determined by FAB-MS, was 1266.6 Da. This corresponded to the  $\alpha_{s1}$ -CN-(f91-100), consequently named  $\alpha$ -casozepine, whose calculated molecular mass is 1266.1 Da. The two aromatic residues of this peptide allow determination of its concentration by spectrophotometry by using an  $\epsilon$  value of 2810 cm<sup>-1</sup>.liter.mol<sup>-1</sup> at 275 nm. The calculated IC<sub>50</sub> value for this peptidic fraction was 88  $\mu$ M, and the Hill number was 0.8. The IC<sub>50</sub> value for diazepam was 8.2 nM, and the Hill number was 1.2 under the same conditions. The peptide corresponding to  $\alpha$ -casozepine was synthesized by using Boc solid-phase peptide-synthesis protocols, and the sequence was checked by Edman sequencing. The synthetic peptide, which displaced [methyl-<sup>3</sup>H]flunitrazepam from the BDZ site of GABA<sub>A</sub> receptor with a calculated IC<sub>50</sub> of 370  $\mu$ M and a Hill number of 0.8, was about four times less potent than the natural one. The others peptides of the  $\alpha_{s1}$ -casein tryptic hydrolysate had no affinity to the BDZ site of the GABA<sub>A</sub> receptor. None of them competed with the [methyl-<sup>3</sup>H]flunitrazepam for this site. Consequently, the  $\alpha_{s1}$ -CN-(f91-100) carries the entire anxiolytic activity of the hydrolysate.

### **$\alpha$ -Casozepeine anxiolytic activity**

$\alpha$ -Casozepeine was tested in the conditioned defensive burying in order to confirm that it carries the anxiolytic activity of the  $\alpha_{s1}$ -casein tryptic hydrolysate *in vivo*. The peptide administered i.p. at 0.4 mg/kg (0.32  $\mu$ mol/kg) decreased significantly the duration of probe burying in comparison with saline (18.8  $\pm$  13.7 s vs. 81.4  $\pm$  19.7 s;  $P < 0.005$ ) (Fig. 4A). Other parameters such as number of head stretchings towards the probe (data not shown) and approaches towards the probe followed by retreats (Fig. 4B) were decreased too ( $P < 0.005$ ). The  $\alpha_{s1}$ -CN-f(91-100) produced significant anxiolytic-like effects with efficiency similar to those of diazepam at 1 mg/kg (3.5  $\mu$ mol/kg) i.p. in the conditioned defensive burying model.

## **$\alpha$ -Casozepine affinity for PBR**

Affinity of  $\alpha$ -casozepine for the MA-10 cell PBR was determined in competition with either [ $^3\text{H}$ ]Ro5-4864 or [ $^3\text{H}$ ]PK 11195. No displacement of  $^3\text{H}$ -labeled ligands was observed, which indicated that  $\alpha$ -casozepine did not bind to PBR and then was specific of the BDZ site of the GABA<sub>A</sub> receptor.

## **DISCUSSION**

Cow's milk has long been considered a tranquilizing beverage with a sleep-inducing role, but the molecular bases of this belief are unknown. Cow's milk contains BDZ-like molecules (32), probably diazepam and its metabolites, but at very low concentration—between 0.5 and 2  $\mu\text{g/liter}$  (33). As it seems unlikely that bovine tissues can synthesize heterocyclic ring with a chlorine atom, these substances would be either of external origin, such as mushroom grazing, or synthesized from plant precursors by rumen microorganisms. Such molecules are also found in human breast milk from women who do not take BDZs (34). The mean concentration of BDZ-like substances in human milk is  $4.3 \pm 2.3 \mu\text{g/liter}$  (35), whereas they are undetectable in women's blood. In the present work, we found evidence of a peptide resulting from bovine  $\alpha_{s1}$ -casein hydrolysis with BDZ-like activity.  $\alpha_{s1}$ -Casein was chosen because it is the major protein from bovine milk (36). Trypsin was used to hydrolyze  $\alpha_{s1}$ -casein to approach an *in vivo* digestion, because it is one of the major proteolytic enzymes of the gastrointestinal tract. Anxiolytic molecules, such as BDZs, contain several aromatic rings. This finding potentially eliminated chymotrypsin (EC 3.4.21.1) and pepsin (EC 3.4.23.1), two other major physiological enzymes, of which preferential cleavage sites include aromatic residues. Activity of pepsin is very low in the newborn during the first 3 weeks (37) due to a stomach pH higher than 5, and trypsin is the only protease of which concentration is similar to that in adults (38).

In the present study, we have shown that i.p. injection of  $\alpha_{s1}$ -casein tryptic hydrolysate significantly reduced PTZ-induced seizures in Wistar rat. PTZ greatly reduces chloride-dependent responses to the iontophoresis of transmitters (39). PTZ modifies GABAergic mediation because it leads to a total or partial inhibition of GABA-induced  $^{36}\text{Cl}^-$  uptake in primary cultures of cortical neurons (40). PTZ also increases the concentration of glutamate and decreases that of GABA (41) because it is involved in the inhibition of glutamate dehydrogenase (EC 1.4.1.3) and aspartate transaminase (EC 2.6.1.1) and in the stimulation of GABA transaminase (EC 2.6.1.19). It has been shown recently that an acute injection of PTZ decreased GABA<sub>A</sub> receptor  $\gamma 2$ ,  $\alpha 1$ , and  $\beta 2$  subunit-mRNAs in cerebral cortex and cerebellum and affected the coupling mechanism between the GABA and BDZ sites of the GABA<sub>A</sub> receptor (42). PTZ administered chronically in rodents induces kindling, and these animals show an enhanced susceptibility to convulsions induced by different inhibitors of central GABAergic function. PTZ may be then associated with a persistent reduction in the inhibitory function of the GABAergic system in the brain (43), and PTZ-induced seizures can be considered as a model of epilepsy mediated through a specific interaction with the GABA-gated chloride ionophore. Diazepam and medazepam have a marked anticonvulsive effect on the clonic-tonic convulsions in PTZ-kindled rats (44). After i.p. injection of  $\alpha_{s1}$ -casein tryptic hydrolysate, we have observed a reduction of the crisis severity and an increase of the crisis latency, which were similar to the effect of

diazepam (2 mg/kg) i.p. injection (data not shown). The hydrolysate would modulate the GABAergic transmission. Although the injected dose of PTZ to nonkindled animals was an acute convulsive dose (the mean stage 4 dose of PTZ in nonkindled rats is 75 mg/kg, 45), it caused seizures in only 63% of the rats. DMI used to dissolve the tested molecules had a slight protective effect against PTZ-induced seizures, so the injected dose of PTZ can be considered as a subconvulsant dose. The repeated injections of such a subconvulsant dose during the five experiments should have produced a progressive sensitization to the effects of PTZ in the animals, which became kindled (46). It has been shown that doses ineffective after the first injection induce tonic seizures after 20 injections (47) and that kindling effect is proportional to the dose of PTZ (48). The decrease of crisis latency and the increase of clonus duration among the control experiments show that kindling increased sensitivity to PTZ convulsant effects. Consequently, the protective effect of the  $\alpha_{s1}$ -casein tryptic hydrolysate against PTZ-induced seizures was probably minimized.

The elevated plus-maze test was chosen to measure anxiolytic effect of the  $\alpha_{s1}$ -casein tryptic hydrolysate. Brain serotonergic, noradrenergic, and GABAergic mechanisms are involved in the regulation of conflict behavior; but the GABA<sub>A</sub>/BDZ receptor complex plays the most central role in this context. In the elevated plus-maze paradigm, diazepam reduces open-arm avoidance; whereas flumazenil, a BDZ antagonist, has no significant behavioral effect (49). In the present experiment, a reduced open-arm avoidance was observed with diazepam and  $\alpha_{s1}$ -casein tryptic hydrolysate because the ratio of entries into the open arms to the total number of arm entries enhanced by comparison with control. This parameter is reliable in assessing the anxiolytic activity of drugs so the elevated plus-maze is commonly used. The standard measures sometimes give false-negative results, as with BDZs injected to animals habituated to gentle handling or tested in less-aversive conditions (50). On the contrary, no false-positive results have been reported, and this finding strongly supports the anxiolytic-like activity of the  $\alpha_{s1}$ -casein tryptic hydrolysate. The conditioned defensive burying paradigm was then chosen to verify the anxiolytic effect of the hydrolysate. When rats are electrically shocked once through a stationary probe, they then bury it with material from the floor of experiment chamber. This “conditioned defensive burying” response is dose-dependent, reduced by various anxiolytic drugs (51). Full and partial BDZ agonists decrease the probe burying duration, which is the crucial parameter (52). BDZ antagonists, such as flumazenil, do not affect the duration of burying (53), whereas BDZ partial inverse agonists lead to a reduction of burying. So, other parameters are needed to differentiate agonists from partial inverse agonists. Full agonists, such as diazepam, increase exploratory approaches to the probe and decrease escape movements from the probe (decrease of the percentage of approaches toward the probe followed by retreats), whereas partial inverse agonists produce a decrease of exploratory approaches to the probe without changing the frequency of retreats (increase of the percentage of approaches towards the probe followed by retreats) (52). A decreased probe-burying duration and a decreased percentage of approaches towards the probe followed by retreats were observed in the  $\alpha_{s1}$ -casein tryptic hydrolysate-treated group. The diazepam-like profile observed in the elevated plus-maze paradigm and in the conditioned defensive burying experiment revealed an anxiolytic-like activity of the  $\alpha_{s1}$ -casein tryptic hydrolysate consistent with its binding to the BDZ site of the GABA<sub>A</sub> receptor. Only one peptide of the tryptic hydrolysate, the  $\alpha_{s1}$ -CN-(f91-100), displaced [methyl-<sup>3</sup>H]flunitrazepam from GABA<sub>A</sub> receptor. The synthetic peptide displayed the same activity, confirming that  $\alpha$ -caseozepine carries the total anxiolytic activity.

The fact that the synthetic peptide was less active *in vivo* and *in vitro* than the natural one has already been observed with biological active peptides prepared from caseins (54), but this finding is not explained. In the conditioned defensive burying paradigm, the  $\alpha_{s1}$ -CN-(f91-100) displayed the anxiolytic activity observed with the  $\alpha_{s1}$ -casein tryptic hydrolysate. An i.p. injection of 0.4 mg/kg of peptide in the rat led to the same behavioral results than an i.p. injection of 1 mg/kg of diazepam. Considering the difference in the molecular weight, the  $\alpha_{s1}$ -CN-(f91-100) might be about 10 times more active than diazepam *in vivo*, whereas it was less affine than diazepam for the BDZ site of the GABA<sub>A</sub> receptor *in vitro*. Some BDZs, such as diazepam or flunitrazepam, exhibit relatively high affinity for the BDZ site of the GABA<sub>A</sub> receptor and for the PBR, which may play a part in anxiolytic response via the steroidogenesis regulation (55). However, the important *in vivo* anxiolytic activity of the  $\alpha_{s1}$ -CN-(f91-100) cannot be explain by such a mechanism because this peptide exhibited no affinity for the PBR and did not significantly affect the progesterone production by MA-10 Leydig cells (data not shown). Changes in peptide conformation due to microenvironment modifications could also be also hypothesized to explain the difference between the *in vivo* and *in vitro* activity of  $\alpha$ -casozepine.

Until now, investigations to identify an endogenous agonist ligand of the BDZ site of the GABA<sub>A</sub> receptor have failed (56). However, an 86-residue peptide called DBI, first isolated from rat brain (57), *in vitro* inhibits competitively the binding of BDZs to their receptor with a  $K_i$  of 4  $\mu$ M. DBI is the precursor of two molecules named octadecaneuropeptide (ODN) (DBI-(f33-50)) and triakontatetrapeptide (TTN) (DBI-(f17-50)) carrying BDZ receptor binding activity. *In vivo*, DBI and its active fragments are anxiogenic (58). DBI also displays epileptogenic activity and seems to be a peripheral marker of epilepsy (59). The comparison of bovine DBI and  $\alpha_{s1}$ -CN-(f91-100) sequences by the CLUSTALW procedure (Fig. 5) leads to an alignment in the carboxy-terminal region of the DBI (residues 73 to 82). Three residues are identical and four residues are homologous. According to Andersen et al. (60), DBI-(f66-83) corresponds to a helical region with potential amphipathic properties. Whereas only 15% of the  $\alpha_{s1}$ -casein residues are in  $\alpha$ -helixes,  $\alpha_{s1}$ -CN-(f91-100) belongs to a helical region (61). Curiously DBI-(f73-82) and  $\alpha_{s1}$ -CN-(f91-100) have a similar primary structure and share the same secondary structure. This DBI region does not belong to the ODN or the TTN that bind on BDZ site of the GABA<sub>A</sub> receptor. The DBI-(f73-82) injected at 1 mg/kg i.p. in the Wistar rat displayed a significant anxiolytic activity in the plus-maze experiment (data not shown).

In conclusion, an anticonvulsant and anxiolytic molecule not structurally related to BDZ could be formed from bovine casein during digestion. The  $\alpha_{s1}$ -CN-(f91-100) peptide bound to the BDZ site of the GABA<sub>A</sub> receptor but not to PBR. The physicochemical characteristics and the pharmacological action of  $\alpha$ -casozepine lead us to hypothesize that this peptide might play a role, as an external ligand, in the regulation of the nervous system of the mammalian newborn and might be involved in the traditional calming properties attributed to milk.

## ACKNOWLEDGMENTS

L. M. was partially supported by a grant of the French Association Nationale de la Recherche Technique and of Ingredia (Arras, France). We gratefully acknowledge Dr. A. Van Dorsselaer

(Centre de Neurochimie, Strasbourg, France) for performing the FAB-MS and ESI-MS analyses. We thank F. Saulnier and Dr. G. Humbert (Service Commun de Séquence des Protéines, Université Henri Poincaré-Nancy 1, France) for sequence analysis. We also thank Dr. M. Messaoudi (ETAP, Nancy, France) for the conditioned defensive burying experiments. We are also grateful to Dr. M. Lecouvey for large-scale purification of the natural peptide. We express our thanks to Martine Culty for reading the manuscript.

## REFERENCES

1. Dial, E. J., Romero, J. J., and Lichtenberger, L. M. (1995) Gastroprotection by dairy foods against stress-induced ulcerogenesis in rats. *Dig. Dis. Sci.* **40**, 2295–2299
2. Laird, D. A., and Drexel, H. (1934) Experimenting with foods and sleep: I. Effects of varying types of foods in offsetting sleep disturbances caused by hunger pangs and gastric distress—children and adults. *J. Am. Diet. Assoc.* **10**, 89–94
3. Brezinova, V., and Oswald, I. (1972) Sleep after a bedtime beverage. *Br. Med. J.* **2**, 431–433
4. Meisel, H. (1997) Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **43**, 119–128
5. Meisel, H. (1986) Chemical characterization and opioid activity of an exorphin isolated from *in vivo* digest of casein. *FEBS Lett.* **196**, 223–227
6. Svedberg, J., De Haas, J., Leimenstoll, G., Paul, F., and Teschemacher, H. (1985) Demonstration of  $\beta$ -casomorphins immunoreactive materials in *in vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adults humans. *Peptides* **6**, 825–830
7. Umbach, M., Teschemacher, H., Praetorius, K., Hirschhäuser, R., and Bostedt, H. (1985) Demonstration of a  $\beta$ -casomorphin immunoreactive material in the plasma of newborn calves after milk intake. *Regul. Pept.* **12**, 223–230
8. Koch, G., Wiedermann, K., Drebes, E., Zimmermann, W., Tink, G., and Teschemacher, H. (1988) Human  $\beta$ -casomorphin-8 immunoreactive materials in the plasma of women during pregnancy and after delivery. *Regul. Pept.* **20**, 107–117
9. Yamamoto, N., Akino, A., and Takano, T. (1994) Antihypertensive effect of peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **77**, 917–922
10. Meisel, H., and Bockelmann, W. (1999) Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties. *Antonie Van Leeuwenhoek* **76**, 207–215
11. File, S. E., Lister R. G., and Nutt D. J. (1982) The anxiogenic action of benzodiazepine antagonists. *Neuropharmacology* **21**, 1033–1037

12. Dorow, R., Horowski, R., Paschelke, G., and Amin M. (1983) Severe anxiety induced by FG 7142, a beta-carboline ligand for benzodiazepine receptors. *Lancet* **2**, 98–99
13. Biggio, G., Concas, A., Corda, M. G., Giorgio, O., Sanna, E., and Serra, M. (1990) GABAergic and dopaminergic transmission in the rat cerebral cortex: effect of stress, anxiolytic and anxiogenic drugs. *Pharmacol. Ther.* **48**, 121–142
14. Luddens, H., and Korpi, E. R. (1995) Biological function of GABAA/benzodiazepine receptor heterogeneity. *J. Psychiatr. Res.* **29**, 77–94
15. Karle, J., Woldbye, D. P., Elster, L., Diemer, N. H., Bolwig, T. G., Olsen, R. W., and Nielsen, M. (1998) Antisense oligonucleotide to GABA(A) receptor gamma-2 subunit induces limbic status epilepticus. *J. Neurosci. Res.* **54**, 863–869
16. Papadopoulos, V., Mukhin, A. G., Costa, E., and Krueger, K. E. (1990) The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J. Biol. Chem.* **265**, 3772–3779
17. Papadopoulos, V., Guarneri, P., Krueger, K. E., Guidotti, A., and Costa, E. (1992) Pregnenolone biosynthesis in C6 glioma cell mitochondria: regulation by a diazepam binding inhibitor mitochondrial receptor. *Proc. Natl Acad. Sci. USA* **89**, 5113–5117
18. Papadopoulos, V. (1993) Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. *Endocr. Rev.* **14**, 222–240
19. Sanogo, T., Paquet, D., Aubert, F., and Linden, G. (1989) Purification of  $\kappa_{s1}$ -casein by Fast Protein Liquid Chromatography. *J. Dairy Sci.* **72**, 2242–2246
20. Krueger K. E., and Papadopoulos V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *J. Biol. Chem.* **265**, 15015–15022
21. Munson P. J., and Rodbard D. (1980) LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**, 220–239
22. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
23. Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Neurosci. Methods* **14**, 149–167
24. Winer, B. J. (1971) In *Statistical Principles in Experimental Design*, McGraw-Hill, Inc.: New York, NY

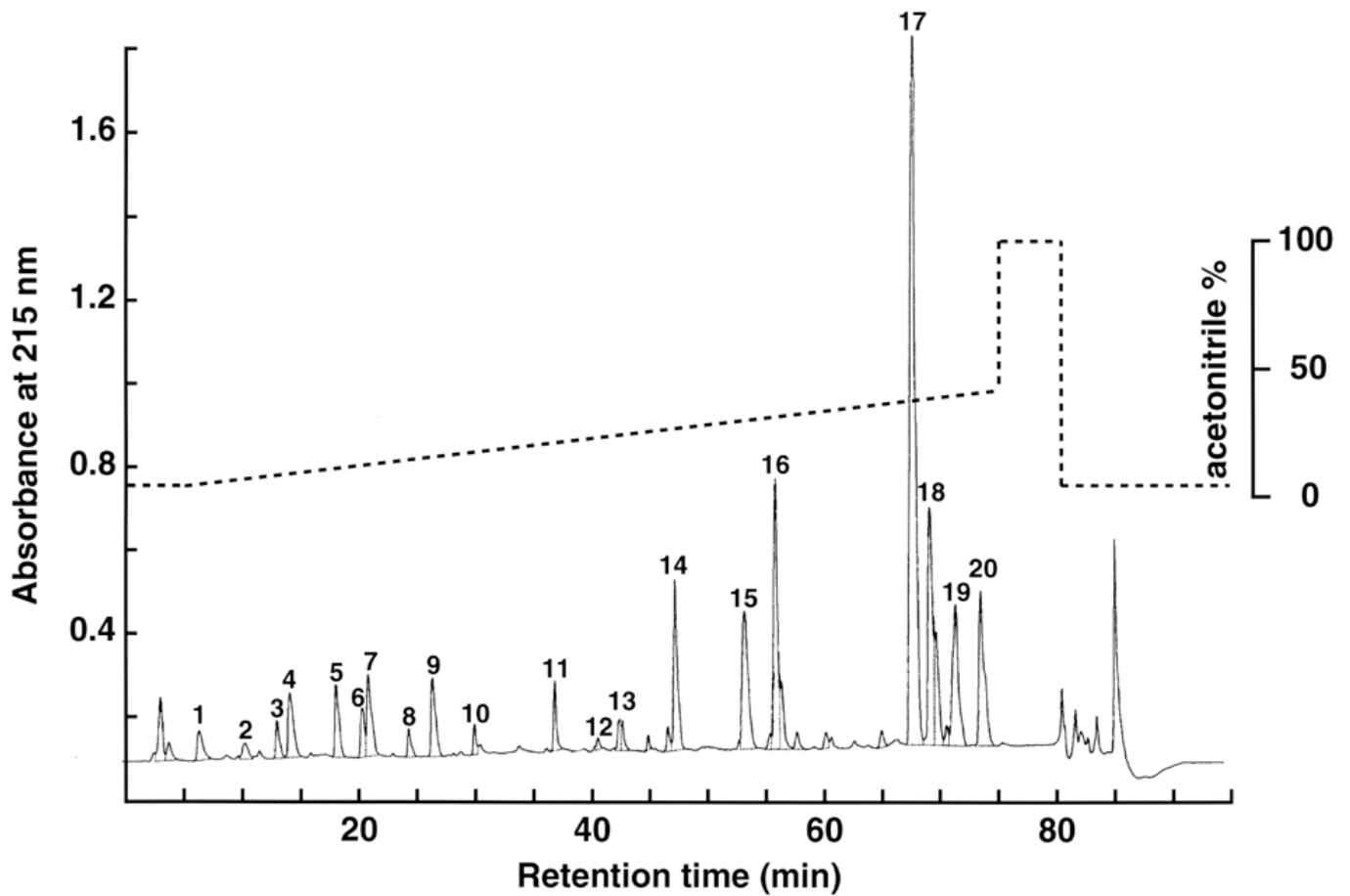
25. Racine, R. J. (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* **32**, 281–294
26. Norusis, M. J. (1994) In *SPSS Advanced Statistics 6.1 Manual* SPSS, Inc.:Chicago, IL; p. 117
27. Pinel, J. P., and Treit D. (1978) Burying as a defensive response in rats. *J. Comp. Physiol. Psychol.* **92**, 708–712
28. Perrin, E., Miclo, L., Driou, A., and Linden, G. (1995) Rapid determination of the ratios of three aromatic residues in peptides by reversed-phase high-performance liquid chromatography with a high-resolution photodiode-array detector. *J. Chromatogr. B Biomed. Appl.* **664**, 267–276
29. Kaminogawa, S., Shimizu, M., Ametani, A., Lee, S. W., and Yamauchi, K. (1987) Proteolysis in structural analysis of  $\alpha_{s1}$ -casein absorbed onto oil surfaces of emulsions and improvement of the emulsifying properties of protein. *J. Am. Oil Chem. Soc.* **64**, 1688–1691
30. Brett, R. R., and Pratt, J.A. (1990) Chronic handling modifies the anxiolytic effect of diazepam in the elevated plus-maze. *Eur. J. Pharmacol.* **178**, 135–138
31. Moser, P. C. (1989) An evaluation of the elevated plus-maze test using novel anxiolytic buspirone. *Psychopharmacology (Berl)* **99**, 48–53
32. Medina, J. H., Peña, C., Piva, M., Paladini, A. C., and De Robertis, E. (1988) Presence of benzodiazepine-like molecules in mammalian brain and milk. *Biochem. Biophys. Res. Commun.* **152**, 534–539
33. Medina, J. H., Peña, C., Piva, M., Wolfman, C., De Stein, M. L., Wasowski, C., Da Cunha, C., Izquierdo, I., and Paladini, A. C. (1992) Benzodiazepines in the brain. Their origin and possible biological roles. *Mol. Neurobiol.* **6**, 377–386
34. Dencker, S. J., and Johansson, G. (1990) Benzodiazepine-like substances in mother's milk. *Lancet* **335**, 413
35. Dencker, S. J., Johansson, G., and Milsom, I. (1992) Quantification of naturally occurring benzodiazepine-like substances in human breast milk. *Psychopharmacology (Berl)* **107**, 69–72
36. Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell Jr, H. M., Harwalkar, V. R., Jenness, R., and Whitney, R. McL. (1984) Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* **67**, 1599–1631
37. Agunod, M., Yamaguchi, N., Lopez, R., Luhby, A. L., and Glass, G. B. (1969) Correlation study of hydrochloric acid, pepsin, and intrinsic factor secretion in newborns and infants. *Am. J. Dig. Dis.* **14**, 400–414
38. Lebenthal, E., Lee, P. C., and Heitlingert, L. A. (1983) Impact of development of the gastrointestinal tract on infant feeding. *J. Pediatr.* **102**, 1–9

39. Pellmar, T. C., and Wilson, W. A. (1977) Synaptic mechanism of pentylenetetrazole: selectivity for chloride conductance. *Science* **197**, 912–914
40. Pomes, A., Rodriguez-Farre, E., and Sunol, C. (1994) Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J. Pharmacol. Exp. Ther.* **271**, 1616–1623
41. Lacoste, L., Bartolucci, S., and Lapointe, J. (1988) Pentylenetetrazole inhibits glutamate dehydrogenase and aspartate aminotransferase, and stimulates GABA aminotransferase in homogenates from rat cerebral cortex. *Can. J. Physiol. Pharmacol.* **66**, 1135–1138
42. Walsh, L. A., Li, M., Zhao, T. J., Chiu, T. H., and Rosenberg, H. C. (1999) Acute pentylenetetrazole injection reduces rat GABAA receptor mRNA levels and GABA stimulation of benzodiazepine binding with no effect on benzodiazepine binding site density. *J. Pharmacol. Exp. Ther.* **289**, 1626–1633
43. Corda, M. G., Orlandi, M., Lecca, D., Carboni, G., Frau, V., and Giorgi, O. (1991) Pentylenetetrazole-induced kindling in rats: effect of GABA function inhibitors. *Pharmacol. Biochem. Behav.* **40**, 329–333
44. Lazarova-Bakarova, M., Petkov, V. V., and Genkova-Papasova, M. (1990) Effect of diazepam and medazepam on pentylenetetrazol kindling in albino rats. *Acta Physiol. Pharmacol. Bulg.* **16**, 37–41
45. Schmidt, J. (1987) Changes in seizure susceptibility in rats following chronic administration of pentylenetetrazol. *Biomed. Biochim. Acta* **46**, 267–270
46. Gramlich, C. A., and Stripling, J. S. (1987) Effect of pentylenetetrazol-induced convulsions on the development and expression of limbic kindled seizures. *Pharmacol. Biochem. Behav.* **26**, 159–165
47. Diehl, R. G., Smialowski, A., Gotwo, T. (1984) Development and persistence of kindled seizures after repeated injections of pentylenetetrazol in rats and guinea pigs. *Epilepsia* **25**, 506–510
48. Karler, R., Murphy, V., Calder, L. D., and Turkanis, S. A. (1989) Pentylenetetrazol kindling in mice. *Neuropharmacology* **28**, 775–780
49. Dalvi, A., and Rodgers, R. J. (1999) Behavioral effects of diazepam in the murine plus-maze: flumazenil antagonism of enhanced head dipping but not the disinhibition of open-arm avoidance. *Pharmacol. Biochem. Behav.* **62**, 727–734
50. Hogg, S. (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol. Biochem. Behav.* **54**, 21–30
51. Treit, D., Pinel, J. P., and Fibiger, H. C. (1981) Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. *Pharmacol. Biochem. Behav.* **15**, 619–626

52. Rohmer, J. G., Di Scala, G., and Sandner, G. (1990) Behavioral analysis of the effects of benzodiazepine receptor ligands in the conditioned burying paradigm. *Behav. Brain Res.* **38**, 45–54
53. Treit, D. (1987) Ro 15-1788, CGS 8216, picrotoxin and pentylentetrazol: do they antagonize anxiolytic drug effects through an anxiogenic action? *Brain Res. Bull.* **19**, 401–405
54. Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., and Klee, W. A. (1983) Opioid activities and structures of  $\alpha$ -casein-derived exorphins. *Biochemistry* **22**, 4567–4573
55. Krueger, K. E. (1991) Peripheral-type benzodiazepine receptors: a second site of action for benzodiazepine. *Neuropsychopharmacology* **4**, 237–244
56. Pelissolo A. (1995) The benzodiazepine receptor: the enigma of the endogenous ligand. *Encephale* **21**, 133–140
57. Guidotti, A., Forchetti, C. M., Corda, M. G., Konkel, D., Bennett, C. D., and Costa E. (1983) Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc. Natl Acad. Sci. USA* **80**, 3531–3535
58. Ferrero, P., Guidotti, A., Conti-Tronconi, B., and Costa, E. (1984) A brain octadecaneuropeptide generated by tryptic digestion of DBI (diazepam binding inhibitor) functions as a proconflict ligand of benzodiazepine recognition sites. *Neuropharmacology* **23**, 1359–1362
59. Ferrarese, C., Cogliati, T., Tortorella, R., Zucca, C., Boglium, G., Beghi, E., Passoni, D., Zoia, C., Begni, B., Airoldi, L., Alho, H., and Frattola, L. (1998) Diazepam binding inhibitor (DBI) in the plasma of pediatric and adult epileptic patients. *Epilepsy Res.* **29**, 129–134
60. Andersen, K. V., Ludrigsen, S., Mandrup, S., Knudsen, J., and Poulsen, F. M. (1991) The secondary structure in solution of acyl-coenzyme A binding protein from bovine liver using <sup>1</sup>H nuclear magnetic resonance spectroscopy. *Biochemistry* **30**, 10654–10663
61. Kumosinski, T. F., Brown, E. M., and Farrell Jr., H. M. (1991) Three-dimensional molecular modeling of bovine caseins:  $\alpha_{s1}$ -casein. *J. Dairy Sci.* **74**, 2889–2895

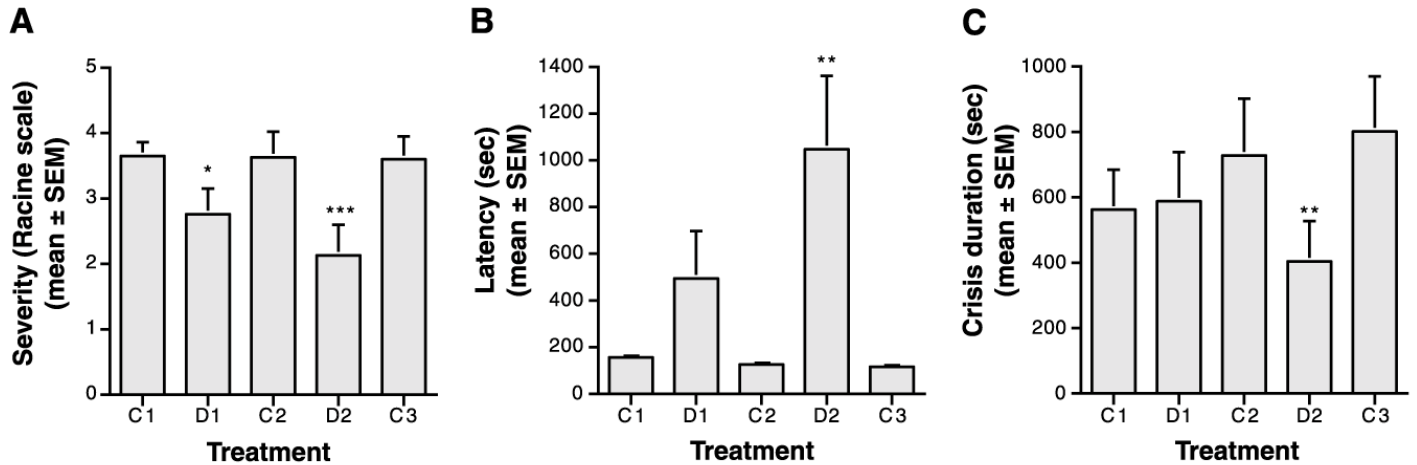
*Received December 11, 2000; revised April 4, 2001.*

Fig. 1



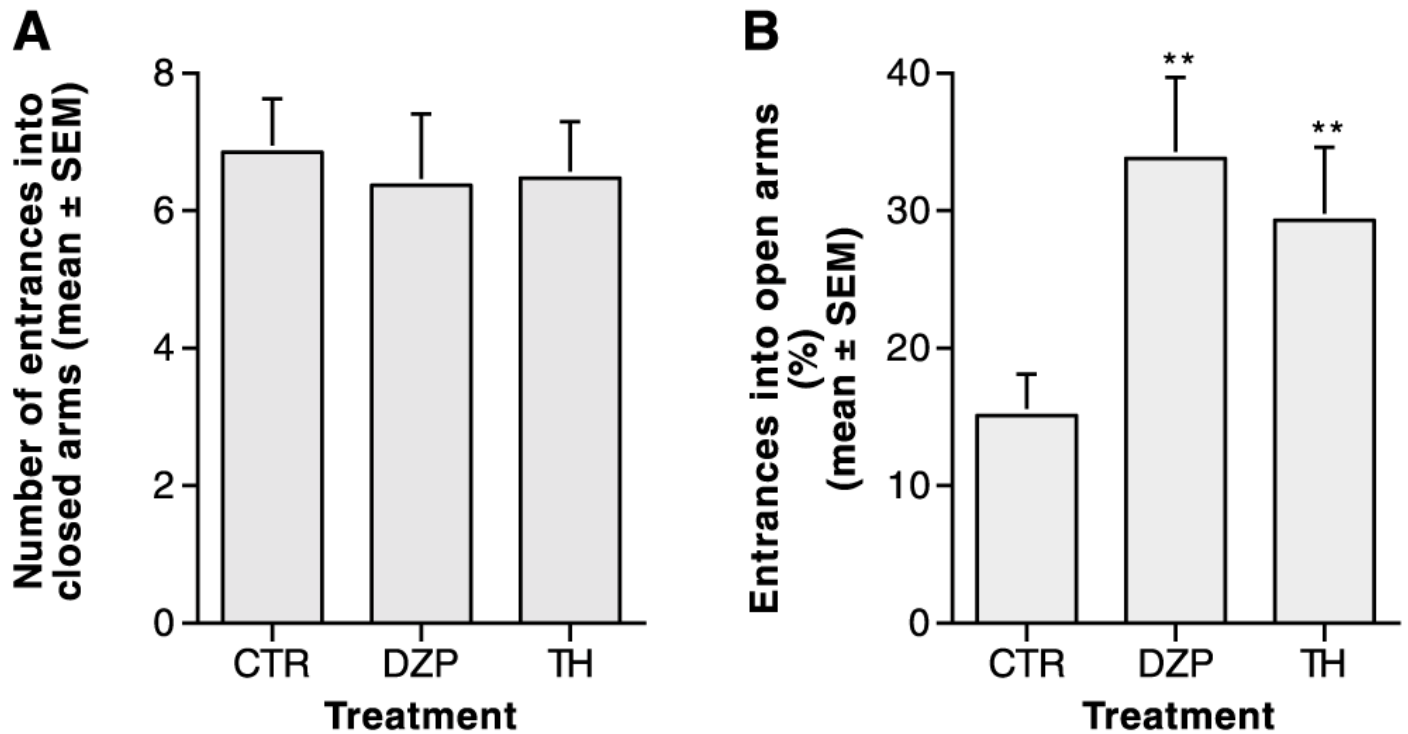
**Figure 1. Separation of  $\alpha_{s1}$ -casein tryptic hydrolysate peptides by reversed-phase HPLC on a C18 column at room temperature.** The gradient (dotted line) was 5%–40% of acetonitrile in water containing 0.1% (v/v) TFA for 70 min at a flow-rate of 1 ml/min. Injection was of 400  $\mu$ g of hydrolysate. Peptide identification: peak 1= $\alpha_{s1}$ -CN-(f1-3), peak 2= $\alpha_{s1}$ -CN-(f101-103), peak 3= $\alpha_{s1}$ -CN-(f80-83), peak 4= $\alpha_{s1}$ -CN-(f4-7), peak 5= $\alpha_{s1}$ -CN-(f125-132), peak 6= $\alpha_{s1}$ -CN-(f84-90), peak 7= $\alpha_{s1}$ -CN-(f120-124), peak 8= $\alpha_{s1}$ -CN-(f35-42), peak 9= $\alpha_{s1}$ -CN-(f80-90), peak 10= $\alpha_{s1}$ -CN-(f59-79), peak 11= $\alpha_{s1}$ -CN-(f43-58), peak 12= $\alpha_{s1}$ -CN-(f35-58), peak 13= $\alpha_{s1}$ -CN-(f106-119), peak 14= $\alpha_{s1}$ -CN-(f104-119), peak 15= $\alpha_{s1}$ -CN-(f8-22), peak 16= $\alpha_{s1}$ -CN-(f194-199), peak 17= $\alpha_{s1}$ -CN-(f91-100) and  $\alpha_{s1}$ -CN-(f152-193), peak 18= $\alpha_{s1}$ -CN-(f23-34), peak 19= $\alpha_{s1}$ -CN-(f125-151), peak 20= $\alpha_{s1}$ -CN-(f133-151).

Fig. 2



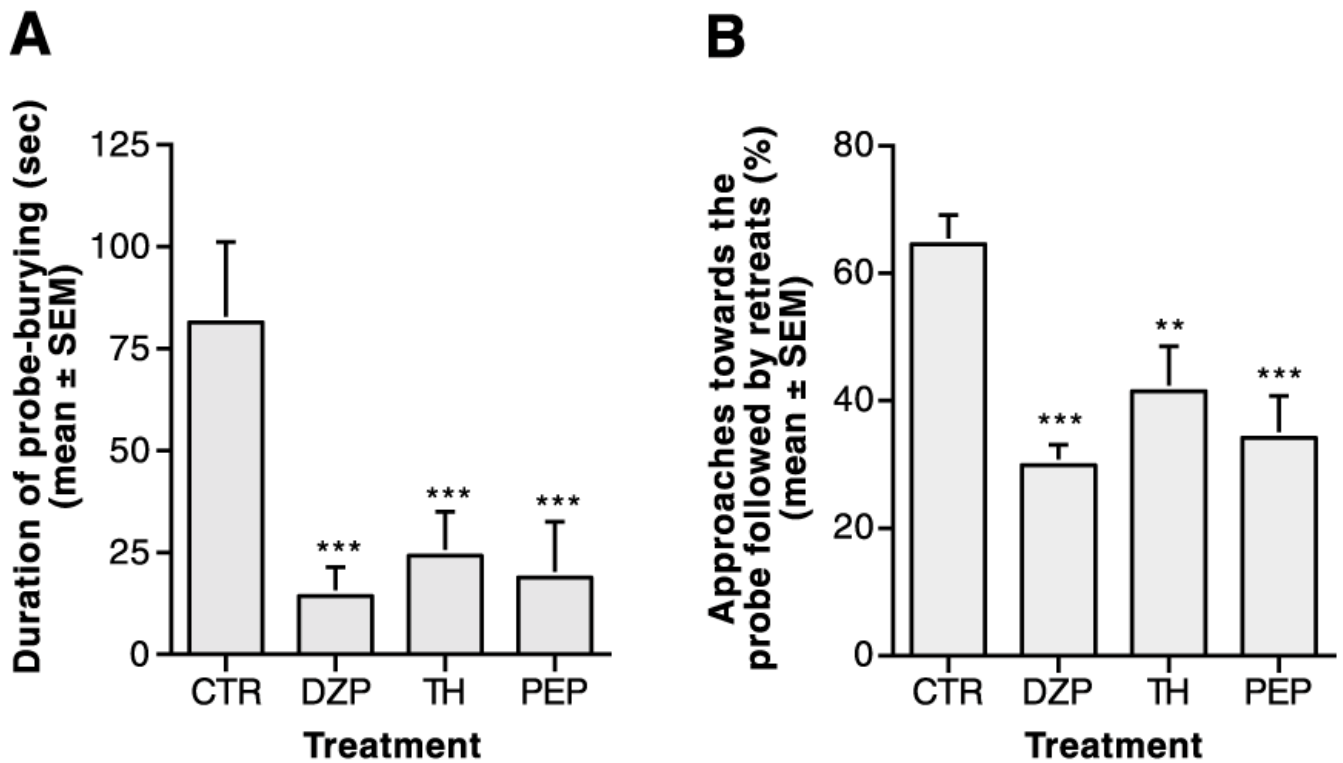
**Figure 2. Effect of the  $\alpha_{s1}$ -casein tryptic hydrolysate on the severity (A), latency (B), and duration (C) parameters of a crisis induced by i.p. injection of 60 mg/kg of pentylentetrazole dissolved in 9% NaCl in Wistar rats.** The same animals ( $n = 17$ ) were used for all the experiments. 2 ml/kg of a solution of 25% (v/v) DMI in water (controls C1, C2, and C3), 1 mg/kg (D1), or 3 mg/kg (D2) of  $\alpha_{s1}$ -casein tryptic hydrolysate were injected by the i.p. route 30 min before the pentylentetrazole injection. For the severity parameter (A), values are the mean of crisis intensity according to Racine SE. For the latency parameter (B), values are the mean of the time (seconds) needed to observe the first parameter of crisis  $\pm$  SE (if no crisis parameter was observed a value of 2700 s was taken in account). For the duration parameter, values are the mean of the total time (seconds) of crisis  $\pm$  SE. \*  $P < 0.02$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.002$  by repeated measures ANOVA procedure corrected by Greenhouse–Geisser epsilon.

Fig. 3



**Figure 3. Effect of the  $\alpha_{s1}$ -casein tryptic hydrolysate on the performance of Wistar rats in the elevated plus-maze paradigm compare to diazepam.** 9% NaCl (CTR), 1 mg/kg of diazepam (DZP) or 3 mg/kg of  $\alpha_{s1}$ -casein tryptic hydrolysate (TH) were injected by i.p. route 30 min. before experiment. Behavior of animals ( $n=20$ , each group) was tape-recorded during 5 min. Values are (A) the mean $\pm$ SE of the number of entrances into the closed arms of the plus-maze, (B) the mean $\pm$ SE of the percentage of entrances into the open arms (entrances into the open arms to the total entrances). \*\*  $P < 0.02$  by ANOVA procedure.

Fig. 4



**Figure 4. Effect of the  $\alpha_{s1}$ -CN-(f91-100) and  $\alpha_{s1}$ -casein tryptic hydrolysate on conditioned defensive burying in the Wistar rat by comparison to diazepam.** 9‰ NaCl (CTR), 1 mg/kg of diazepam (DZP), 3 mg/kg of  $\alpha_{s1}$ -casein tryptic hydrolysate (TH) or 0.4 mg/kg of  $\alpha_{s1}$ -CN-(f91-100) (PEP) were administered by i.p. way ( $n=12$ , each group), 30 min before the burying test commenced. Behavior of animals was tape-recorded during 5 min. Values are (A) the mean  $\pm$  SE of time (s) spent to bury the probe, (B) the mean  $\pm$  SE of the percentage of approaches towards the probe followed by retreats (number of retreats to number of approaches). \*\*  $P < 0.01$ ; \*\*\*  $P < 0.005$  by ANOVA procedure.

