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Diazepam-binding Inhibitor-like Activity in Rat Cerebrospinal Fluid after Stimulation by an Aversive Quinine Taste

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Abstract

Cerebrospinal fluid (CSF) taken from rats after stimulation by an aversive quinine taste (hereafter called quinine CSF) administered into the fourth ventricle of mice suppressed their intake of 5% sucrose solution. We examined the effects of CSF on glutathione-induced tentacle ball formation (TBF) of hydra to determine the change in CSF components associated with aversive taste stimuli. The suppressive activity of quinine CSF on TBF in the presence of 3 μM *S*-methyl-glutathione (GSM) was markedly lower than that of CSF obtained from control rats (control CSF). Pronase-treated quinine CSF had suppressive activity similar to that of control CSF. The active principle passed through an ultrafiltration membrane, with a molecular weight cut-off of 30 kDa, but not through one with a cut-off of 3 kDa. A peptide fragment of diazepam-binding inhibitor (DBI) nullified the suppression of TBF at 3 μM GSM by control CSF. The nullifying activity of quinine CSF was not observed after treatment with a benzodiazepine receptor preparation that was able to bind DBI. When flumazenil, a benzodiazepine receptor antagonist, was given to mice, the suppression of the intake of 5% sucrose solution by quinine CSF was partially reversed. It is suggested that quinine CSF contains a DBI-like substance.

Introduction

Taste plays an important role in the regulation of food and fluid intake in animals. Dopamine (Schneider, 1989; Mark *et al.*, 1994; Martel and Fantino, 1996), opioids (Levine *et al.*, 1985; Doyle *et al.*, 1993; Rideout and Parker, 1996) and benzodiazepines (Cooper, 1982; Berridge and Pecina, 1995; Gray and Cooper, 1995) affect the palatability of foods. Morphine and benzodiazepine agonists enhance not only feeding but also the ingestive reaction as judged by a taste reactivity test (Berridge and Pecina, 1995; Rideout and Parker, 1996). Dopamine could be involved in incentive motivation of food reward rather than assessment of palatability (Mark *et al.*, 1994). Neuroactive substances thus seem to be involved in the intake of palatable food. However, studies of substances released in the brain after stimulation by an aversive taste are quite limited. Calcitonin gene-related peptide (CGRP)-like immunoreactivity levels in the gustatory insular cortex increased significantly after strong aversive taste stimuli were given (Yamamoto *et al.*, 1990). It has been suggested that acetylcholine is released into the insular gustatory cortex after aversive taste stimuli (Shimura *et al.*, 1995), but acetylcholine could be related mainly to formation of a memory of the taste aversion (Yamamoto *et al.*, 1998; Miranda and Bermudez-Rattoni, 1999). Thus, little is known about substances released in the brain after aversive taste stimuli, though information about aversive tastes may be transmitted via a humoral factor in the brain.

We searched for a substance released in the brain from cerebrospinal fluid (CSF) of rats after they had experienced an unpleasant taste (quinine). An aversive quinine solution was placed in the mouths of rats; CSF obtained from these rats ('quinine CSF') was injected into the brains of mice and subsequent intake of a highly palatable sucrose solution by these mice was studied.

We then examined a candidate for the active substance in the quinine CSF using the hydra behavioral test, which determines tentacle ball formation (TBF), a component of the feeding response of hydra, elicited by *S*-methyl-glutathione (GSM) (Hanai, 1981). TBF elicited at five different GSM concentrations showed a specific pattern of modulation for each biologically active substance tested. GSM-elicited TBF was modified by many biologically active

peptides at low concentrations, but not by neurotransmitters with lower molecular weights, such as catecholamines (Hanai *et al.*, 1987, 1989; Manabe *et al.*, 2000). This system is useful for examining biologically active peptides in a small amount of biological material (Inoue *et al.*, 1999). In the present study, we compared the modulation of GSM-elicited TBF by quinine CSF with a panel of known biologically active peptides. We examined the effects of a substance transmitted in quinine CSF to mice, and determined a candidate responsible for the effects using hydra behavioral test.

Experiment 1

Materials and methods

CSF sampling

Eight-week-old male Wistar rats (Nihondobutsu, Osaka, Japan) were kept at 22 ± 2°C in a humidity-controlled environment in a 12 h light/12 h dark cycle. Food and water were available *ad libitum* until the day before the experiment. Rats were randomly divided into two groups: a quinine group and a control group. In rats in the quinine group, an intraoral cannula was implanted at least 3 days before injection of quinine solution (Shimura *et al.*, 1995) and rats were given quinine–HCl dissolved in distilled water (10^{-4} M) through the cannula for 20 min after fasting overnight. CSF was collected from cisterna magna (quinine CSF) in the rats under pentobarbital anesthesia 1.5 h after the beginning of quinine infusion (Yamamoto *et al.*, 2000). Control CSF was collected from untreated rats after fasting overnight to avoid the influence of feeding to the hydra response (Hanai *et al.*, 1989) in the same manner.

CSF injection into mice

Five-week-old male Std ddY mice (Japan SLC, Hamamatsu, Japan) were housed individually in standard cages (33 cm × 23 cm × 12 cm) under controlled conditions of temperature (22 ± 2°C) and a 12 h light/12 h dark cycle. Food and water were available *ad libitum* until the day before the experiment.

To train mice to drink sucrose solution, they were deprived water and food for 1 h from the beginning of the dark period, and were given only 5% (w/w) sucrose solution for 1 h once a day for three consecutive days. On day 4, mice were anesthetized with pentobarbital and a guide cannula was surgically implanted in the fourth ventricle. Coordinates were: anteroposterior, –6.0 mm from bregma; lateral to right, 0.2 mm; dorsoventral, –4 mm (Franklin and Paxinos, 1997). After surgery, a dummy cannula was placed in the guide cannula to prevent occlusion. The sucrose intake training continued for another 5 days to allow recovery from surgery.

The effects of both the control CSF and quinine CSF were examined on each mouse. First, control CSF (2 µl) was injected, and then 2 days later, quinine CSF (2 µl) was injected using a microsyringe through the cannula over 1 min. The mice were given 5% sucrose solution just after CSF injection, and the intake in a 30 min period was determined.

All animals received humane care as outlined in the *Guide for the Care and Use of Laboratory Animals* (Kyoto University Animal Care Committee according to NIH 86-23; revised 1985).

Hydra culture and behavioral test

Hydra japonica was cultured as described previously (Hanai and Matsuoka, 1995; Hanai, 1998; Manabe *et al.*, 2000). The hydra behavioral test has been described previously (Manabe *et al.*, 2000). Briefly, 10 hydras were incubated with a test sample in a dish containing 2 ml of PIPES solution (1 mM PIPES, 1 mM $CaCl_2$, pH 6.2). Then, GSM was added at various final concentrations (0.1, 0.3, 3, 10 and 50 µM) to stimulate the TBF. The number of hydras exhibiting TBF was counted each minute from 6 to 10 min after stimulation under a binocular microscope (×8). The response was expressed as the number of hydras exhibiting the TBF every minute from 6 to 10 min divided by the total number of hydras (10) (Hanai and Matsuoka, 1995). A response of 3.0 was judged to indicate lack of suppression of activity, while a response of <2.4 was considered to indicate a suppressive compound.

The effect of rat CSF after quinine stimulus on TBF

TBF was suppressed by control CSF, which contained various biologically active substances, so CSF samples were diluted with 0.2% PRIONEX (Merck, Darmstat, Germany) in PIPES solution until no suppressive effect was observed. The suppressive activity of a sample was expressed as the maximum dilution at which TBF was still suppressed.

An equal mixture of quinine CSF and control CSF was subjected to the hydra behavioral test. When TBF was suppressed by the original sample, it was diluted with 0.2% PRIONEX until the effect disappeared.

Pronase treatment of CSF and molecular size fractionation

Quinine CSF (50 µl) or control CSF (50 µl) was incubated with 100 µg of pronase (Calbiochem Novabiochem Corp, La Jolla, CA) in 0.1 M phosphate-buffered saline in a final volume of 100 µl at 37°C for 3 h. Control reaction mixtures were incubated without pronase. To stop the reaction, 0.1 N HCl (80 µl) was added. These samples (1 µl) were subjected to the hydra assay and TBF was examined at 3 µM GSM. Quinine CSF was fractionated into low and high molecular weight fractions by ultrafiltration membranes, with cut-off molecular weights of 3000, 10 000 and 30 000 (Microcon 3, 10 and 30, respectively; Millipore Corp., Bedford, MA). All size fractions were subjected to the hydra behavioral test.

Statistics

All statistical tests were done using StatView (SAS Institute Inc., Cary, NC). The data were analyzed by one-way

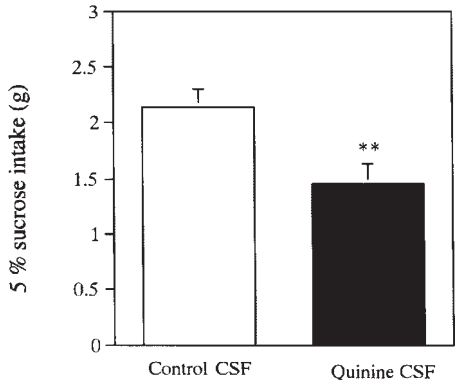

Figure 1 Effect of injection of rat CSF on intake of 5% sucrose in mice. Control CSF or quinine CSF (2 μl) was injected into the fourth ventricle of mice and sucrose intake was examined for 30 min after the injection. Quinine CSF significantly decreased the intake of sucrose solution (**P < 0.05; paired t-test). Values are means ± SEM (n = 16).

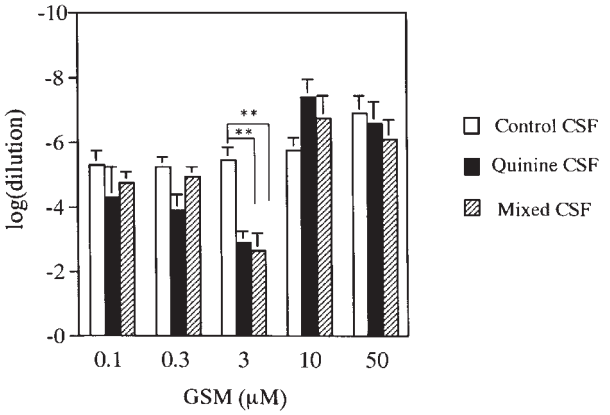

Figure 2 Suppression of TBF by control CSF, quinine CSF and a mixture of control CSF and quinine CSF. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. The upper bar indicates stronger suppression of the hydra behavioral response. Values are means ± SEM (n = 7). The data were analyzed by ANOVA and the *post hoc* Tukey test. **P < 0.01.

ANOVA followed by the *post hoc* Tukey test. Comparison between two groups was done using Student's t-test.

Results

Sucrose intake by mice injected with CSF

Sucrose intake by mice injected with quinine CSF was significantly suppressed compared with that by mice injected with control CSF (Figure 1). When control CSF was used instead of quinine CSF for the second injection, the intake of sucrose solution was not significantly different from that after the first control CSF injection. This implies that a substance in the quinine CSF suppressed sucrose intake and that this substance also participated in the suppression of intake by quinine.

Effect of quinine CSF on TBF

The suppressive effect of quinine CSF on TBF was weaker than that of control CSF at 3 μM GSM [F(2,18) = 12.146; P = 0.0005] (Figure 2). TBF is modulated independently by many peptides (Hanai *et al.*, 1987, 1989; Manabe *et al.* 2000) and monoclonal antibodies (Sakaguchi *et al.*, 1991) at different GSM concentrations. We then analyzed TBF data at each GSM concentration by one-way ANOVA followed by multiple comparisons. The effects of the mixture of quinine CSF and control CSF on TBF were similar to that of quinine CSF (Figure 2). These results suggested that there was a substance in quinine CSF that specifically nullified the suppressive activity in response to GSM, especially at 3 μM.

Effect of CSF pretreated with pronase and molecular size fractionation

The nullifying effect of quinine CSF on TBF at 3 μM GSM disappeared when the quinine CSF was pretreated with pronase [F(3,16) = 29.397; P = 0.0001] (Figure 3), suggesting that the substance responsible for the nullifying effect is a peptide. Size-fractionation of quinine CSF revealed

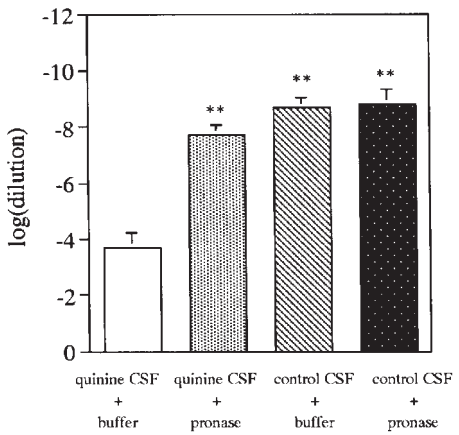

Figure 3 Suppression of TBF response after treatment with pronase. After treatment of quinine CSF with pronase, suppressive activity was increased, whereas treatment of control CSF did not change the suppressive activity. Suppression of the TBF response was examined after stimulation with 3 μM GSM. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. 0.1 M PBS was used as the buffer. Values are means ± SEM (n = 5). The data were analyzed by ANOVA and the *post hoc* Tukey test. **P < 0.01.

that the nullifying activity was present larger than 3 kDa (Figure 4A) but smaller than 30 kDa (Figure 4C). Similar amounts of activity were found in the fractions above and below 10 kDa (Figure 4B). Thus, the substance in quinine CSF responsible for the nullification appeared to be a peptide of molecular weight ~10 kDa.

We have previously examined the effect of a number of peptides, including CGRP, on TBF (Hanai *et al.*, 1989; Manabe *et al.*, 2000), but no peptide showed the same effect as quinine CSF. Therefore, in the next study, we focused on the possible contribution of the benzodiazepine receptor and its endogenous ligand to the effects of the substance in

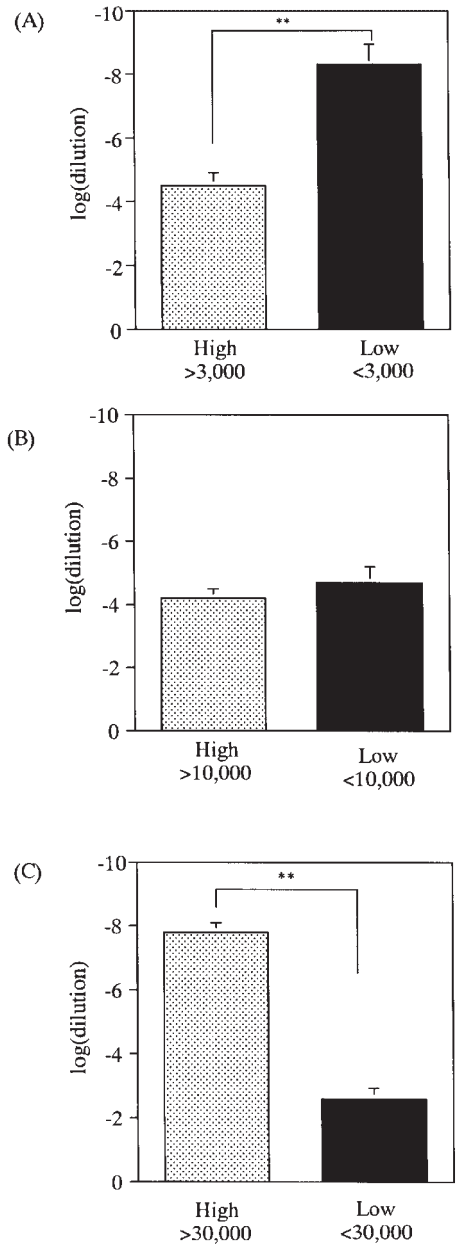

Figure 4 Effect of size-fractionation of quinine CSF on TBF. **(A)** Mol. wt 3000 fractionation. **(B)** Mol. wt 10 000 fractionation. **(C)** Mol. wt. 30 000 fractionation. Suppression of the TBF response stimulated with 3 μM GSM was examined. The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. Values are means ± SEM. The data were analyzed using the unpaired *t*-test. $^{**}P < 0.01$.

quinine CSF, since benzodiazepines are known to be involved in the palatability of foods (Cooper, 1982; Berridge and Pecina, 1995; Gray and Cooper, 1995).

Experiment 2

Benzodiazepines have been reported to be related to an increase in the intake of palatable foods (Cooper, 1982; Berridge and Pecina, 1995; Gray and Cooper, 1995), although their effects are only exogenous. The benzodiazepine receptor is widely distributed in the brain, but an endogenous benzodiazepine-like ligand is unknown. Diazepam-binding inhibitor (DBI) is the only known endogenous ligand for this receptor, which is a 10 kDa neuropeptide present in the brain of rats and humans (Guidotti *et al.*, 1983; Shoyab *et al.*, 1986). It binds to the benzodiazepine-binding site on the $GABA_A$ receptor complex (Bormann, 1991; Guidotti, 1991) and acts as an inverse agonist, eliciting effects opposite to those of benzodiazepines (Guidotti *et al.*, 1983; Ferrero *et al.*, 1986; Costa, 1991). Injection of DBI into the brain reportedly produced anxiogenic effects in the conflict test (Guidotti, 1991). Further, increases in DBI-like immunoreactive compounds have been reported in depressive patients (Ferrero *et al.*, 1988; Roy, 1991) and in alcohol-dependent rats (Adinoff *et al.*, 1996; Katsura *et al.*, 1998). Katsura *et al.* also reported that expression of DBI mRNA was elevated in the brains of ethanol-withdrawn mice after chronic treatment with ethanol (Katsura *et al.*, 1995). Taken together, these results indicate that DBI might be related to an aversive sensation as a biologically active molecule. Since injection of benzodiazepine agonists into the brain enhances palatability, DBI, a benzodiazepine inverse agonist, could be involved in aversive taste. Therefore, we examined the effects of DBI on TBF and whether it could be the peptide suppressor found in experiment 1.

Materials and methods

Methods of CSF sampling and injection into mice were as for experiment 1. Hydra culture and the behavioral test were also as described for experiment 1.

Effect of DBI on TBF

We examined the effect of a DBI peptide fragment (Bachem AG, Bubendorf, Switzerland) on TBF in the presence of 1 fg of the DBI fragment and 1 μl of control CSF. The DBI peptide fragment, which is composed of 20 amino acid residues, from Gln51 to Lys70 of the human DBI sequence, has been reported to be biologically active (Dong *et al.*, 1999). A stock solution of the peptide was prepared in distilled water, and added to the TBF assay system after dilution with 0.2% PRIONEX. Control CSF, which contained various biologically active peptides, strongly suppressed TBF induced by GSM at all concentrations examined (Hanai *et al.*, 1989; Inoue *et al.*, 1999). The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

Effect of benzodiazepine receptor preparation

Benzodiazepine receptor preparation [Research Biochemicals, Inc. (RBI), Natick, MA] was dissolved at a concentration of 50 mg/ml in 0.25 M NaH_2PO_4 buffer (pH 7.4) (Imaizumi *et al.*, 1994). Quinine CSF (5 μl) or the control CSF (5 μl) and the receptor preparation (20 μl) were mixed and incubated for 1 h on ice. Control reaction mixtures were incubated without receptor preparation. The mixture was filtered through an ultrafiltration membrane (Microcon 30)

and subjected to the TBF assay at 3 μM GSM. The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

N-Methyl-β-carboline-3-carboximide (FG7142) (RBI), which binds to the benzodiazepine receptor (Cooper, 1986; Cooper *et al.*, 1988), was used to examine the specificity of binding of the substance in quinine CSF to the receptor preparation. FG7142 solution (2 mg/ml, 10 μl) and the benzodiazepine receptor preparation (20 μl) were mixed and incubated for 15 min on ice. Then, quinine CSF (5 μl) was added and incubated for another 30 min on ice. The resultant mixture (1 μl) was subjected to the hydra behavioral test and observed at 3 μM GSM. The sample was diluted with 0.2% PRIONEX until the suppressive effect disappeared.

Flumazenil pretreatment

The selective benzodiazepine receptor antagonist, flumazenil (a gift from Yamanouchi Pharmaceutical Co. Ltd, Tokyo, Japan), was suspended in physiological saline containing 0.5 % carboxymethyl cellulose (CMC saline). Flumazenil (20 mg/kg) was administered intraperitoneally (i.p.) 20 min before CSF injection and CMC saline was used as control.

Mice were treated and trained as in experiment 1. On the first day of injection, CMC saline was administered i.p. 20 min before control CSF (2 μl) was injected through the cannula. The sucrose intake was determined 30 min after the injection of CSF, and then mice were divided into two groups, the CMC saline–quinine CSF group and the flumazenil–quinine CSF group. Two days after the first injection, CMC saline or flumazenil was administered i.p. 20 min before quinine CSF (2 μl) was injected into the fourth ventricle through the cannula and the intake of 5% sucrose was determined.

Statistics

Statistical analysis was done as described in experiment 1.

Results

Effect of DBI on TBF

The DBI fragment itself did not suppress TBF (data not shown). To examine the ability of DBI to nullify the suppressive effect of quinine CSF, we examined the activity of a mixture of the DBI fragment and control CSF. Suppression of TBF at 3 μM GSM by CSF was greatly reduced by the mixture of DBI and control CSF (Figure 5), which was similar to that by quinine CSF.

Effect of treating quinine CSF with the benzodiazepine receptor preparation

CSF was incubated with the benzodiazepine receptor preparation, which bound DBI. The mixture of quinine CSF and the receptor preparation suppressed TBF at 3 μM GSM to a similar extent as control CSF [$F(3,20) = 11.033$; $P = 0.0002$] (Figure 6), indicating that the nullifying effect of the original quinine CSF had disappeared. When the benzodiazepine receptor preparation was preincubated with

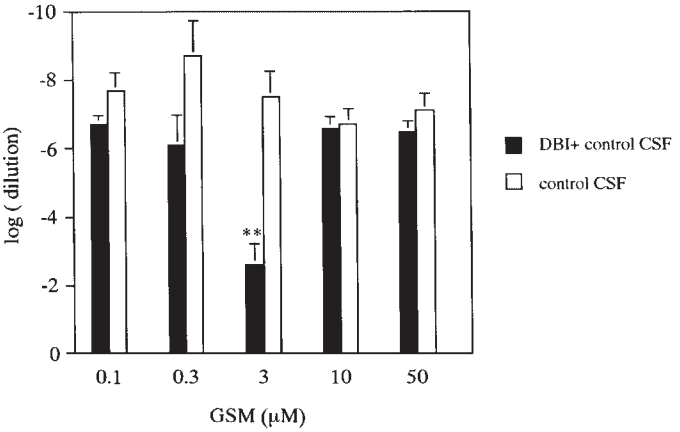

Figure 5 Suppression of GSM response by control CSF and by a mixture of control CSF and DBI (1 fg). The vertical axis is the logarithm of the maximum dilution at which suppression of the TBF response was observed. Values are means ± SEM ($n = 5$). The data were analyzed using the unpaired *t*-test $^{**}P < 0.01$.

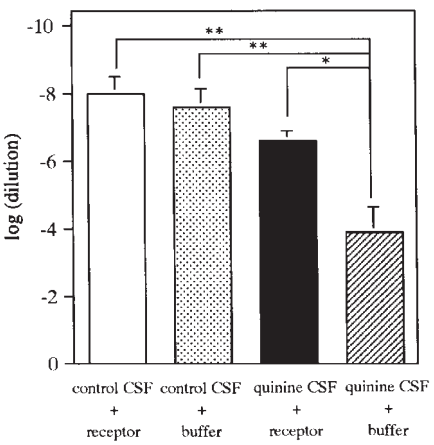

Figure 6 Suppression of TBF response after treatment with the benzodiazepine receptor preparation. Treatment of quinine CSF with the benzodiazepine receptor preparation increased the suppressive activity, whereas the same treatment of control CSF did not change the suppressive activity. Suppression of the TBF response was examined after stimulation with 3 μM GSM. The vertical axis is the maximum dilution. Values are means ± SEM ($n = 6$). The data were analyzed by ANOVA and the *post hoc* Tukey test. $^{*}P < 0.05$; $^{**}P < 0.01$.

FG7142, the nullifying effect did not disappear [$F(2,12) = 12.118$; $P = 0.013$] (Figure 7). These results indicate that the nullifying activity in the quinine CSF was removed by the specific binding of the active substance to the benzodiazepine receptor in a manner similar to that of DBI.

Flumazenil antagonizes the effect of quinine CSF on sucrose intake

We confirmed that quinine CSF suppressed the intake of 5% sucrose intake in mice pretreated with CMC saline for 30 min after the injection ($F(2,41) = 4.786$; $P = 0.01368$) (Figure 8). Pretreatment with flumazenil, a benzodiazepine

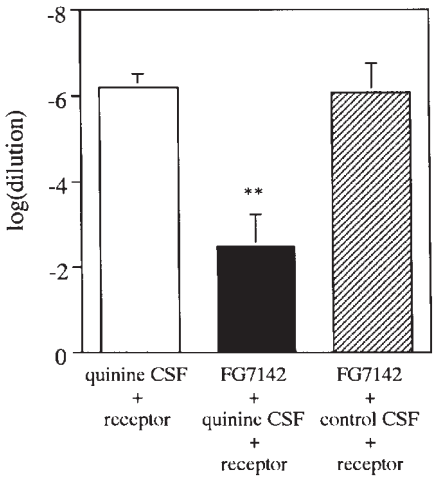

Figure 7 Effect on TBF response of the mixture of quinine CSF and the benzodiazepine receptor preparation after pretreatment with FG7142. After pretreatment of quinine CSF with FG7142, the nullifying effect of benzodiazepine receptor preparation on quinine CSF activity disappeared. FG7142 did not change the suppressive activity of the mixture of control CSF and the receptor preparation. Suppression of the TBF response was examined after stimulation with 3 μM GSM. The vertical axis is the maximum dilution. Values are means ± SEM ($n = 5$). The data were analyzed by ANOVA and the *post hoc* Tukey test. $^{**}P < 0.01$.

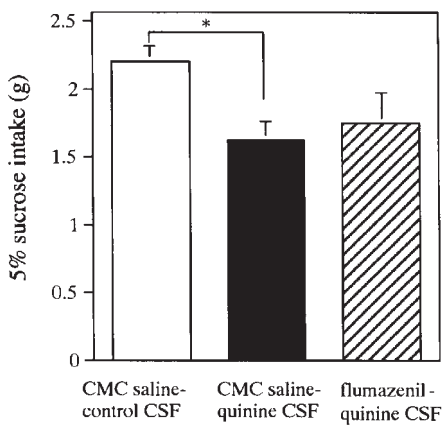

Figure 8 The effect of pretreatment with flumazenil (20 mg/kg, i.p.) on the intake of 5% sucrose solution. Flumazenil or CMC saline was given 20 min before injection of CSF. Sucrose intake for 30 min after the CSF injection was examined. Data are means ± SEM (CMC saline + control CSF, $n = 22$; CMC saline + quinine CSF, $n = 11$; flumazenil + quinine CSF, $n = 11$). The data were analyzed by ANOVA and the *post hoc* Tukey test. $^{*}P < 0.05$.

receptor antagonist, reduced the suppressive effect of quinine CSF on sucrose intake so that it was no longer statistically significant (Figure 8).

Discussion

Sucrose intake was significantly suppressed in mice injected with quinine CSF, indicating the presence of an active substance in quinine CSF. The hydra behavioral test detected a change in suppression of TBF by CSF that was accompanied by aversive quinine taste stimuli. Control CSF was suppressive at all concentrations of GSM, while quinine CSF was not as suppressive at 3 μM GSM. TBF was not suppressed by a mixture of quinine CSF and control CSF, suggesting that a substance in quinine CSF nullified the suppression of TBF caused by various substances in control CSF at 3 μM GSM. By means of pronase treatment and size-fractionation of quinine CSF, the nullifying effect of the suppressive activity in quinine CSF disappeared, suggesting that a proteinaceous substance of ~10 kDa was responsible for this effect.

We examined the effect of a number of peptides on TBF. No peptides examined previously, other than the DBI fragment, selectively nullified suppression of the response at 3 μM GSM (Hanai *et al.*, 1989; Manabe *et al.*, 2000). In this study, we focused on DBI, an inverse agonist of the benzodiazepine receptor, which elicits effects opposite to those of benzodiazepines (Guidotti *et al.*, 1983; Ferrero *et al.*, 1986; Costa, 1991). DBI is the only known endogenous ligand of the benzodiazepine receptor. No relationship between DBI and feeding has yet been reported, while benzodiazepines are reported to be involved in palatability (Cooper, 1982; Berridge and Pecina, 1995; Gray and Cooper, 1995). The results of experiment 2 suggest that the nullifying effect on TBF at 3 μM GSM in the hydra behavioral test is mediated principally by an increase in a DBI-like activity in the CSF. The effect of a mixture of the DBI peptide fragment and control CSF on TBF in the present study resembled that of quinine CSF. Quinine CSF treated with the benzodiazepine receptor preparation gave TBF similar to that induced by control CSF, indicating the presence of DBI-like activity in the quinine CSF. Further, the suppression of sucrose intake by quinine CSF was reduced by pretreatment with flumazenil. Our results suggest that a DBI-like peptide is released by aversive quinine stimuli, and that it acts via benzodiazepine receptors.

Flumazenil did not completely antagonize the suppressive effects of quinine CSF on sucrose intake. It is likely that factors other than DBI are involved in the suppression of sucrose intake by quinine CSF. The DBI fragment alone only suppressed sucrose intake when injected at a high concentration (unpublished observation).

In this study, DBI-like activity increases in CSF after rats are given aversive quinine taste stimuli. The meaning of this increase is not clear, but higher CSF levels of DBI may be related to the feeling of disgust after intake of disliked foods. Further studies are necessary to determine if DBI is specific to this feeling of disgust.

In conclusion, we suggest that the aversive sensation experienced after quinine stimulation could be transmitted by a peptide (experiment 1), which may be a DBI-like molecule that binds benzodiazepine receptors (experiment 2).

Acknowledgements

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