Characterization of α-casozepine, a tryptic peptide from bovine α_{s1}-casein with benzodiazepine-like activity[1]

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SPECIFIC AIMS

According to folk wisdom, milk intake improves sleeping or has a calming role, a belief supported by some scientific studies. As caseins contain peptides with physiological roles, we hypothesized that one of their peptides can carry this anxiolytic activity. In this study, α_{s1}-casein tryptic hydrolysate (α_{s1}-CnTH) was tested in vitro on benzodiazepine (BDZ) receptors and checked in vivo for its anticonvulsant and anxiolytic effects in the rat.

PRINCIPAL FINDINGS

1. α_{s1}-CnTH inhibits pentylenetetrazole (PTZ) -induced seizures in Wistar rat

Bovine α_{s1}-CnTH was injected intraperitoneally (i.p.) in a Wistar rat 30 min before an i.p. injection of 60 mg/kg of PTZ to evaluate anticonvulsant activity. Crisis severity parameters, crisis latency, and clonus duration were determined after 45 min observation of behavior. The i.p. injection of 1 mg/kg of α_{s1}-CnTH reduced crisis severity ($P<0.02$). Other parameters were not significantly different from the control. The i.p. injection of 3 mg/kg of α_{s1}-CnTH increased crisis latency ($P<0.005$), decreased crisis severity ($P<0.002$), and decreased clonus duration ($P<0.005$) of PTZ-induced seizures. The anticonvulsant action of the α_{s1}-CnTH was underestimated because of a sensitization of animals to PTZ during successive experiments.

2. α_{s1}-CnTH displays an anxiolytic activity in the elevated plus maze paradigm

Elevated plus maze test was used to evaluate the anxiolytic effect of the α_{s1}-CnTH. Diazepam (1 mg/kg) and α_{s1}-CnTH (3 mg/kg) enhanced ($P<0.02$) the percentage of entries in the open arms whereas general activity, represented by entries in closed arms, was not modified (**Fig. 1**).

3. α_{s1}-CnTH displays an anxiolytic activity in the conditioned defensive burying (CDB) experiment

When a rat is shocked once through an electrical probe mounted on a wall of a test cage, it returns to the probe and buries it with bedding material from the floor of the cage. This CDB response is reduced by anxiolytic drugs. Some behavioral sequences such as exploratory approaches to the probe and escape movements from the probe are needed to differentiate agonists from partial inverse agonists. Similar to diazepam, the α_{s1}-CnTH, administrated at 3 mg/kg i.p., decreased the duration of probe burying ($P<0.005$) (**Fig. 2**). The ratio of the retreats to the approaches decreased in rats treated with α_{s1}-CnTH or diazepam. The CDB paradigm indicates that the α_{s1}-CnTH exerts an anxiolytic activity over rats.

4. A peptide (α-casozepine) of the α_{s1}-CnTH binds on the BDZ site of the $GABA_A$ receptor

Affinity for the BDZ site of the $GABA_A$ receptor from bovine cerebral cortex membranes was measured in competition with [methyl-^{3}H]-flunitrazepam. α_{s1}-CnTH competed with the radioligand for binding on the BDZ site of the $GABA_A$ receptor with an IC_{50} of 72

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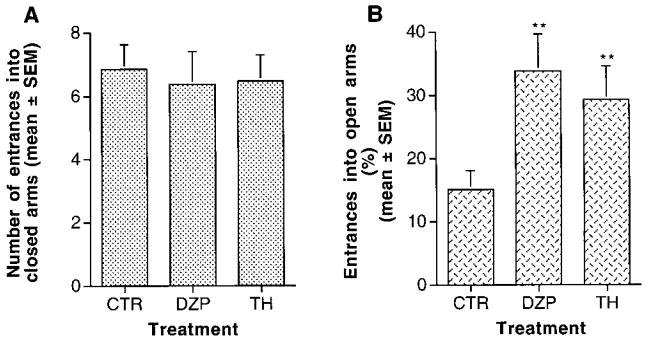

Figure 1. Effect of the α_{s1}-CnTH on the performance of Wistar rats in the elevated plus maze paradigm compared with diazepam. 9‰ NaCl (CTR), 1 mg/kg of diazepam (DZP), or 3 mg/kg of α_{s1}-CnTH (TH) were injected by i.p. route 30 min before the experiment. Behavior of animals (n=20, each group) was tape-recorded for 5 min. Values are *A)* the mean ± SE of the number of entrances into the closed arms of the plus maze; *B)* the mean ± SE of the percentage of entrances into the open arms (entrances into the open arms to the total entrances). $^{**}P < 0.02$ by ANOVA.

μM and a Hill number of 0.8. All peptides of the α_{s1}-CnTH were purified and tested in competition with the radioligand. Only one peptide exhibited affinity for the BDZ site of the $GABA_A$ receptor. The sequence and mass of this peptide corresponded to those of the α_{s1}-casein-(f91–100); it has consequently been named α-casozepine. This purified peptide competed against [methyl-^{3}H]-flunitrazepam in binding assays with an IC_{50} of 88 μM and a Hill number of 0.8 compared to an IC_{50} value for diazepam of 8.2 nM determined under the same conditions. The synthetic α-casozepine also displaced the radioligand from the BDZ site of $GABA_A$ receptor, but was fourfold less potent than the milk-purified peptide. These findings indicate that the α_{s1}-casein-(f91–100) carries the activity of the α_{s1}-CnTH.

5. The α-casozepine exhibits an anxiolytic activity in vivo

α-Casozepine was tested in the CDB paradigm. This peptide administrated i.p. at 0.4 mg/kg (0.32 μmol/kg) decreased the duration of probe burying compared to saline (P<0.005) (Fig. 2). Other behavioral parameters show the same modifications as observed with the α_{s1}-CnTH. Effects of α-casozepine were comparable to those induced by diazepam injected i.p. at 1 mg/kg (3.5 μmol/kg). The α-casozepine seems to be ~10-fold more active than diazepam in vivo despite the lower binding affinity of the tryptic fragment for the BDZ site of the $GABA_A$ receptor in vitro.

6. Effects of α-casozepine are not mediated by the peripheral-type BDZ receptor (PBR)

PBR are a second class of BDZ binding receptor with predominant mitochondrial localization. The possibility that the peptide would act via the PBR was examined. The binding ability of α-casozepine to PBR was tested on MA-10 mouse Leydig cell mitochondrial preparation in competition experiments against two specific ligands of PBR. No displacement of these ligands was observed, indicating that α-casozepine did not bind to PBR. Moreover, α-casozepine did not significantly affect progesterone production by MA-10 mouse Leydig cells. These results exclude the possibility for PBR to be a target of the α-casozepine and suggest that this peptide is specific for the BDZ site of the $GABA_A$ receptor.

CONCLUSIONS

Cow's milk has long been considered a tranquilizing beverage with sleep-inducing capacity, but the molecular bases of this potential action are not known. Cow or human breast milks contain BDZ-like molecules, even in the absence of BDZ administration. However, the concentrations of these milk BDZ-like compounds are very low—only a few micrograms per liter. This study demonstrated that a peptide stemming from the hydrolysis of α_{s1}-casein by trypsin, the only protease of the gastrointestinal tract that is in equal concentration and activity in the newborn and adult, exhibited a BDZ-like activity. In two behavioral tests, elevated plus maze test and CDB paradigm, α_{s1}-CnTH exhibited the characteristic profile of full BDZ agonists such as diazepam. Moreover, α_{s1}-CnTH suppressed the inhibitory effect of PTZ on the GABAergic system. These results were consistent with the binding of one of the peptides of the α_{s1}-CnTH to the BDZ site of the $GABA_A$ receptor. This peptide, named α-casozepine, corresponds to the α_{s1}-casein-(f91–100) (**Fig. 3**). Despite a lower in vitro affinity than diazepam for the BDZ site of the $GABA_A$ receptor, α-casozepine might be ~10-fold more active

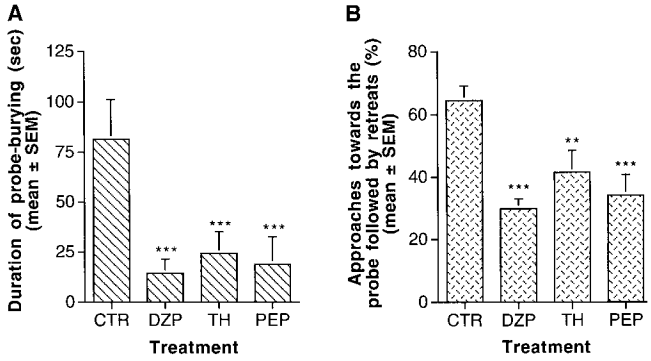

Figure 2. Effect of the α_{s1}-casein-(f91–100) and α_{s1}-CnTH on conditioned defensive burying in the Wistar rat vs. diazepam. 9‰ NaCl (CTR), 1 mg/kg of diazepam (DZP), 3 mg/kg of α_{s1}-CnTH (TH), or 0.4 mg/kg of α_{s1}-casein-(f91–100) (PEP) were administered i.p. (n=12, each group) 30 min before the burying test began. Animals' behavior was tape-recorded for 5 min. Values are *A)* the mean ± SE of time (seconds) spent to bury the probe; *B)* the mean ± SE of the percentage of approaches toward the probe followed by retreats (number of retreats to number of approaches). $^{**}P < 0.01$; $^{***}P < 0.005$ by ANOVA.

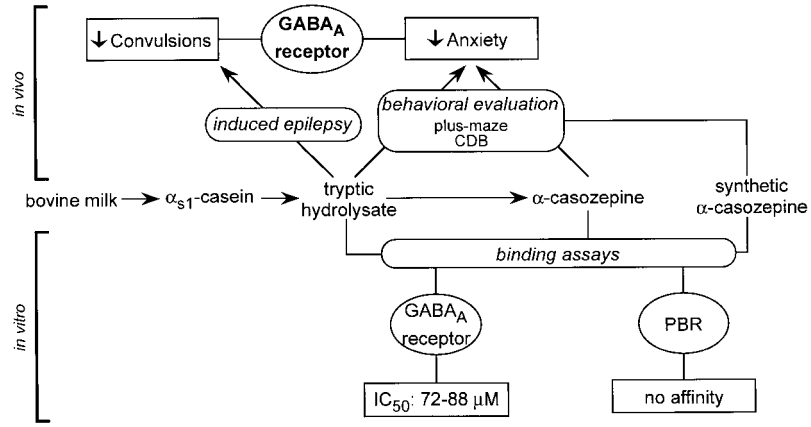

Figure 3. Assessment of biological activity of bovine α_{s1}-CnTH and of α-casozepine.

in vivo than the BDZ. Although α-casozepine displays the characteristic anxiolytic and anticonvulsant effects of BDZ, it does not trigger any side effects. The lack of affinity of α-casozepine for PBR and its small effect on steroidogenesis exclude this way of action. Whereas the present results clearly show that α-casozepine is acting via the $GABA_A$ receptor, the difference of efficiency between in vivo and in vitro experiments remains unclear. A physiological relevance of modulation of the GABAergic system through the BDZ site of the $GABA_A$ receptor implies the presence of an endogenous ligand.

Until now, attempts to identify an endogenous agonist ligand of the BDZ site of the $GABA_A$ receptor have failed. A 86 residue endogenous peptide called diazepam binding inhibitor (DBI) inhibits competitively the binding of BDZ to the $GABA_A$ receptor with an IC_{50} of ~4 μM. DBI is the precursor of two peptides, ODN [DBI-(f33–50)] and TTN [DBI-(f17–50)], that carry the BDZ receptor binding activity. However, DBI and its fragments have effects opposite to those of BDZ-like agonists. In behavioral paradigms, DBI and its fragments are anxiogenic, which means they are anxiety factors. Moreover, DBI displays epileptogenic activity. A comparison of the carboxyl-terminal region of DBI (residues 73 to 82) from different species and bovine α-casozepine shows a great homology of sequence and structure. This region of DBI does not relate to the ODN or the TTN peptides. The region 73–82 of DBI belongs to the fragment 66–83, which is predicted to form a helix. The fragment 91–100 of the bovine α_{s1}-casein is predicted to insert in a helical region, whereas only 15% of the α_{s1}-casein residues belong to α-helix structures. We have shown that the bovine DBI-(f73–82) injected i.p. at 1 mg/kg in Wistar rats displayed an anxiolytic activity in the plus maze experiment. At this time, we cannot conclude whether the DBI-(f73–82) can be generated by in vivo hydrolysis of DBI.

Casein from bovine milk contains a latent peptide with BDZ-like properties that may be released upon proteolytic hydrolysis, which could explain the calming properties attributed to this beverage. This peptide, which displays strong in vivo anxiolytic activity in the rat, is homologous to a peptide of the carboxyl-terminal region of DBI. Our results also raised the possibility that the carboxyl-terminal part of DBI could act as an agonist of physiological importance in regulating anxiety. Moreover, this suggests that bovine α-casozepine, or the equivalent in other mammal species, may act as an exogenous ligand for the BDZ site of the $GABA_A$ receptor in newborn brain, thus regulating anxiety in the newborn. FJ