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RESEARCH PAPER

PEGylated cholecystokinin is more potent in inducing anorexia than conditioned taste aversion in rats

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Background and purpose: The physiological involvement of endogenous cholecystokinin (CCK) in the termination of feeding has been challenged by evidence of aversive effects of exogenous CCK_8. We previously prolonged the anorectic effect of CCK by conjugation to polyethylene glycol (PEGylation) to produce PEG-CCK_9. In this study, we investigated the ability of different doses of PEG-CCK_9 to induce conditioned taste aversion (CTA) and satiety and identified the receptors involved in CTA induction.
Experimental approach: Induction of CTA, measured by the saccharin preference ratio determined in a two-bottle CTA procedure, and of satiety in adult male Wistar rats after intraperitoneal (i.p.) injection of different doses of PEG-CCK_9 (1, 2, 4, 8, 16 or 32 $\mu g\,kg^{-1}$) was compared. Devazepide (100 $\mu g\,kg^{-1}$) and 2-NAP (3 $mg\,kg^{-1}$), two selective CCK_1-receptor antagonists, were co-administered i.p. with PEG-CCK_9 (8 $\mu g\,kg^{-1}$) and the CTA effects monitored.
Key results: PEG-CCK_9 dose-dependently induced CTA, with a minimal effective dose of 8 $\mu g\,kg^{-1}$, whereas the minimal effective dose to induce satiety was 1 $\mu g\,kg^{-1}$. The CTA effects of PEG-CCK_9 were completely abolished by i.p. administration of devazepide prior to PEG-CCK_9 treatment and only partially abolished by administration of 2-NAP.
Conclusions and implications: Although PEG-CCK_9-induced satiety and PEG-CCK_9-induced CTA both increased with dose, the conjugate was more potent in inducing satiety, suggesting that the anorexia could not be completely attributed to the aversiveness of the drug. As observed with induction of satiety, PEG-CCK_9-induced CTA was mediated by CCK_1-receptors.
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Keywords: cholecystokinin; PEGylated cholecystokinin; conditioned taste aversion; satiety; malaise; 2-NAP; devazepide

Abbreviations: CCK_9, cholecystokinin$_9$; 2-NAP, 2-naphthalenesulphonyl 1-aspartyl-(2-phenethyl)amide; PEG, polyethylene glycol; PEG-CCK_9, PEGylated cholecystokinin nonapeptide

Introduction

Gibbs *et al*. (1973) identified the active form of cholecystokinin$_{26–33}$ (CCK_8) and showed that exogenous CCK_8 dose-dependently reduced food intake, but not water intake. These results led to the hypothesis that endogenous CCK, released in the small intestine during a meal, caused satiety through normal physiological processes. However, the satiety role of exogenous CCK was challenged by the demonstration of marked conditioned taste aversion (CTA) after CCK administration, which suggested that CCK inhibited food intake by inducing non-specific aversiveness, rather than satiety (Deutsch and Hardy, 1977). CTA is a well-established paradigm in which animals or humans learn to associate a novel taste (conditioned stimulus) with nausea, discomfort or malaise (unconditioned stimulus) and, as a consequence, avoid drinking fluid with this specific taste (Welzl *et al*., 2001). In rats, dose–response studies and comparison with known aversive substances, such as lithium chloride (LiCl), showed that CCK can induce dose-related anorexia at doses that cause no taste aversion and that, even at high concentrations, CCK-induced taste aversion is mild (Ervin and Teeter, 1986; Perez and Sclafani, 1991; Ervin *et al*., 1995a; Mosher *et al*., 1998). The short plasma half-life of CCK was suggested to be the reason for this mild induction of CTA (Ervin *et al*., 1995b).

We previously circumvented the difficulties of this short plasma half-life of CCK by conjugating the peptide to polyethylene glycol (PEGylation). We covalently coupled CCK to a mixture of polyethylene glycol polymers (MW of

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the PEG fraction 5–30 kDa) and showed that intraperitoneal (i.p.) bolus injection of this conjugate, PEG-CCK$_9$, into rats resulted in a prolonged anorectic effect compared with injection of the unmodified molecule, with the 10 kDa PEG-CCK$_9$ being the most active (Leon-Tamariz *et al.*, 2007). The prolonged anorectic effect resulting from PEGylation of the peptide was confirmed in a study which demonstrated that PEG-CCK$_9$ showed a clear dose–response effect on both the duration and initial intensity of anorexia and that this was owing to stimulation of peripheral CCK$_1$-receptors (Verbaeys *et al.*, 2007).

The purpose of the present study was to determine whether PEG-CCK$_9$ would induce CTA in rats. In the first experiment, the CTA-inducing effect of different doses of PEG-CCK$_9$ (1, 2, 4, 8, 16 or 32 $\mu g\,kg^{-1}$; i.p.) was examined in rats using a two-bottle CTA procedure. The PEG-CCK$_9$-induced CTA effects were then compared with the food intake reduction caused by the same doses of PEG-CCK$_9$ to investigate the anorexia/taste aversion correlation. Finally, the effects of devazepide and 2-NAP, two selective CCK$_1$-receptor antagonists with different blood–brain barrier (BBB) penetration capabilities, on the CTA response induced by 10 kDa PEG-CCK$_9$ were studied to identify the receptors involved in induction of aversion.

Methods

Animals

This research protocol was approved by the Ethical Commission for Experimental Use of Animals of the Katholieke Universiteit Leuven. Male Wistar rats (Janvier, Le Genest Saint Isle, France), with body weights between 150–174 g on arrival at the laboratory, were housed individually in iron wire cages (dimensions: 20–20–58 cm) under standardized conditions (room temperature of 21 ± 0.2 °C, 40–60% relative humidity, 12:12 h light–dark cycle with lights on at 0900 h and lights off at 2100 h), following European guidelines on animal care. In the conditioned taste aversion procedure, complete rodent food, presented as pellets (Sniff, Bioservices, Schaijk, The Netherlands), was provided *ad libitum* during the entire experimental period. Identical rodent food, but in a powdered form was presented in experiment 2. The animals were allowed to adapt to laboratory conditions, handling and i.p. injections for 2 weeks prior to the experimental sessions.

Conditioned taste aversion procedure

In a subsequent 7-day adaptation period, the rats were accustomed to water access for only 1 h a day (1000–1100 h), during which the animals learned to drink a reasonable amount of water in a short period of time, thus establishing a relatively stable baseline of water intake per day. To determine whether PEG-CCK$_9$ induced CTA, two consecutive conditioning weeks were needed; a control conditioning week and a drug conditioning week, each conditioning week consisting of a conditioning day, a washout day, a conditioning day, a washout day, a test day and 2 washout days. On washout days the rats received no treatment and were able to drink water from 1000 to 1100 h. On the conditioning days, the rats received a bottle filled with 0.1% $w\,v^{-1}$ saccharin solution from 1000 to 1100 h, after which they were immediately injected i.p. with the control compound mPEG-OH during the control conditioning week or the test drug during the drug conditioning week (the drugs are specified in experiments 1, 3 and 4). After the second of the single washout days in both conditioning weeks, the rats were subjected to a two-bottle test with water and saccharin for 1 h, and, by measuring the amount of fluid consumed from each bottle separately, the individual control- or drug-induced saccharin preference ratio could be determined. The saccharin preference ratio was the intake of saccharin divided by the total intake (saccharin plus water) multiplied by 100. To control for location bias in the two-bottle test, half of the animals received saccharin in the left bottle and the other half in the right bottle. Drug-induced CTA was calculated for each rat by comparison of its drug-induced saccharin preference ratio determined in the drug conditioning week to its corresponding control saccharin preference ratio determined in the preceding control conditioning week.

Experiment 1: dose-related CTA caused by PEGylated CCK$_9$. To assess the dose-related relative aversiveness of PEG-CCK$_9$, the intensity of the CTA induced by different doses of PEG-CCK$_9$ was determined using the above-described CTA procedure. Forty-five rats were divided into seven treatment groups. In the drug conditioning period, each group received an i.p. injection of either 1, 2, 4, 8, 16 or 32 $\mu g\,kg^{-1}$ of PEG-CCK$_9$ dissolved in 0.5 mL of saline ($n = 6$) or 170.5 $\mu g\,kg^{-1}$ of mPEG-OH (control injection) dissolved in 0.5 mL of saline ($n = 9$). The amount of mPEG-OH given in the control injection was the same as the amount of PEG injected at the highest dose of PEG-CCK$_9$.

Experiment 2: relationship between PEG-CCK$_9$-induced food intake reduction and the corresponding CTA. Fifty rats not previously used in a CTA procedure were divided into five groups of 10, after their adaptation period to experimental conditions. The experimental conditions were the same as those described above except for the light–dark cycle, which was reversed (lights off at 1100 h and lights on at 2300 h), and the start of food intake was synchronized by 1 h fasting before lights off. Water and complete powdered rodent food (Sniff, Bioservices, Schaijk, The Netherlands) were provided *ad libitum*. Food intake was measured by weighing the mangers, which are specially designed to avoid spillage (Scholz, Overijse, Belgium). On the first test day, all five groups were injected i.p. with 0.5 mL of 85.25 $\mu g\,kg^{-1}$ of mPEG-OH at 1045 h, 15 min before the start of food intake measurement. To equate groups, treatment groups were made based on the control food intake. At 1045 h the next day, all treatment groups received an i.p. injection of different doses of PEG-CCK$_9$ (1, 2, 4, 8 or 16 $\mu g\,kg^{-1}$). On both days, food intake was measured at time zero (1100 h), after 30 min and after 6 h. The PEG-CCK$_9$-induced food intake reduction was calculated by comparing food intake after injection of a given dose of PEG-CCK$_9$ to the corresponding control food intake measured the day before. To determine the correlation between PEG-CCK$_9$-induced food intake reduction and CTA, the dose-related effects of PEG-CCK$_9$ on CTA (experiment 1) and on food intake

(experiment 2) were presented as a percentage of the control value on the same graph and approximated by linear regression. The food intake data measured after 30 min were used for this comparison. The food intake measured after 6 h only served to ascertain that our current results were in accordance to the earlier published dose–response data (Verbaeys *et al*., 2007) and these data were therefore not used for further analysis.

The amount of mPEG-OH given in the control injection in experiments 2 and 3 was the same as the amount of PEG injected at the different doses of PEG-CCK$_9$.

Experiment 3: effect of devazepide on PEG-CCK$_9$-induced CTA. To test whether PEGylated CCK$_9$-induced CTA involved activation of CCK$_1$-receptors, devazepide, a specific CCK$_1$-receptor antagonist able to cross the BBB, was co-administered with PEGylated CCK$_9$ in the above-mentioned CTA procedure. Thirty-two rats were divided into four groups of eight. Immediately after saccharin presentation, the four groups received an i.p. injection of, respectively, mPEG-OH (42.6 $\mu g\,kg^{-1}$), devazepide (100 $\mu g\,kg^{-1}$), PEG-CCK$_9$ (8 $\mu g\,kg^{-1}$) or devazepide (100 $\mu g\,kg^{-1}$), followed immediately by i.p. injection of PEG-CCK$_9$ (8 $\mu g\,kg^{-1}$). The dose of 8 $\mu g\,kg^{-1}$ of PEG-CCK$_9$ was used, as experiment 1 demonstrated a significant CTA at this dose. No second control injection was given to the groups receiving only one substance as we are convinced that in this protocol (and in the similar protocol of experiment 4) the stress of the manipulation with two injections is equal to the stress caused by the manipulation with only one injection and that this has no effect at all on the data itself.

Experiment 4: effect of 2-NAP on PEG-CCK$_9$-induced CTA. To assess whether central and/or peripheral CCK$_1$-receptors were involved in the PEG-CCK$_9$-induced CTA, the effect of 2-NAP on the PEGylated CCK$_9$-induced CTA was investigated using the above-mentioned CTA protocol. In contrast to devazepide, 2-NAP, which is also a specific CCK$_1$-receptor antagonist, does not cross the BBB (Hull *et al*., 1993; Baldwin *et al*., 1994, 1998). The four treatment groups ($n=8$) received an i.p. injection of, respectively, mPEG-OH (42.6 $\mu g\,kg^{-1}$), 2-NAP (3 $mg\,kg^{-1}$), PEG-CCK$_9$ (8 $\mu g\,kg^{-1}$) or 2-NAP (3 $mg\,kg^{-1}$), followed immediately by i.p. injection of PEG-CCK$_9$ (8 $\mu g\,kg^{-1}$). Our recent results showed that a dose of 8 $\mu g\,kg^{-1}$ of PEG-CCK$_9$ results in food intake inhibition lasting 6 h owing to activation of peripheral CCK$_1$-receptors and that the inhibitory effect of 2-NAP (3 $mg\,kg^{-1}$) on the CCK$_9$-induced anorexia is halved within 2 h (Verbaeys *et al*., 2007). The i.p. injection of 2-NAP (3 $mg\,kg^{-1}$) was therefore repeated every 2 h for 6 h, and the two other groups received an i.p. injection of saline to create the same injection-induced stress.

Data analysis and statistical procedures

The food intake data and the saccharin preference ratio of each rat relative to the control preference ratio of the same rat were analysed by one-way ANOVA followed by a *post hoc* Tukey test. A *P*-value <0.05 was considered statistically significant.

The saccharin preference ratio for each PEG-CCK$_9$ dose was log-transformed and fitted to a 4 parameter dose–response model with a constraint (bottom = 0), giving the half-maximal effective concentration (EC_{50}), that is, the dose of PEG-CCK$_9$ at which 50% of its maximal CTA effect is observed. The lowest dose resulting in a statistically significant reduction in the control saccharin preference ratio using ANOVA and Dunnett's multiple comparison test was considered to be the minimal effective dose (MED).

A linear regression model was used to determine the correlation between dose and PEG-CCK$_9$-induced food intake reduction or CTA. If the slopes were not significantly different, the food intake reduction and CTA induced by the same doses of PEG-CCK$_9$ were considered functionally related. The MED for the food intake dose–response curve was determined in the same way as that for the CTA dose–response curve and both were indicated on the same graph.

All the statistical procedures were carried out using GraphPad Prism (version 4, San Diego, California, USA).

Drugs, chemical reagents and other materials

The 10 kDa PEG-CCK$_9$ conjugate was prepared as described by Leon-Tamariz *et al*. (2007). The PEG-CCK$_9$ conjugate was quantified by analytical HPLC, calibrated using pure CCK$_9$ (Bachem, Bubendorf, Switzerland). The amounts of PEG-CCK$_9$ ($\mu g\,kg^{-1}$) mentioned in the experiments indicate the amount of peptide present in the conjugate. The 10 kDa non-active linear methoxy polyethylene glycol (mPEG-OH or CH3-(OCH2CH2)n-OH), used for control injections, was purchased from Nektar Therapeutics (Huntsville, AL, USA). The PEG-CCK$_9$ conjugate consisted of 84.2% PEG and 15.8% CCK$_9$ by weight. Devazepide, kindly provided by Merck, Sharp and Dohme (New Jersey, USA), was dissolved as a 1 $mg\,mL^{-1}$ stock solution in a 7:3 ($v\,v^{-1}$) mixture of PEG 400 and glycerine. The stock solution was stored at 4 °C and was diluted in 0.5 mL of saline prior to injection. 2-NAP, kindly provided by the James Black Foundation (London, UK), was dissolved directly in saline. Receptor nomenclature conforms to that recommended by Alexander *et al*. (2008).

Results

Experiment 1: dose-related CTA caused by PEGylated CCK$_9$

Administration of PEG-CCK$_9$ produced dose-dependent CTA (Figure 1a). The saccharin preference ratio was reduced with increasing doses of PEG-CCK$_9$. The MED was 8 $\mu g\,kg^{-1}$ ($P<0.05$). The dose–response curve is shown in Figure 1b. The fitted dose–response relationship revealed an EC_{50} of 14.3 $\mu g\,kg^{-1}$ with a 95% confidence interval of 8.9–22.9.

Experiment 2: relationship between PEG-CCK$_9$-induced food intake reduction and the corresponding CTA

Linear regression analysis (Figure 2) revealed that the slopes of the food intake dose–response curve and the CTA dose–response curve were not significantly different ($P>0.05$), but the MED of PEG-CCK$_9$ able to significantly reduce food intake after 30 min was 1 $\mu g\,kg^{-1}$, whereas that to induce significant CTA was 8 $\mu g\,kg^{-1}$ PEG-CCK$_9$.

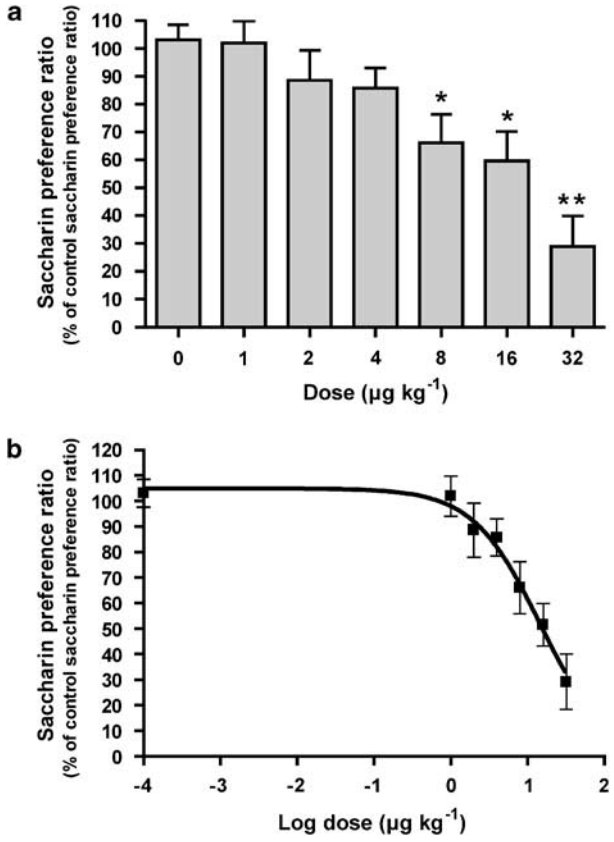

Figure 1 Dose-dependency of conditioned taste aversion (CTA) effects in rats treated with PEG-CCK$_9$ (1, 2, 4, 8, 16 and 32 µg kg^{-1}), determined using a two-bottle test procedure. Graph (**a**) shows the saccharin preference ratios of the PEG-CCK$_9$-treated rats, presented as a percentage of their own control saccharin preference ratio (mean + s.e.mean; $n=6$). ANOVA analysis ($F=9,136$, d.f. = (6, 38)) with the *post hoc* Tukey test revealed significant different results compared with controls (dose 0 µg kg^{-1}). *$P<0.05$; **$P<0.001$. Graph (**b**) depicts the PEG-CCK$_9$-induced saccharin preference ratio data presented as a percentage of their own control saccharin preference ratio, log-transformed and fitted to a standard dose–response model with constraint; bottom = 0 (mean ± s.e.mean; $n=6$). The fitted dose–response relationship revealed an EC_{50} of 14.3 µg kg^{-1} with a 95% confidence interval of 8.9–22.9.

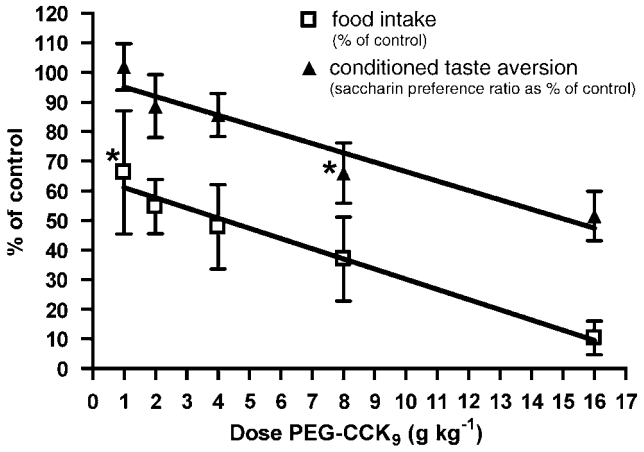

Figure 2 Relationship between PEG-CCK$_9$-induced food intake reduction and the corresponding conditioned taste aversion (CTA). The dose-related effects of PEG-CCK$_9$ (1, 2, 4, 8 and 16 µg kg^{-1}) on food intake and on CTA, both presented as a percentage of control (mean ± s.e.mean; food intake data: $n=10$; CTA data: $n=6$), were plotted on the same graph and approximated with linear regression to determine the relationship between the two behavioural measures. The 95% confidence intervals of the two regression slopes overlap (slope 95% confidence interval for the food intake dose–response curve: −5.7 to −1.2; slope 95% confidence interval for the CTA dose–response curve: −4.7 to −1.6), indicating that the two functions are parallel and thus the food intake reduction and CTA induced by the same doses of PEG-CCK$_9$ are functionally related. ANOVA analysis of the food intake data ($F=13,75$, d.f. = (5, 124)) and the CTA-data ($F=9,136$, d.f. = (6, 38)) with the *post hoc* Tukey test revealed significant different results compared with controls (dose 0 µg kg^{-1}). The minimal effective dose (MED) for the two parameters is indicated by an asterisk.

Experiment 3: effect of devazepide on PEG-CCK$_9$-induced CTA

Figure 3a shows the effect of devazepide (100 µg kg^{-1}) on PEG-CCK$_9$-induced CTA. A statistically significant difference in saccharin preference ratio was observed between the treatment groups ($P<0.0001$). The saccharin preference ratio of PEG-CCK$_9$-treated rats (8 µg kg^{-1}) was significantly reduced compared with the control saccharin preference ratio, indicating the presence of a marked CTA. Administration of devazepide alone did not affect the control saccharin preference ratio, whereas devazepide pretreatment of PEG-CCK$_9$-treated rats completely antagonized the PEG-CCK$_9$-induced CTA, as the saccharin preference ratio of these rats was not significantly different from the control value.

Experiment 4: effect of 2-NAP on PEG-CCK$_9$-induced CTA

Figure 3b shows the effect of 2-NAP (3 mg kg^{-1}) on PEG-CCK$_9$-induced CTA. Statistical analysis of the data showed significantly different saccharin preference ratios for the different treatments ($P=0.0008$). The PEG-CCK$_9$-treated rats (8 µg kg^{-1}) displayed a significantly lower saccharin preference ratio than the control animals, revealing a PEG-CCK$_9$-induced CTA. Repeated injections of 2-NAP alone (3 mg kg^{-1} every 2 h for 6 h) did not change the saccharin preference ratio compared with control-treated rats, but partially abolished the CTA effect of PEG-CCK$_9$, as the saccharin preference ratio of the co-administration group was not significantly different from the ratio for the control group or the PEG-CCK$_9$-treated group.

Discussion

The present study demonstrated that i.p. injection of rats with PEGylated CCK$_9$ (PEG-CCK$_9$) dose-dependently induced CTA in a two-bottle testing procedure. A dose of 8 µg kg^{-1} of PEG-CCK$_9$ was the minimal effective dose, significantly reducing the saccharine preference ratio to 66% of control. Taste aversion caused by unmodified CCK$_8$ is also dose-dependent, and it has been generally accepted that doses lower than 8 µg kg^{-1} of CCK$_8$, given i.p., do not produce learned taste aversion in rats and are therefore not aversive (Ervin and Teeter, 1986). The CTA effects after i.p. administration of high doses of CCK$_8$ (>8 µg kg^{-1}) are mild in comparison to the CTA effects induced by known aversive agents, such as LiCl (Ervin *et al*., 1995a; Mosher *et al*., 1998).

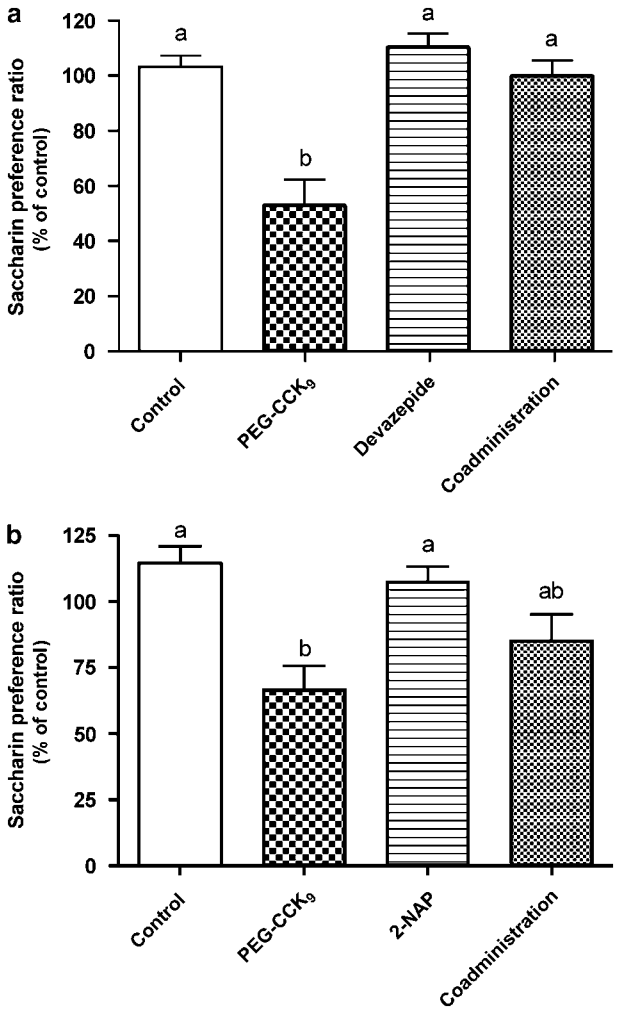

Figure 3 Effect of devazepide (**a**) or 2-NAP (**b**) on PEG-CCK$_9$-induced conditioned taste aversion (CTA). The saccharin preference ratios, determined in a two-bottle CTA procedure, are presented as a percentage of their own control (mean ± s.e.mean; $n = 8$). The letters above the bars indicate statistically significant differences among treatment groups ($P < 0.05$) (ANOVA followed by Tukey's multiple comparison test: devazepide; F = 17,97, d.f. = (3, 28), 2-NAP; F = 7,567, d.f. = (3, 27)).

Whether the comparison of the ability of LiCl and CCK$_8$ to cause CTA is appropriate has been disputed in several studies, as the pharmacokinetic properties of those two compounds are very different and may affect the ability to induce CTA (Ervin and Teeter, 1986; Ervin *et al*., 1995a; Mosher *et al*., 1998). In rats, CCK$_8$ has a plasma half-life of only a few minutes (Rehfeld, 1978; Koulischer *et al*., 1982), whereas LiCl has a 6 h plasma half-life (Wood *et al*., 1986). Taste aversion effects may be very sensitive to the duration of drug effects, and this may possibly explain the marked CTA differences between LiCl and CCK$_8$ (Ervin *et al*., 1995a; Mosher *et al*., 1998). Ervin *et al*. (1995b) artificially prolonged the presence and action of unmodified CCK$_8$ by 10 half-hourly injections of the peptide and compared its CTA effect with a single dose injection. They concluded that multiple small doses of CCK$_8$ were more effective at inducing CTA than a single large dose, sustaining the hypothesis that an increase in circulation time of a CTA-inducing drug increases its potency in inducing CTA. As PEGylation of CCK$_9$ prolongs the *in vivo* anorectic action by increasing proteolytic resistance and shielding of the immunoreactive sites by the PEG polymers, thus increasing the circulation time of the conjugate (Leon-Tamariz *et al*., 2007; Verbaeys *et al*., 2007), one would expect the PEGylated peptide to show increased potency in inducing CTA. Our observations did not sustain this hypothesis and showed that, despite the prolonged anorectic action due to the increased plasma half-life of the conjugate, PEGylated CCK$_9$ was not more potent in inducing CTA than the unmodified peptide. Comparison of the PEG-CCK$_9$-induced CTA strengths observed in our two-bottle procedure to the published CTA strength induced by unmodified CCK$_8$ in either a two- or a one-bottle test showed that, for both compounds, the strengths were mild and not significant at doses lower than 8 μg kg^{-1} (Deutsch and Hardy, 1977; Ervin and Teeter, 1986; Ervin *et al*., 1995a; Mosher *et al*., 1998). CTA strengths vary with rat strain and protocol implementation, such as the use of a one-or two-bottle procedure (Ervin *et al*., 1995b), making comparison between different studies difficult. In our study, we used the two-bottle test, which is known to be more sensitive in measuring CTA effects than a one-bottle test (Deutsch and Hardy, 1977). To have a suitable conjugation site for the synthesis of the PEGylated conjugate, the nonapeptide CCK$_9$ was used instead of the octapeptide CCK$_8$. Both peptides express full biological activity (Martinez, 1990) and we therefore believe that, if CTA differences exist between CCK$_8$ and PEG-CCK$_9$, they are not because of the extra amino acid.

The main goal of our study was to investigate whether the dose-dependent PEG-CCK$_9$-induced anorexia, described in a previous report (Verbaeys *et al*., 2007), was because of normal physiological satiety processes or to malaise, discomfort or nausea. The anorexia/taste aversion correlation was therefore determined by comparison of the CTA and the anorexia induced by the same doses of PEG-CCK$_9$ (1, 2, 4, 8 and 16 μg kg^{-1}). The regression lines for the two functions were parallel, indicating that the size of the PEG-CCK$_9$-induced food intake reduction was functionally related to the CTA strengths of PEG-CCK$_9$. Thus, a higher PEG-CCK$_9$ dose results in a proportional increase of both anorexia and CTA. These results make it tempting to conclude that the dose-dependent increase in anorexia is because of the proportional dose-dependent increase in CTA and not due to a more pronounced physiological satiety effect. However, we question this, as the observation of a functional relation is no proof at all of a causal connection between PEG-CCK$_9$-induced anorexia and CTA. Moreover, comparison of the MEDs for induction of anorexia and CTA revealed that low doses of PEG-CCK$_9$ (1, 2 and 4 μg kg^{-1}), which were able to significantly reduce food intake, did not have a significant CTA effect. This observation provides direct evidence that PEG-CCK$_9$ at doses lower than 8 μg kg^{-1} causes food intake reduction by a specific physiological satiety effect and not merely as a result of aversiveness. It was accepted in the first CTA studies that a compound which reduces feeding without inducing CTA causes anorexia through physiological satiety mechanisms, not by non-specific aversive effects, whereas a compound which induces both feeding inhibition and CTA is assumed to cause anorexia by aversiveness (Deutsch and

Hardy, 1977; Krahn *et al*., 1986). This simple relationship between the ability of a compound to induce CTA and decrease feeding has been challenged in several studies (Gamzu *et al*., 1985; Hunt and Amit, 1987; Ervin *et al*., 1995a). A study by Ervin *et al*. (1995a) evaluated several compounds for their potential to produce feeding inhibition and CTA, and found that the patterns of the response to these compounds in these two behavioural assays varied greatly. They observed that the anorexia/taste aversion correlation was not clear-cut and therefore concluded that the ability of a drug to induce anorexia and taste aversion is no proof that the anorexia necessarily results from aversion or malaise.

Our previous report demonstrated that the anorectic effect of PEG-CCK$_9$ is mediated through activation of peripheral CCK$_1$-receptors (Verbaeys *et al*., 2007). Two selective CCK$_1$-receptor antagonists with different BBB penetration capacities were therefore used to investigate whether the receptors mediating the anorexic effect of PEG-CCK$_9$ were also involved in the PEG-CCK$_9$-induced CTA. Devazepide can cross the BBB, whereas 2-NAP cannot (Hull *et al*., 1993; Baldwin *et al*., 1994; Baldwin *et al*., 1998). The PEG-CCK$_9$-induced CTA (8 $\mu g\,kg^{-1}$) was completely antagonized by a single i.p injection of devazepide (100 $\mu g\,kg^{-1}$), indicating that CCK$_1$-receptors are critical in inducing CTA after PEG-CCK$_9$ administration. 2-NAP (3 $mg\,kg^{-1}$ every 2 h for 6 h), on the other hand, only partially abolished PEG-CCK$_9$-induced CTA in our experimental set-up. We cannot therefore completely exclude the involvement of central CCK$_1$-receptors in CTA induction.

The CTA effects after administration of CCK$_8$ are completely blocked by the CCK$_1$-receptor antagonist MK-329 and only partially blocked by the CCK$_2$-receptor antagonist L-365.260 (Mosher *et al*., 1996). The partial antagonistic effect of L-365.260 was ascribed to an anxiolytic effect of the antagonist, as it is also able to partially antagonize LiCl-induced CTA. We therefore concluded that CCK$_8$-induced CTA is mediated through CCK$_1$-receptor activation, the same receptor subtype mediating the anorexia. CCK$_1$-receptor involvement in inducing CTA is also supported by the demonstration that CCK$_2$-receptor agonists, such as CCK$_4$ and unsulphated CCK$_8$, do not induce CTA after i.p. or i.c.v. administration, questioning whether CCK$_2$-receptors are involved in the production of CTA (Ervin *et al*., 1995a; Mosher *et al*., 1998). Furthermore, Ervin *et al*. (1995a) strongly suggested that the CTA induced by CCK$_8$ is due to peripheral CCK$_1$-receptor mediation and not central CCK$_1$-receptors, as CCK$_8$ is not able to cross the BBB (Passaro *et al*., 1982). However, this hypothesis was never tested by the use of selective CCK$_1$-receptor antagonists with different BBB penetration capacities. Thus, PEG-CCK$_9$-induced CTA and CCK$_8$-induced CTA are mediated by the same CCK receptor subtype, the CCK$_1$-receptor and further investigation is necessary to distinguish between a central or peripheral mode of action.

In conclusion, we demonstrated that PEG-CCK$_9$ induces both anorexia and CTA and that both effects increase with dose. However, the PEGylated conjugate is more potent in inducing satiety, suggesting that the anorexia cannot be completely attributed to the aversiveness of the drug. Devazepide, a selective CCK$_1$-receptor antagonist, completely blocks PEG-CCK$_9$-induced CTA, indicating that anorexia and taste aversion are mediated by the same CCK$_1$-receptor subtype. Further investigations are needed to distinguish between central or peripheral CCK$_1$-receptors in the mediation of these responses.

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Conflict of interest

The authors state no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Baldwin BA, de la Riva C, Gerskowitch VP (1994). Effect of a novel CCKA receptor antagonist (2-NAP) on the reduction in food intake produced by CCK in pigs. *Physiol Behav* **55**: 175–179.

Baldwin BA, Parrott RF, Ebenezer IS (1998). Food for thought: a critique on the hypothesis that endogenous cholecystokinin acts as a physiological satiety factor. *Prog Neurobiol* **55**: 477–507.

Deutsch JA, Hardy WT (1977). Cholecystokinin produces bait shyness in rats. *Nature* **266**: 196.

Ervin GN, Birkemo LS, Johnson MF, Conger LK, Mosher JT, Menius Jr JA (1995a). The effects of anorectic and aversive agents on deprivation-induced feeding and taste aversion conditioning in rats. *J Pharmacol Exp Ther* **273**: 1203–1210.

Ervin GN, Mosher JT, Birkemo LS, Johnson MF (1995b). Multiple, small doses of cholecystokinin octapeptide are more efficacious at inducing taste aversion conditioning than single, large doses. *Peptides* **16**: 539–545.

Ervin GN, Teeter MN (1986). Cholecystokinin octapeptide and lithium produce different effects on feeding and taste aversion learning. *Physiol Behav* **36**: 507–512.

Gamzu E, Vincent G, Boff E (1985). A pharmacological perspective of drugs used in establishing conditioned food aversions. *Ann NY Acad Sci* **443**: 231–249.

Gibbs J, Young RC, Smith GP (1973). Cholecystokinin decreases food intake in rats. *J Comp Physiol Psychol* **84**: 488–495.

Hull RA, Shankley NP, Harper EA, Gerkowitch VP, Black JW (1993). 2-Naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide (2-NAP) a selective cholecystokinin CCKA-receptor antagonist. *Br J Pharmacol* **108**: 734–740.

Hunt T, Amit Z (1987). Conditioned taste aversion induced by self-administered drugs: paradox revisited. *Neurosci Biobehav Rev* **11**: 107–130.

Koulischer D, Moroder L, Deschodt-Lanckman M (1982). Degradation of cholecystokinin octapeptide, related fragments and analogs by human and rat plasma *in vitro*. *Regul Pept* **4**: 127–139.

Krahn DD, Gosnell BA, Levine AS, Morley JE (1986). The effect of calcitonin gene-related peptide on food intake involves aversive mechanisms. *Pharmacol Biochem Behav* **24**: 5–7.

Leon-Tamariz F, Verbaeys I, Van Boven M, De Cuyper M, Buyse J, Clynen E *et al*. (2007). PEGylation of cholecystokinin prolongs its anorectic effect in rats. *Peptides* **28**: 1003–1011.

Martinez J (1990). Gastrointestinal regulatory peptide receptors. In: Hansch C, Taylor JB, Sammens PG (eds). *Comprehensive Medical Chemistry: The Rational Design, Mechanistic Study and Therapeutic Application of Chemical Compounds*. Pergamon: Oxford. pp 925–959.

Mosher JT, Birkemo LS, Johnson MF, Ervin GN (1998). Sulfated cholecystokinin (26–33) induces mild taste aversion conditioning in rats when administered by three different routes. *Peptides* **19**: 849–857.

Mosher JT, Johnson MF, Birkemo LS, Ervin GN (1996). Several roles of CCKA and CCKB receptor subtypes in CCK-8-induced and LiCl-induced taste aversion conditioning. *Peptides* **17**: 483–488.

Passaro Jr E, Debas H, Oldendorf W, Yamada T (1982). Rapid appearance of intraventricularly administered neuropeptides in the peripheral circulation. *Brain Res* **241**: 335–340.

Perez C, Sclafani A (1991). Cholecystokinin conditions flavor preferences in rats. *Am J Physiol* **260**: 179–185.

Rehfeld JF (1978). Immunochemical studies on cholecystokinin II. Distribution and molecular heterogeneity in the central nervous system and small intestine of man and hog. *J Biol Chem* **253**: 4022–4030.

Verbaeys I, Leon-Tamariz F, Buyse J, De Cuyper M, Pottel H, Van Boven M *et al*. (2007). PEGylated cholecystokinin prolongs satiation in rats: dose dependency and receptor involvement. *Br J Pharmacol* **152**: 396–403.

Welzl H, D'Adamo P, Lipp HP (2001). Conditioned taste aversion as a learning and memory paradigm. *Behav Brain Res* **125**: 205–213.

Wood AJ, Goodwin GM, De Souza R, Green AR (1986). The pharmacokinetic profile of lithium in rat and mouse; an important factor in psychopharmacological investigation of the drug. *Neuropharmacology* **25**: 1285–1288.