

## RESEARCH PAPER

# PEGylated cholecystokinin prolongs satiation in rats: dose dependency and receptor involvement

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**Background and purpose:** Acute intraperitoneal (i.p.) administration of cholecystokinin (CCK) is known to induce a significant, but short-lasting, reduction in food intake, followed by recovery within hours. Therefore, we had covalently coupled CCK to a 10 kDa polyethylene glycol and showed that this conjugate, PEG-CCK<sub>9</sub>, produced a significantly longer anorectic effect than unmodified CCK<sub>9</sub>. The present study assessed the dose-dependency of this response and the effect of two selective CCK<sub>1</sub> receptor antagonists, with different abilities to cross the blood-brain barrier (BBB), on PEG-CCK<sub>9</sub>-induced anorexia.

**Experimental approach:** Food intake was measured, for up to 23 h, after i.p. administration of different doses (2, 4, 8, 16 and 32  $\mu\text{g kg}^{-1}$ ) of CCK<sub>9</sub> or PEG-CCK<sub>9</sub> in male Wistar rats. Devazepide (100  $\mu\text{g kg}^{-1}$ ), which penetrates the BBB or 2-NAP (3 mg  $\text{kg}^{-1}$ ), which does not cross the BBB, were coadministered i.p. with PEG-CCK<sub>9</sub> (6  $\mu\text{g kg}^{-1}$ ) and food intake was monitored.

**Key results:** In PEG-CCK<sub>9</sub>-treated rats, a clear dose-dependency was seen for both the duration and initial intensity of the anorexia whereas, for CCK<sub>9</sub>, only the initial intensity was dose-dependent. Intraperitoneal administration of devazepide or 2-NAP, injected immediately prior to PEG-CCK<sub>9</sub>, completely abolished the anorectic effect of PEG-CCK<sub>9</sub>.

**Conclusions and implications:** The duration of the anorexia for PEG-CCK<sub>9</sub> was dose-dependent, suggesting that PEGylation of CCK<sub>9</sub> increases its circulation time. Both devazepide and 2-NAP completely abolished the anorectic effect of i.p. PEG-CCK<sub>9</sub>, indicating that its anorectic effect was solely due to stimulation of peripheral CCK<sub>1</sub> receptors.

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**Keywords:** cholecystokinin; PEGylated cholecystokinin; anorexia; dose-response; CCK<sub>1</sub> receptor antagonists; devazepide 2-NAP

**Abbreviations:** BBB, blood-brain barrier; CCK<sub>9</sub>, cholecystokinin<sub>9</sub>; 2-NAP, 2-naphthalenesulphonyl 1-aspartyl-(2-phenethyl) amide; PEG, polyethylene glycol; PEG-CCK<sub>9</sub>, PEGylated cholecystokinin<sub>9</sub>

## Introduction

It is generally accepted that the gut peptide, cholecystokinin (CCK), is involved in the short-term regulation of the energy balance. CCK has been subject to many studies since its first description in 1927 (Ivy and Oldberg, 1927). However, there is still no consensus about how endogenous CCK causes meal termination. Studies in a number of animal species indicate that pretreatment with devazepide (L-364,718), a specific antagonist of both central and peripheral CCK<sub>1</sub> receptors, completely abolishes the inhibitory effects of exogenous CCK on food intake (Weller *et al.*, 1990; Smith and Gibbs, 1992, 1994; Weatherford *et al.*,

1992). Furthermore, systemic administration of devazepide increases meal size in several species (Hewson *et al.*, 1988; Ebenezer *et al.*, 1990; Bado *et al.*, 1991; Reidelberger *et al.*, 1991; Weatherford *et al.*, 1993; Covasa and Forbes, 1994; Ebenezer and Baldwin, 1995). These results suggest that endogenous CCK, released from the small intestine during a meal, results in satiety via the activation of CCK<sub>1</sub> receptors. Other studies have shown that systemic administration of the specific CCK<sub>1</sub> receptor antagonists, 2-naphthalenesulphonyl 1-aspartyl-(2-phenethyl) amide (2-NAP) (Baldwin *et al.*, 1994; Ebenezer and Baldwin, 1995) or A70104 (Ebenezer, 2003), which do not cross the blood-brain barrier (BBB) (Baldwin *et al.*, 1994), does not increase meal size, although pretreatment with these drugs blocks the anorectic effect of intravenous CCK. These studies support the hypothesis that endogenous CCK plays a role in the regulation of food intake via activation of central CCK<sub>1</sub> receptors (Baldwin *et al.*, 1998). However, Reidelberger

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*et al.* (2003) showed that doses of A70104 did increase food intake in rats, challenging the above-mentioned hypothesis.

We have examined previously the physiological effects of simmondsin, an anorectic glucoside extracted from the jojoba plant (*Simmondsia chinensis*), and hypothesized that its anorectic effect might be caused by interference with the CCK signalling pathway (Cokelaere *et al.*, 1995; Flo *et al.*, 1998, 1999, 2000; Lievens *et al.*, 2003). A direct comparison of simmondsin- and CCK-induced anorexia is hampered by the short anorectic action of CCK. To compare the effects of simmondsin with those of CCK, we prolonged the anorectic effect of CCK by coupling the hormone covalently to distearoylphosphatidylethanolamine via a poly(ethylene glycol) (PEG) spacer (De Cuyper *et al.*, 2004). More recently, we successfully PEGylated CCK<sub>9</sub> (molecular weight of the PEG fraction from 5 to 30 kDa) and we demonstrated that intraperitoneal (i.p.) bolus injection of rats with PEGylated CCK<sub>9</sub> resulted in a prolonged anorectic effect compared to the unmodified molecule, the 10 kDa PEG-CCK<sub>9</sub> being the most active (León-Tamariz *et al.*, 2007). PEGylation is a recently developed method, in which a flexible strand or strands of PEG is covalently attached to a protein or small drug molecule, improving the pharmacokinetics of the PEGylated molecule (Abuchowski *et al.*, 1977a; Veronese and Pasut, 2005). This newly synthesized PEG-CCK<sub>9</sub> provides the means for investigating the importance of CCK in the control of feeding behaviour and whether endogenous peripheral CCK acts as a satiety signal.

In the present study, we first examined the anorectic potency of different doses of PEGylated CCK<sub>9</sub> (10 kDa) given i.p. to free-feeding rats. We also investigated whether 10 kDa PEG-CCK<sub>9</sub> induced its anorectic effect by stimulating peripheral and/or central CCK<sub>1</sub> receptors by comparing the antagonistic effects of devazepide and 2-NAP on the 10 kDa PEG-CCK<sub>9</sub>-induced anorexia.

## Methods

### Animals

Male Wistar rats (Janvier, Le Genest Saint Isle, France), weighing  $268 \pm 16$  g at the start of the experiment, were housed individually in iron-wired cages (room temperature of  $21 \pm 0.2^\circ\text{C}$ , 40–60% relative humidity, reversed 12:12 h light-dark cycle with lights on at 2100 hours and lights off at 0900 hours), following national guidelines on animal care. Water and complete powdered rodent food (Sniff, Bioservices, Schaijk, The Netherlands) were provided *ad libitum*. Food intake was measured by weighing the manglers, which are specially designed to avoid spillage (Scholz, Overijse, Belgium). Animals were adapted to the experimental conditions and i.p. injections at least 10 days before the start of the experiment.

### Experimental design

#### Experiment 1: dose-dependent effects of unmodified and PEGylated CCK<sub>9</sub> on food intake in rats

Two series of experiments were performed. The first determined the dose-response relationship of unmodified

CCK<sub>9</sub> and the second that of PEGylated CCK<sub>9</sub>. The same rats were used in both series with 1 week of recovery between both series.

**Dose-response relationship of unmodified CCK<sub>9</sub>.** The rats were divided into three groups of 10. The start of the food intake was synchronized by 1 h fasting before lights off. To test all five doses, two test periods were needed; these were separated by a 1 week recovery period during which the rats received no treatment. Each test period consisted of two consecutive days, the control day, on which all three groups received an i.p. injection of mPEG-OH dissolved in 0.5 ml saline, and the treatment day, on which each group received a different dose of i.p. injected unmodified CCK<sub>9</sub> (2, 4 and  $8 \mu\text{g kg}^{-1}$  in the first test period; 8, 16 and  $32 \mu\text{g kg}^{-1}$  in the second test period). The test on the  $8 \mu\text{g kg}^{-1}$  dose was repeated to check the reproducibility and to determine if there was a time effect caused by the recovery week. The data from the second test of the dose of  $8 \mu\text{g kg}^{-1}$  were not included in subsequent analyses. All injections were given at 0845 hours, 15 min before the start of the measurements. Food intake was measured at time zero (0900 hours), after 30 min and 1 h, then every 1 h for 8 h. As there was no significant difference in control food intake between the three groups, the food intake data of the preceding control day were pooled and compared with the food intake data for each group on the treatment day.

**Dose-response relationship for PEGylated CCK<sub>9</sub>.** The experimental design was identical to the design used in the preceding experiment except for one extra measurement at 23 h.

#### Experiment 2: effect of devazepide on PEGylated CCK<sub>9</sub>-induced food intake inhibition

To test whether PEGylated CCK<sub>9</sub> exerts its anorectic effect by activation of CCK<sub>1</sub> receptors, devazepide, a specific CCK<sub>1</sub> receptor antagonist, was coadministered with PEGylated CCK<sub>9</sub>. A dose of  $6 \mu\text{g kg}^{-1}$  PEG-CCK<sub>9</sub> was used, since experiment 1b indicated that its anorectic effect on cumulative food intake lasted for at least 6 h. Four groups of eight rats were used. One received an i.p. injection of mPEG-OH ( $32 \mu\text{g kg}^{-1}$ ), one an i.p. injection of devazepide ( $100 \mu\text{g kg}^{-1}$ ), one an i.p. injection of PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ) and one an i.p. injection of devazepide ( $100 \mu\text{g kg}^{-1}$ ) immediately followed by an i.p. injection of PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ). Devazepide was administered as a single bolus injection, as it has a relatively long plasma half-life of over 4 h (Reidelberger *et al.*, 2003). To synchronize the start of food intake, the rats were fasted 1 h before the start of the dark period. All test substances were injected at 0845 hours, 15 min before lights off and renewed access to food. Food intake was recorded at 1, 6 and 23 h after the start of the dark period.

#### Experiment 3: effect of 2-NAP on PEGylated CCK<sub>9</sub>-induced food intake inhibition

**Duration of the in vivo effect of 2-NAP.** The biological half-life of 2-NAP *in vivo* had not been determined previously. This series of experiments was performed to establish the

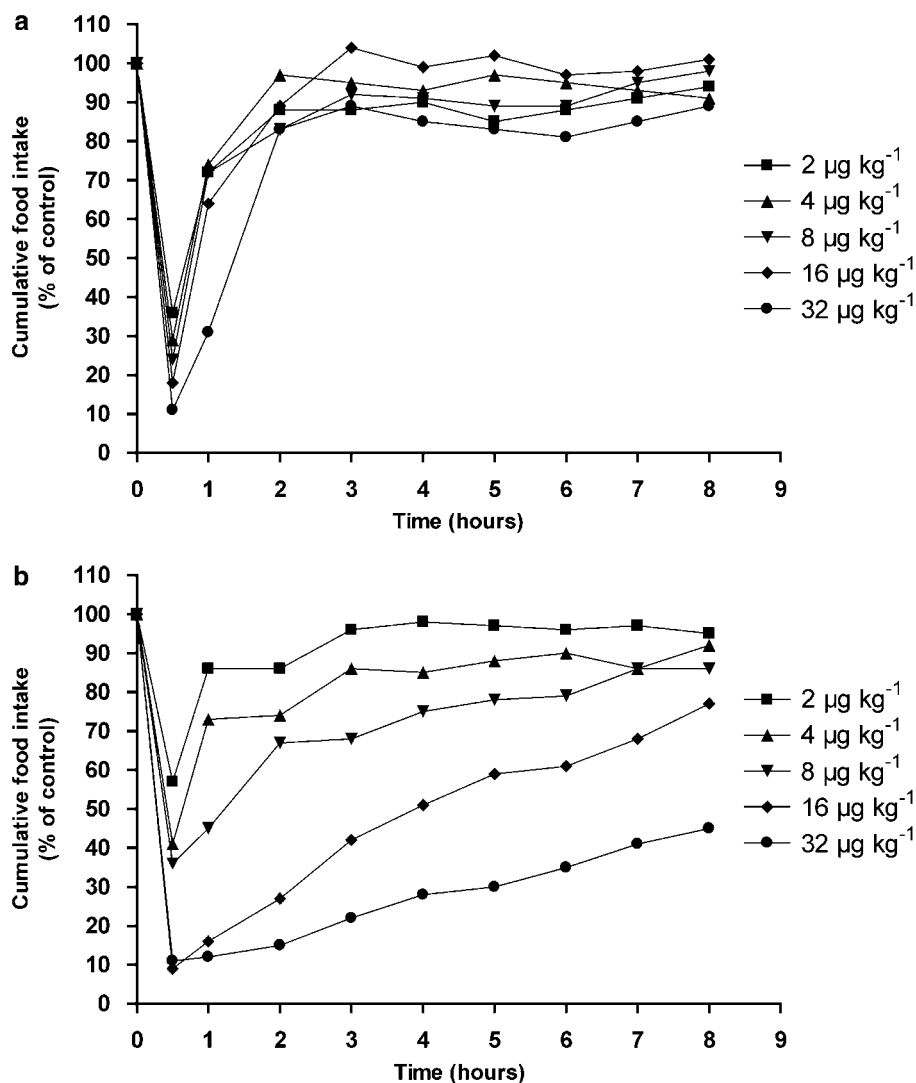
time course of the ability of 2-NAP to inhibit CCK<sub>9</sub>- or PEG-CCK<sub>9</sub>-induced anorexia *in vivo*. Test rats were deprived of food for 6 h (from 0800 hours until 1400 hours), then were given free access to food. Four groups of eight rats received an i.p. injection of 2-NAP ( $3 \text{ mg kg}^{-1}$ ), 6, 4, 2 or 0 h before an i.p. injection of CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ) or PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ), given 10 min before renewed access to food. Food intake was measured 30 min later and compared with that in 6 h food-deprived rats given either an i.p. injection of mPEG-OH ( $32 \mu\text{g kg}^{-1}$ ) or CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ) or PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ), as appropriate, 10 min before food was re-supplied.

**Effect of 2-NAP on PEGylated CCK<sub>9</sub>-induced food intake inhibition.** To distinguish between a central or a peripheral mode of action of PEGylated CCK<sub>9</sub>, the effect of 2-NAP on the PEGylated CCK<sub>9</sub>-induced anorexia was investigated using the same protocol as in the devazepide experiment (experiment 2), except that, since the previous experiment (3a) showed that the antagonistic effect of 2-NAP on CCK<sub>9</sub>-

or PEGylated CCK<sub>9</sub>-induced anorexia was halved within 2 h, the i.p. 2-NAP injection ( $3 \text{ mg kg}^{-1}$ ) was repeated every 2 h for 6 h and the two other groups received an i.p. saline injection to create the same injection-induced stress. The four treatments were (i) i.p. injection of mPEG-OH ( $32 \mu\text{g kg}^{-1}$ ), (ii) i.p. injection of 2-NAP ( $3 \text{ mg kg}^{-1}$ ), (iii) i.p. injection of PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ) or (iv) i.p. injection of 2-NAP ( $3 \text{ mg kg}^{-1}$ ) followed immediately by i.p. injection of PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ).

#### Data analysis and statistical procedures

The dose dependency of the initial intensity of anorexia was analysed by plotting food intake (g) measured at 0.5 h (Y) vs log dose (X) and was fitted by linear regression (Figure 2). To examine the effect of dose on the time course of the anorectic effect, mean cumulative food intake was represented as a percentage of control food intake, resulting in dose-response curves over time (Figures 1a and b). Each



**Figure 1** Dose-response curves for the intraperitoneal injection of unmodified CCK<sub>9</sub> (a) or PEGylated CCK<sub>9</sub> (b) on food intake in rats over the dose range of 2–32  $\mu\text{g kg}^{-1}$ . The results are expressed as a percentage of the control food intake (100%). CCK, cholecystokinin; PEG, polyethylene glycol.

response curve was fitted with a sigmoidal curve (four-parameter logistic model) using Prism software (GraphPad Software Inc., San Diego, CA, USA), which provided  $\tau_{50}$  estimates for every concentration used;  $\tau_{50}$  is the time needed to reach a response halfway between the baseline and maximum of the cumulative food intake. To compare the dose-response curves over time between unmodified CCK<sub>9</sub> and PEG-CCK<sub>9</sub>, the relationship between dose ( $X$ ) and  $\tau_{50}$  ( $Y$ ) for both unmodified CCK<sub>9</sub> and PEG-CCK<sub>9</sub> was approximated using linear regression (Figure 3).

The effects of devazepide and 2-NAP on PEGylated CCK<sub>9</sub>-induced inhibition of food intake were analysed by one-way analysis of variance (ANOVA) using GraphPad Prism (version

4, San Diego, CA, USA). Significant differences among treatment means were analysed by Tukey's multiple comparison test, with  $P$ -value  $< 0.05$  considered statistically significant.

#### Drugs, chemical reagents and other materials

The PEG-CCK<sub>9</sub> conjugate was prepared as described by León-Tamariz *et al.* (2007). The PEG-CCK<sub>9</sub> conjugates were quantified by analytical high-performance liquid chromatography, calibrated using pure CCK<sub>9</sub> (Bachem, Bubendorf, Switzerland). The amounts of PEG-CCK<sub>9</sub> ( $\mu\text{g kg}^{-1}$ ) mentioned in the experiments indicate the amount of peptide present in the conjugate. The non-active linear methoxy-polyethylene glycol (mPEG-OH or CH<sub>3</sub>-(OCH<sub>2</sub>CH<sub>2</sub>) $n$ -OH), used for control injections, was purchased from Nektar Therapeutics (Huntsville, AL, USA). The PEG-CCK<sub>9</sub> conjugate consisted of 84.2% PEG and 15.8% CCK<sub>9</sub> by weight. The amount of mPEG-OH given in the control injection was the same as the amount of PEG injected at the different doses of PEG-CCK<sub>9</sub>. Devazepide, kindly provided by Merck, Sharp and Dohme (Whitehouse Station, NJ, USA), was dissolved as a 1 mg ml<sup>-1</sup> stock solution in a 7:3 (v/v) mixture of PEG 400 and glycerine. The stock solution was stored at 4°C and was diluted in 0.5 ml of saline before injection. 2-NAP was kindly provided by the James Black Foundation (London, UK) and was dissolved directly in saline.

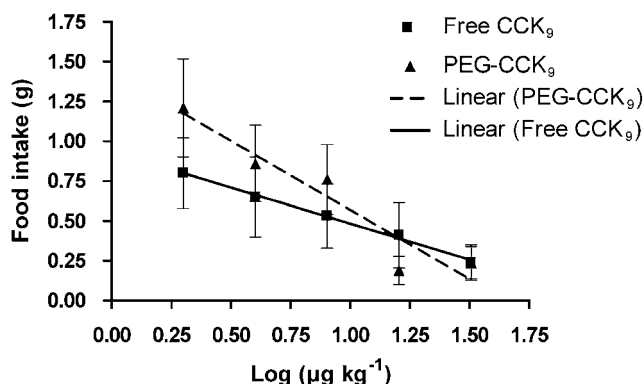
## Results

### Experiment 1: dose-response effects of unmodified and PEGylated CCK<sub>9</sub> on food intake in rats

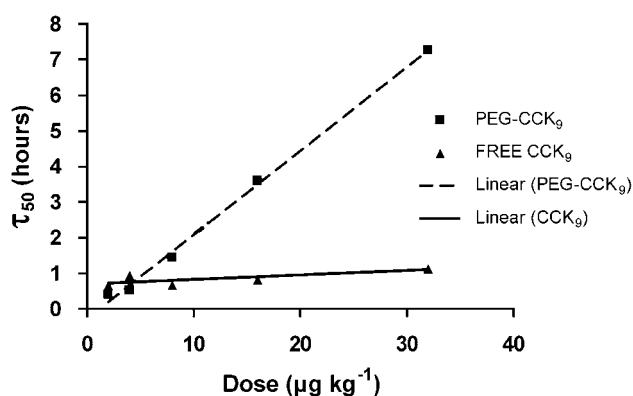
The food intake reduction induced by a dose of 8  $\mu\text{g kg}^{-1}$  was examined in both test periods for unmodified CCK<sub>9</sub> and PEGylated CCK<sub>9</sub> and no difference was observed between the results for the two test periods ( $P = 0.3370$  and  $P = 0.2562$ ). The data from the two test series could therefore be directly compared by statistical analysis.

Food intake, measured 0.5 h after dark onset, was reduced compared to control food intake, by 67, 71, 79, 83 and 92% by injection of 2, 4, 8, 16 and 32  $\mu\text{g kg}^{-1}$ , respectively for unmodified CCK<sub>9</sub>, whereas the same doses of PEG-CCK<sub>9</sub> reduced food intake by 43, 59, 64, 91 and 89%, respectively (Figures 1a and b). Linear regression analysis revealed an inverse relationship between log dose and the 0.5 h food intake for both unmodified and PEGylated CCK<sub>9</sub> (Figure 2). Both slopes were significantly different from zero, indicating a dose dependency of the initial intensity of anorexia for both drugs (CCK<sub>9</sub>; slope =  $-0.4526$ ,  $P = 0.0304$ ; PEG-CCK<sub>9</sub>; slope =  $-0.8670$ ,  $P = 0.0002$ ). There was no significant difference between the two slopes ( $P = 0.1665$ ).

Figure 3 shows the relationship between dose ( $X$ ) and  $\tau_{50}$  (time for half-maximal food intake;  $Y$ ) for both unmodified CCK<sub>9</sub> and PEG-CCK<sub>9</sub>, approximated by linear regression. A significant linear relationship between the  $\tau_{50}$  and the dose was seen for PEG-CCK<sub>9</sub>, but not for CCK<sub>9</sub> (CCK<sub>9</sub>;  $P = 0.1285$ , PEG-CCK<sub>9</sub>; slope =  $0.2357$ ,  $P < 0.0001$ ,  $r^2 = 0.9970$ ). This shows that the duration of the anorectic effect of PEG-CCK<sub>9</sub>, but not that of CCK<sub>9</sub>, increased with dose.



**Figure 2** Dose dependency of the initial anorectic intensity in rats treated with unmodified CCK<sub>9</sub> or PEGylated CCK<sub>9</sub>. The relationship between the log dose ( $X$ ) and food intake measured at 0.5 h ( $Y$ ) for unmodified CCK<sub>9</sub> and PEG-CCK<sub>9</sub> was approximated by linear regression. Both slopes were significantly different from zero (CCK<sub>9</sub>: slope =  $-0.4526$ ,  $P = 0.0304$ ; PEG-CCK<sub>9</sub>: slope =  $-0.8670$ ,  $P = 0.0002$ ). There was no significant difference between the two slopes ( $P = 0.1665$ ). CCK, cholecystokinin; PEG, polyethylene glycol.



**Figure 3** Dose response over time in rats treated with unmodified CCK<sub>9</sub> or PEGylated CCK<sub>9</sub>. The relationship between dose ( $X$ ) and  $\tau_{50}$  ( $Y$ ) for unmodified CCK<sub>9</sub> and PEG-CCK<sub>9</sub> was approximated by linear regression. The response curves as shown in Figures 1a and 1b were fitted with a sigmoidal curve (four-parameter logistic model), which provided  $\tau_{50}$  estimates for every concentration used.  $\tau_{50}$  is defined as the time for the half-maximal response. The slope of the line from the PEG-CCK<sub>9</sub> results is significantly different from zero, indicating a clear correlation between dose and  $\tau_{50}$ , whereas that for CCK<sub>9</sub> is not (CCK<sub>9</sub>:  $P = 0.1285$ ; PEG-CCK<sub>9</sub>: slope =  $0.2357$ ,  $P < 0.0001$ ,  $r^2 = 0.9970$ ). CCK, cholecystokinin; PEG, polyethylene glycol.

**Experiment 2: effect of devazepide on PEGylated CCK<sub>9</sub>-induced food intake inhibition**

A statistically significant difference in food intake was observed between the different treatments used (ANOVA;  $P < 0.0001$  at 1 h,  $P < 0.0001$  at 6 h,  $P < 0.0001$  at 23 h). An i.p. injection of  $100 \mu\text{g kg}^{-1}$  of devazepide stimulated cumulative food intake across the 23 h period, cumulative food intake at 1, 6 and 23 h being increased by 39, 19 and 18% respectively, compared to mPEG-OH-treated controls (Table 1). I.p. injection of  $6 \mu\text{g kg}^{-1}$  PEGylated CCK<sub>9</sub> significantly reduced cumulative food intake for 6 h, while simultaneous administration of devazepide and PEG-CCK<sub>9</sub> restored cumulative food intake to control levels.

**Experiment 3: effect of 2-NAP on PEGylated CCK<sub>9</sub>-induced food intake inhibition**

**Duration of the in vivo effect of 2-NAP.** Table 2 shows that the antagonistic effect of 2-NAP on CCK<sub>9</sub>- and PEG-CCK<sub>9</sub>-induced anorexia decreased with increasing time between the injection of 2-NAP and the injection of CCK<sub>9</sub> or PEG-CCK<sub>9</sub>. I.p. injection of 2-NAP ( $3 \text{ mg kg}^{-1}$ ), followed immediately by i.p. injection of CCK<sub>9</sub> or PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ), was able to completely restore food intake to control levels, whereas the same dose of 2-NAP, injected 2 h or more before CCK<sub>9</sub> or PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ), was not able to completely antagonize their anorectic effects. The food intake of the groups receiving the 2-NAP injection, 6 h before injection of CCK<sub>9</sub> or PEG-CCK<sub>9</sub>, was similar to that of the groups receiving only the CCK<sub>9</sub> or PEG-CCK<sub>9</sub> injection.

**Table 1** Effect of devazepide on PEGylated CCK<sub>9</sub>-induced food intake inhibition

Treatment	Cumulative food intake (g)		
	1 h	6 h	23 h
mPEG-OH ( $32 \mu\text{g kg}^{-1}$ )	$2.8 \pm 0.2^a$	$12.5 \pm 0.3^b$	$27.1 \pm 0.7^{bc}$
Devazepide ( $100 \mu\text{g kg}^{-1}$ )	$3.9 \pm 0.6^a$	$14.8 \pm 0.5^a$	$31.9 \pm 0.8^a$
PEG-CCK <sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ )	$1.1 \pm 0.2^b$	$8.8 \pm 0.3^c$	$26.3 \pm 0.7^c$
Devazepide ( $100 \mu\text{g kg}^{-1}$ ) and PEG-CCK <sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ )	$2.9 \pm 0.3^a$	$13.4 \pm 0.7^{ab}$	$29.8 \pm 0.8^{ab}$

Abbreviations: CCK, cholecystokinin; PEG, polyethylene glycol. Values are expressed as mean  $\pm$  s.e.m. ( $n = 8$ ). The different superscripts indicate statistically significant differences within columns ( $P < 0.05$ ) (ANOVA followed by Tukey's multiple comparison test).

**Table 3** Effect of 2-NAP on PEGylated CCK<sub>9</sub>-induced anorexia in rats

	Cumulative food intake (g)		
	1 h	6 h	23 h
mPEG-OH ( $32 \mu\text{g kg}^{-1}$ )	$2.5 \pm 0.4^a$	$13.1 \pm 0.5^a$	$27.3 \pm 0.4^a$
2-NAP ( $3 \text{ mg kg}^{-1}$ )	$2.2 \pm 0.3^a$	$13.5 \pm 0.7^a$	$26.9 \pm 0.5^a$
PEG-CCK <sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ )	$1.2 \pm 0.1^b$	$10.4 \pm 0.3^b$	$26.4 \pm 0.6^a$
2-NAP ( $3 \text{ mg kg}^{-1}$ ) and PEG-CCK <sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ )	$2.2 \pm 0.3^a$	$13.2 \pm 0.9^a$	$27.4 \pm 0.7^a$

Abbreviations: CCK, cholecystokinin; 2-NAP, 2-naphthalenesulphonyl 1-aspartyl-(2-phenethyl) amide; PEG, polyethylene glycol. Values are expressed as the mean  $\pm$  s.e.m. ( $n = 8$ ). The different superscripts indicate statistically significant differences within columns ( $P < 0.05$ ) (ANOVA followed by Tukey's multiple comparison test).

**Effect of 2-NAP on PEGylated CCK<sub>9</sub>-induced food intake inhibition.** The effect of 2-NAP ( $3 \text{ mg kg}^{-1}$ ) on PEG-CCK<sub>9</sub>-induced food intake inhibition is shown in Table 3. Statistical analysis of the data showed significant effects on the cumulative, 1 h and 6 h, but not the 23 h, food intake for the different treatments ( $P = 0.0017$  at 1 h;  $P = 0.0001$  at 6 h;  $P = 0.5655$  at 23 h). Repeated injections of 2-NAP alone ( $3 \text{ mg kg}^{-1}$  every 2 h for 6 h) were not able to stimulate food intake, compared to mPEG-OH-treated controls. A single i.p. bolus injection of PEG-CCK<sub>9</sub> ( $6 \mu\text{g kg}^{-1}$ ) induced a significant food intake reduction for at least 6 h and repeated injections of 2-NAP completely abolished the suppressant effect of PEG-CCK<sub>9</sub>.

**Discussion**

Conjugation of PEG to peptides and proteins has become a popular approach to improve their therapeutic potential. With a minimal loss of biological activity, PEGylation results in an increase in the effective size of proteins, decreasing the kidney filtration rate, and protects the proteins from enzymatic degradation, thereby increasing their circulation time. It also renders proteins non-immunogenic and non-antigenic (Abuchowski *et al.*, 1977b; Harris *et al.*, 2001; Roberts *et al.*, 2002; Harris and Chess, 2003; Veronese and Pasut, 2005). Previously, we successfully PEGylated CCK<sub>9</sub>

**Table 2** Reduction over time of the antagonistic effect of 2-NAP ( $3 \text{ mg kg}^{-1}$ ) on CCK<sub>9</sub>- and PEG-CCK<sub>9</sub>-induced anorexia ( $6 \mu\text{g kg}^{-1}$ ) in rats

Time before refeeding				Food intake 30 min after refeeding (g)	
6 h	4 h	2 h	10 min	X = CCK <sub>9</sub>	X = PEG-CCK <sub>9</sub>
			mPEG-OH	$5.3 \pm 0.2^a$	$5.0 \pm 0.3^a$
			X	$1.2 \pm 0.4^c$	$2.4 \pm 0.2^b$
			2-NAP/X	$4.5 \pm 0.7^{ab}$	$5.2 \pm 0.5^a$
		2-NAP	X	$2.7 \pm 0.6^{bc}$	$3.2 \pm 0.3^b$
	2-NAP		X	$2.1 \pm 0.4^c$	$2.8 \pm 0.4^b$
2-NAP			X	$1.6 \pm 0.4^c$	$2.0 \pm 0.2^b$

Abbreviations: CCK, cholecystokinin; 2-NAP, 2-naphthalenesulphonyl 1-aspartyl-(2-phenethyl) amide; PEG, polyethylene glycol. Values are expressed as the mean  $\pm$  s.e.m. ( $n = 8$ ). The different letters indicate significant differences in food intake within columns (ANOVA followed by Tukey's multiple comparison test). The significance level was set at  $P < 0.05$ .

(molecular weight of the PEG fraction from 5 to 30 kDa) and demonstrated that an i.p. bolus injection of PEGylated CCK<sub>9</sub> in rats resulted in a prolonged anorectic effect compared with unmodified CCK<sub>9</sub>, the 10 kDa PEG-CCK<sub>9</sub> being the most active conjugate (León-Tamariz *et al.*, 2007).

The present study showed that both the duration and initial intensity of the anorexia caused by PEGylated CCK<sub>9</sub> displayed a clear dose-response effect (Figure 1b). In contrast, only the initial intensity of unmodified CCK<sub>9</sub>-induced anorexia showed a dose-related effect. To compare the dose-response curves over time of PEGylated CCK<sub>9</sub> with unmodified CCK<sub>9</sub>, we used a four-parameter logistic model, which generated the quantitative parameter  $\tau_{50}$ , indicating the time at which the response was half-maximum. The  $\tau_{50}$  showed that the anorectic effect of unmodified CCK<sub>9</sub> was half-maximal within 1.15 h at every concentration used, whereas that of PEG-CCK<sub>9</sub> was linearly dependent on the dose. The findings for free CCK<sub>9</sub> are consistent with the earlier findings of Koulischer *et al.* (1982), who showed that CCK<sub>9</sub> analogues are rapidly converted into their corresponding octapeptides, which are then degraded within 17 min in rat plasma. In general, PEGylation increases the circulation time in the body by protecting the active molecule from proteolytic enzymes and shielding immunoreactive sites (Harris *et al.*, 2001; Harris and Chess, 2003). The fact that  $\tau_{50}$  for PEGylated CCK<sub>9</sub> showed a dose response whereas that for unmodified CCK<sub>9</sub> did not suggests that PEG-CCK<sub>9</sub> remains in the body longer than free CCK<sub>9</sub>. This confirms a previous report of León-Tamariz *et al.* (2007), who found that CCK<sub>9</sub> incubated in fresh rat plasma at 37°C has a half-life between 2 and 3 min, whereas PEGylated CCK<sub>9</sub> incubated under the same conditions is not degraded over a 24 h period. This higher resistance of PEGylated CCK<sub>9</sub> to proteolysis suggests that the observed prolonged anorectic effect of the conjugate could be due to an increased circulation time. Further research is needed to substantiate this. The process of PEGylation can be accompanied by some loss of biological activity through steric hindrance (Veronese and Pasut, 2005) and this may explain the lower initial anorectic activity observed with PEG-CCK<sub>9</sub> than with similar doses of free CCK<sub>9</sub>, although these differences were not statistically different.

Devazepide and 2-NAP, two selective CCK<sub>1</sub> receptor antagonists with different abilities to penetrate the BBB, were used to examine whether PEG-CCK<sub>9</sub> induced its anorectic effect by acting on central and/or peripheral CCK<sub>1</sub> receptors. Devazepide can cross the BBB whereas 2-NAP cannot (Baldwin *et al.*, 1994). In our experimental system, administration of devazepide alone resulted in a significant 23 h augmentation of food intake compared to controls. This observation is consistent with the hyperphagia originally reported in a variety of animal species under a number of different feeding schedules and sustains the hypothesis that endogenous CCK plays a role in the control of food intake through the activation of CCK<sub>1</sub> receptors (Hewson *et al.*, 1988; Ebenezer *et al.*, 1990; Bado *et al.*, 1991; Weatherford *et al.*, 1993; Covasa and Forbes, 1994; Ebenezer and Baldwin, 1995). The present study also showed that coadministration of devazepide (100 µg kg<sup>-1</sup>) with the PEGylated CCK<sub>9</sub> (6 µg kg<sup>-1</sup>) completely abolished the anor-

ectic effect of PEG-CCK<sub>9</sub> over the 23 h measurement period, showing that CCK<sub>1</sub> receptors are involved in food intake reduction induced by PEGylated CCK<sub>9</sub>. The fact that devazepide was able to stimulate food intake and abolished the anorectic effect of PEG-CCK<sub>9</sub> over a period of 23 h suggests that devazepide (100 µg kg<sup>-1</sup>) is at least active for 6 h, the active period of a dose of 6 µg kg<sup>-1</sup> PEG-CCK<sub>9</sub>. Unpublished data (Dr Jiunn Lin, Merck Sharp and Dohme) reported in two studies (Reidelberger *et al.*, 1991, 2003) give the half-life of devazepide as 3.5 ± 1.8 or 4 h, although studies using [<sup>3</sup>H]devazepide have suggested that the half-life time may be longer than this (Reidelberger *et al.*, 2003). It should be noted that the present study did not focus on the kinetics of binding of PEGylated CCK<sub>9</sub> to the receptor and further research is needed to compare the binding kinetics of unmodified and PEGylated CCK<sub>9</sub>. Future competitive binding studies could provide useful information concerning the change of bioactivity by the process of PEGylation. As devazepide is known to block both central and peripheral CCK<sub>1</sub> receptors (Pullen and Hodgson, 1987; Woltman *et al.*, 1999), these results with devazepide could not discriminate between a peripheral or central mode of action of i.p.-injected PEG-CCK<sub>9</sub>. PEGylation of conjugates improves penetration into central nervous system (CNS) tissue (Wu and Pardridge, 1999; Ankeny *et al.*, 2001; Calvo *et al.*, 2001; MacKay *et al.*, 2005) and it is possible that PEG-CCK<sub>9</sub> can enter the brain, in contrast to free CCK<sub>9</sub>, which cannot (Passaro *et al.*, 1982). To test this issue, further studies were performed using a specific CCK<sub>1</sub> receptor antagonist 2-NAP, which cannot cross the BBB (Abuchowski *et al.*, 1977a; Hull *et al.*, 1993; Baldwin *et al.*, 1994).

To investigate the effect of 2-NAP on the long-lasting anorectic effect of PEGylated CCK<sub>9</sub>, we first determined the reduction with time of the antagonistic effect of 2-NAP on CCK<sub>9</sub>- and PEGylated CCK<sub>9</sub>-induced anorexia *in vivo* and found that the antagonistic effect on unmodified CCK<sub>9</sub>- and on PEGylated CCK<sub>9</sub>-induced food intake reduction was significantly decreased within 2 h. Consequently, 2-NAP was injected every 2 h in the subsequent experiment. The observed reduction of the antagonistic effect of 2-NAP with time could probably be ascribed to the natural clearance rate of the peptide, although the ultimate reason for this decrease in antagonistic effect remains to be established as no published data are available on this point.

In our experimental system, it was observed that injections of 2-NAP alone (3 mg kg<sup>-1</sup>, every 2 h for 6 h) did not stimulate food intake. This observation is consistent with the report of Ebenezer and Baldwin (1995), promoting the role of endogenous CCK in satiety through central nervous system CCK<sub>1</sub> receptors and questioning the hypothesis that endogenous peripheral CCK acts as a satiety signal. In contrast, Reidelberger *et al.* (2003) found that food intake in rats increases in response to systemic administration of A-70104, another CCK<sub>1</sub> receptor antagonist that does not cross the BBB. On the other hand, Ebenezer and Parrot could not find a stimulating effect of A-70104 on food intake in pigs (Ebenezer and Parrot, 1993) and rats (Ebenezer, 2003). Reidelberger *et al.* (2003) suggested that the ineffectiveness of A-70104 in these studies of Ebenezer and Parrot could be ascribed to the small doses used. In the present study, we

used a dose of 3 mg kg<sup>-1</sup> of 2-NAP, which is reported to be sufficient to block the effects of exogenous peripheral CCK (Ebenezer and Baldwin, 1995), suggesting that this dose should also be sufficient to block the effects of the endogenous peripheral CCK. Using concentrations of 2-NAP up to 21 mg kg<sup>-1</sup>, identical observations were made (data not shown). However, the goal of our study was to unravel the pathway used by exogenous PEGylated CCK<sub>9</sub> and not to determine whether endogenous CCK<sub>9</sub> has a central or peripheral mode of action.

In the present studies, administration of 2-NAP (3 mg kg<sup>-1</sup>, every 2 h for 6 h) blocked the anorectic effect of PEG-CCK<sub>9</sub>, suggesting that the PEG-CCK<sub>9</sub>-induced anorexia is completely due to stimulation of peripheral CCK<sub>1</sub> receptors. We cannot rule out the possibility that the PEGylated molecule is able to cross the BBB, but, if so, the present study suggests that it does not cause a significant anorectic effect by stimulation of central CCK<sub>1</sub> receptors. Further research is needed to explore the possible movement of PEG-CCK<sub>9</sub> across the BBB.

In conclusion, we demonstrate that PEGylated CCK<sub>9</sub> shows a clear dose dependency of both the duration and initial intensity of anorexia, confirming the prolonged anorectic effect of PEGylated CCK<sub>9</sub> compared with unmodified CCK<sub>9</sub>. Our results also show that the anorectic effect of PEGylated CCK<sub>9</sub> is completely abolished by administration of devazepide, a CCK<sub>1</sub> receptor antagonist that can cross the BBB, suggesting that the anorectic effect of PEGylated CCK<sub>9</sub> is due to activation of CCK<sub>1</sub> receptors. Repeated administration of 2-NAP, which does not cross the BBB, also completely inhibits the anorectic effect of PEG-CCK<sub>9</sub>, indicating that PEG-CCK<sub>9</sub>-induced anorexia is solely due to stimulation of peripheral CCK<sub>1</sub> receptors.

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## Conflict of interest

The authors state no conflict of interest.

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