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Role for the cholecystokinin-A receptor in fever: a study of a mutant rat strain and a pharmacological analysis

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The involvement of the cholecystokinin (CCK)-A receptor in fever was studied. The polyphasic febrile responses to lipopolysaccharide (LPS; 10 μg kg^{-1}, I.V.) were compared between wild-type Long-Evans (LE) rats and the CCK-A-receptor-deficient Otsuka LE Tokushima Fatty (OLETF) rats. The response of the wild-type rats was biphasic, which is typical for LE rats. Phases 1 and 2 of the response of the OLETF rats were similar to those of the LE rats, but the OLETF rats also developed a robust phase 3. This late enhancement of the febrile response could reflect either the absence of the A receptor *per se* or a secondary trait of the mutant strain. To distinguish between these possibilities, we conducted a pharmacological analysis. We studied whether the normally low phase 3 of LE rats can be enhanced by a CCK-A-receptor antagonist, sodium lorglumide (4.3 μg kg^{-1} min^{-1}, 120 min, I.V.), and whether the normally high phase 3 of Wistar rats can be attenuated by a CCK-A receptor agonist, sulphated CCK-8 (up to 0.17 μg kg^{-1} min^{-1}, 120 min, I.V.). The dose of sodium lorglumide used was sufficient to increase food intake (to block satiety), but it did not affect the fever response. In both febrile and afebrile rats, CCK-8 induced dose-dependent skin vasodilatation and decreased body temperature, but it failed to produce any effects specific for phase 3. We conclude that the exaggeration of phase 3 in OLETF rats reflects a secondary trait of this strain and not the lack of the CCK-A receptor *per se*. None of the three known phases of the febrile response of rats to LPS requires the CCK-A receptor.

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Cholecystokinin (CCK) acts both as a gastrointestinal hormone that regulates food intake and as a neuropeptide involved in the processes of memory, learning and anxiety (Fink *et al.* 1998). Such functional dualism reflects the existence of two pools of CCK, peripheral and central, and two subtypes of the CCK receptor, A and B (Wank, 1995; Shulkes & Baldwin, 1997). The A ('alimentary') subtype is localized primarily in the gastrointestinal system where it mediates the hormonal effects of CCK. The B ('brain') subtype is the main target for CCK in the central nervous system (CNS). However, the topographical separation of the two receptor subtypes is not absolute: the B subtype is also present in the periphery, and the A subtype is found in the CNS (Wank, 1995; Shulkes & Baldwin, 1997).

Whereas central CCK is known to affect the hypothalamic control of fever and cause hyperthermia by acting on CCK-B receptors (Szelényi *et al.* 1994; Sugimoto *et al.* 1999; Szelényi, 2001), involvement of the peripheral pool of CCK in fever mechanisms remains unclear. For example, there is no agreement on whether blockade of predominant peripheral receptors to CCK, the A subtype, affects fever. In a preliminary study by Székely (1995), S.C. administration of the selective CCK-A receptor antagonist L-364,718 attenuated phase 2 of the polyphasic febrile response to I.V.-administered lipopolysaccharide (LPS). However, these data have not been published in the peer-reviewed literature. In a study by Martin *et al.* (2000), the same antagonist did not affect the febrile response of rats to I.P.-administered LPS or interleukin (IL)-1β, an endogenous mediator of the pyrogenic action of LPS; this study remains largely inconclusive because the efficacy of the antagonist was not verified, even though all observed effects were negative.

Interestingly, a notion that peripheral CCK may participate in LPS-induced fever is supported by indirect evidence. It is known that pyrogens (e.g. IL-1β) increase the blood concentration of CCK (Daun & McCarthy, 1993; Kurosawa *et al.* 1997). Furthermore, peripheral CCK (Rinaman *et al.* 1993; Li & Rowland, 1995) induces a neuronal activation pattern (expression of an immediate-early gene product, Fos protein, in neuronal nuclei) similar to that induced by LPS (Wan *et al.* 1994; Hermann *et al.* 2001). Among other brain areas, both substances induce the Fos response in the dorsal vagal complex, particularly in the nucleus of the

solitary tract (the major collector of afferent signals from the vagus nerve) and the dorsal motor nucleus of the vagus (the major efferent nucleus of the vagal system).

The vagus nerve mediates a variety of biological responses to peripherally administrated pyrogens (LPS, proinflammatory cytokines) as well as to a peripherally administrated CCK. Receptors for fever mediators, e.g. IL-1β and prostaglandin (PG) E_2 (Ek *et al.* 1998), and CCK receptors (Zarbin *et al.* 1981) are present on the vagus nerve. Experiments involving subdiaphragmatic vagotomy have revealed vagal involvement in the febrile response to LPS and IL-1β, even though such an involvement seems to be limited to phase 1 of the response (for review, see Romanovsky, 2000). Subdiaphragmatic vagotomy also attenuates LPS- or IL-1β-induced Fos expression in the CNS, behavioural depression, hyperalgesia/allodynia, and activation of the pituitary–adrenal axis (for review, see Maier *et al.* 1998), as well as the satiation effect of exogenous CCK (Smith *et al.* 1981) and CCK-induced enhancement of memory (Flood *et al.* 1987). Moreover, peripheral administration of either IL-1β or CCK increases electrical activity of vagal neurons via a CCK-A-receptor-mediated mechanism (Kurosawa *et al.* 1997, 1999), and the response of vagal afferents to CCK is sensitized by IL-1β (Bucinskaite *et al.* 1997). Given these data, we propose that CCK-A receptors on vagal afferents may convey peripheral pyrogenic signals to the brain and thus trigger the earliest phase (phase 1) of the febrile response.

Several genetic and pharmacological tools have been developed to study the physiological role of the CCK-A receptor. These tools include the Long-Evans (LE)-derived, spontaneous CCK-A receptor gene knockout rat strain, Otsuka LE Tokushima Fatty (OLETF; Funakoshi *et al.* 1994), and a variety of pharmacological agonists and antagonists. CCK-8 sulphate and sodium lorglumide (also known as CR-1409) can be considered a relatively selective CCK-A receptor agonist and antagonist, respectively; both have 100–150 times higher affinity for the A than for the B receptor (Shulkes & Baldwin, 1997). Sodium lorglumide has an important advantage over other selective A-receptor antagonists: it is soluble in aqueous solutions, which makes it the antagonist of choice for many *in vivo* experiments. The present study utilized both genetic (OLETF rats) and pharmacological (sulphated CCK-8 and sodium lorglumide) tools.

METHODS

Animals

Male OLETF, LE and Wistar rats (2 months old) were used in this study. The CCK-A receptor-deficient OLETF rats were a gift from the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). A group of wild-type LE rats for experiments (Expts) 1 and 2 was also donated by Otsuka Pharmaceutical. LE rats for Expts 3 and 4 and Wistar rats for Expt 5 were purchased from Harlan (Indianapolis, IN, USA). The rats were housed under the standard conditions and extensively habituated to stay in artificial 'rat holes' (cylindrical confiners made of stainless-steel wire; the same confiners were used later in experiments), as described elsewhere (Ivanov *et al.* 2002). At the time of the experiments, the rats weighed 389 ± 13 g (OLETF), 281 ± 9 g (LE, Otsuka Pharmaceutical), 296 ± 7 g (LE, Harlan) and 339 ± 11 g (Wistar). At the end of the study they were killed with sodium pentobarbital (20 mg kg^{-1}, I.V.). The protocols were approved by the Institutional Animal Care and Use Committees of Legacy Clinical Research and Technology Center, Portland, OR, and St Joseph's Hospital and Medical Center, Phoenix, AZ, USA.

Surgery

The surgical preparation of the donated OLETF and LE rats included installation of an acrylic platform on the skull and jugular catheterization. The acrylic platform was used to protect the exteriorized portion of the jugular catheter. Such protection was necessary because the donated rats had to be used in experiments twice (i.e. they served as their own controls; see Experimental protocols, below). No catheter protection was required for the commercially available LE and Wistar rats, which were used in the experiment only once. All surgery was performed under ketamine–xylazine–acepromazine (55.6, 5.5 and 1.1 mg kg^{-1}, respectively, I.P.) anaesthesia and antibiotic (enrofloxacin, 12 mg kg^{-1}, s.c.) prophylaxis. Acrylic platform installation onto the skull was performed in a stereotaxic instrument (model 900, David Kopf Instruments, Tujunga, CA, USA) according to Ivanov & Romanovsky (2002). Due to the small size (~1 cm × 1 cm × 0.3 cm) and weight (~1 g) of the platform, it was well tolerated by the rats and did not interfere with their normal behaviour. Immediately after the platform installation (donated rats) or skipping this step (rats purchased from Harlan), a silicone catheter (i.d. 0.5 mm, o.d. 0.9 mm) containing heparinized (50 U ml^{-1}) saline was passed into the superior vena cava through the jugular vein, as described elsewhere (Ivanov & Romanovsky, 2002). In rats without a platform, the catheter was exteriorized at the nape and left unprotected; these rats were allowed to recover for 2 days before experiments. In rats with a platform, the catheter was exteriorized at its caudal edge and placed into a polypropylene vial, which was affixed to the platform with dental acrylic and protected with a screwed-on cap; these rats were allowed to recover for 5 days. The catheters were flushed with heparinized saline every other day.

Instrumentation

For an experiment, each rat was instrumented with thermocouples to record its colonic temperature (T_c; a measure of deep body temperature) and tail skin temperature (T_{sk}; a measure of skin vasomotor tone). The thermocouples were made of TT-T-36 insulated copper–constantan wire (Omega Engineering, Stamford, CT, USA). To measure deep body temperature, a lubricated colonic thermocouple was inserted 9–10 cm past the anal sphincter (Donhoffer, 1980). A skin thermocouple was placed on the lateral surface of the tail, approximately at the boundary between the distal and middle thirds. Both thermocouples were attached to the tail with a loop of tape. The thermocouples were connected to a data logger (model AI-24, Dianachart, Rockaway, NJ, USA) and personal computer. The rat was then placed in a wire confiner and transferred to a climatic chamber (Forma Scientific, Marietta, OH, USA) set to an ambient temperature (T_a) of 29.5 °C and 50 % relative humidity; these conditions are truly thermoneutral for the experimental set-up used (Romanovsky *et*

al. 2002). Although T_c measured by thermocouples in this set-up is usually higher (by ~1 °C) than the abdominal temperature measured in freely moving rats by telemetry at a subneutral T_a, this difference reflects the well-established dependence of rat body temperature on T_a (e.g. Yang & Gordon, 1996) and the fact that T_c (one of the highest temperatures of the whole body; see Donhoffer, 1980) substantially exceeds the abdominal temperature; a detailed analysis of this issue is presented elsewhere (Romanovsky *et al.* 1998*b*). The exteriorized portion of the jugular catheter was pulled through a wall port and connected to a syringe. After a 1 h stabilization period, the measurements were begun, and T_c, T_{sk} and T_a were sampled every 2 min for 5–7 h. The set-up used is adequate for studying all three febrile phases (Romanovsky *et al.* 1998*a*).

Experimental protocols

Experiment 1. This experiment was designed to compare the febrile responses to *Escherichia coli* 0111:B4 LPS (lot no. 35H4086; Sigma, St Louis, MO, USA) of the CCK-A-receptor-deficient OLETF rats and the control LE rats. The animals were instrumented as described above. One hour after the beginning of recording, they were injected I.V. with LPS (10 μg kg^{-1}) in pyrogen-free saline (pH = 7.0; 1.0 ml kg^{-1}) or saline alone. Two tests were performed in each animal 3 days apart, and the order of the injections was alternated (i.e. half of the animals received LPS during the first test and saline during the second, whereas the other half received saline first and LPS second).

Experiment 2. This experiment was performed for functional verification of the CCK-A receptor deficiency (suppression of satiety) in the OLETF as compared to the LE rats. Two weeks after Expt 1, the rats were deprived of food, but not water, for 18 h. Thereafter, they were allowed to have an immediate (0–30 min postdeprivation) followed by a delayed (30–180 min) access to a known amount of standard food pellets. After each period, the food remaining was weighed and the food consumption calculated.

Experiment 3. This experiment was designed based on the observed enhancement of phase 3 of LPS-induced fever in OLETF rats in Expt 1. The aim of Expt 3 was to investigate whether pharmacological blockade of CCK-A receptors with sodium lorglumide (RBI, Natick, MA, USA) would exaggerate phase 3. To increase the chance of revealing such an effect, Expt 3 was conducted in LE rats. This strain and its offspring strain (Zucker) normally respond to LPS with a low (Romanovsky *et al.* 1998*b*; Ivanov & Romanovsky, 2002) or no (this study, Expt 1) phase 3. The rats were instrumented, injected with LPS (10 μg kg^{-1}, I.V.), and infused I.V. with either sodium lorglumide (4.3 μg kg^{-1} min^{-1}) in alkalinized saline (pH = 8.0; 17.2 μl kg^{-1} min^{-1}) or alkalinized saline alone by using a multichannel Infusion Syringe Pump 53220 (Stoelting, Wood Dale, IL, USA). The infusion was started 120 min after the LPS injection and continued for 120 min. In a control experiment, afebrile rats received a similar infusion of sodium lorglumide or alkalinized saline.

Experiment 4. In Expt 4, the same dose of sodium lorglumide as that in Expt 3 was used. Experiment 4 was designed to verify the efficacy of that dose under the given experimental conditions to inhibit the satiety effect of endogenous CCK. LE rats were deprived of food but not water for 18 h. Thereafter, the rats were placed in enclosures and transferred to the climatic chamber, as in Expt 3. One hour later, they were infused with either sodium lorglumide (4.3 μg kg^{-1} min^{-1}, I.V.) or alkalinized saline for 120 min. Immediately after the infusion, the animals were released from their enclosures, transferred to their home cages, and subjected to the same food-consumption test as in Expt 2.

Experiment 5. This experiment was designed to study the effect of sulphated CCK-8 (RBI), an A receptor agonist, on phase 3 of LPS-induced fever. Based on the results of Expt 1, the expected outcome was an inhibition of phase 3. To increase the chance of revealing such an effect, Expt 5 was conducted in Wistar rats; this rat strain responds to LPS with a robust phase 3 (Romanovsky *et al.* 1998*a*, *b*). To choose appropriate doses of CCK-8, its effects on afebrile body temperature were studied first. After a 1 h stabilization period, Wistar rats were infused I.V. with either CCK-8 (0.03, 0.17 or 0.83 μg kg^{-1} min^{-1}) in saline (pH = 7.0; 16.7 μl kg^{-1} min^{-1}) or saline alone for 120 min, and their thermal responses were recorded for 3 h after cessation of the infusion. The two lower doses (0.03 and 0.17 μg kg^{-1} min^{-1}) appeared to be thermally ineffective and moderately hypothermizing, respectively. These two doses were used in subsequent fever experiments. The rats were injected with LPS (10 μg kg^{-1}, I.V.) and 2 h later infused I.V. with either CCK-8 (0.03 or 0.17 μg kg^{-1} min^{-1}) or saline for 120 min; their T_c and T_{sk} responses were recorded.

Data processing and analysis

To evaluate the thermal response, the change in T_c (ΔT_c) was calculated as a deviation from the mean T_c for the 60 min preceding the drug administration. To evaluate the thermoeffector response of tail skin vasculature, the heat loss index (HLI) was calculated according to the formula: $HLI = (T_{sk} - T_a)/(T_c - T_a)$. The use of the HLI is justified elsewhere (Romanovsky *et al.* 2002). The index changes between 0 (maximal vasoconstriction) and 1 (maximal dilatation), with a decrease in the HLI corresponding to a decrease in Newtonian heat loss from the tail. The ΔT_c and HLI curves were analysed by a statistical simulation (Monte Carlo) method. As in a previous study (Ivanov & Romanovsky, 2002), the curves were randomized between the treatment groups and subjected to 10^4 repetitions of the analysis of variance. An empirical distribution of the *F*-statistic was created and compared to the original statistic. Data on food consumption were analysed by Student's *t* test. All data are presented as means ± S.E.M.

RESULTS

Experiment 1: effect of genetic deletion of the CCK-A receptor on LPS-induced fever

The OLETF rats responded to LPS (10 μg kg^{-1}, I.V.) with a triphasic febrile response (T_c maxima at ~60, 170 and 280 min postinjection), whereas the LE rats developed a biphasic fever (Fig. 1*A*). Phase 1 seemed to be slightly lower (by 0.2 °C) and peaked ~10 min later in the OLETF rats than in the LE rats, but these differences were not statistically significant. The fever curves started to differ between the two strains immediately after the peak of phase 2 in the LE rats, but the maximal difference (as great as 1.6 °C) occurred at the time of phase 3 and made the whole response curves easily distinguishable ($P = 5.0 \times 10^{-4}$). In the saline test (Fig. 1*B*), the T_c of the LE rats progressively decreased throughout the experiment, while it was maintained at a relatively stable level in the OLETF rats. Although the progressive decrease in T_c of the saline-treated LE rats from Otsuka Pharmaceutical was rather unusual, this observation does not affect the interpretation

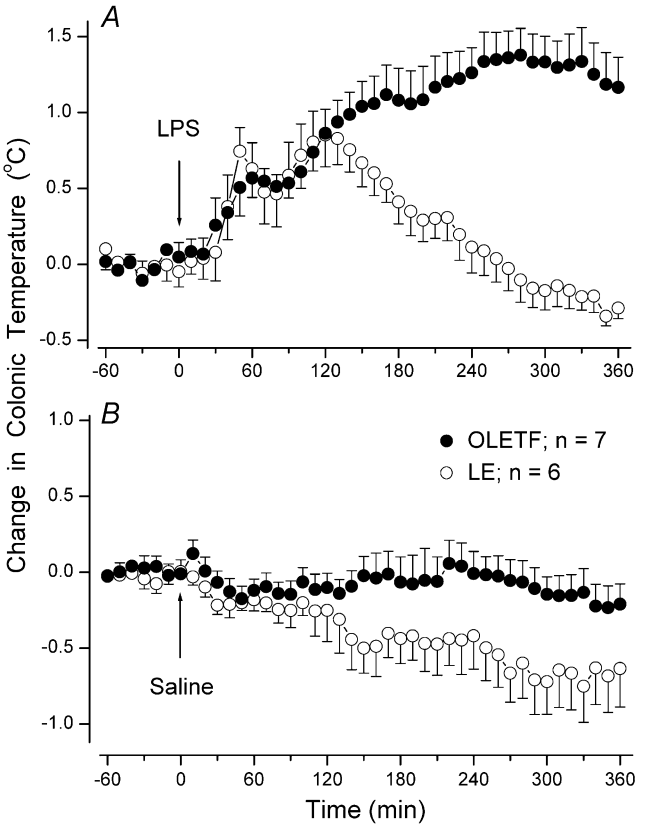

Figure 1. Thermal responses of Otsuka Long-Evans (LE) Tokushima Fatty (OLETF; a CCK-A-receptor-deficient strain) and LE (wild-type) rats to lipopolysaccharide (LPS)

The animals were injected I.V. (arrow) with either LPS (10 μg kg^{-1}; *A*) or saline (1.0 ml kg^{-1}; *B*). The initial colonic temperature (T_c) of OLETF and LE rats in the LPS experiment was 38.4 ± 0.2 and 38.6 ± 0.1 °C, respectively. The initial T_c of OLETF and LE rats in the saline experiment was 38.3 ± 0.2 and 38.6 ± 0.1 °C, respectively.

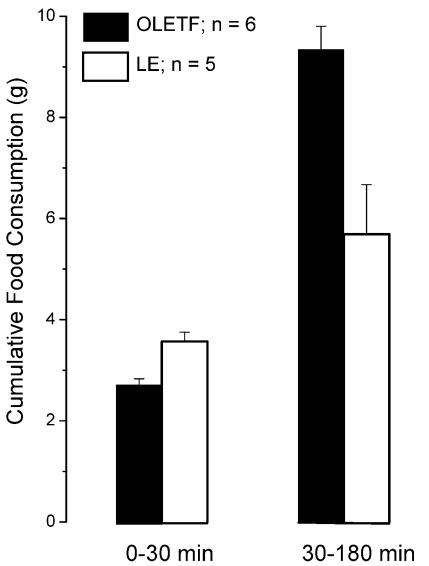

Figure 2. Cumulative food intake of the food-deprived OLETF and LE rats 0–30 and 30–180 min after food presentation

of the results obtained with LPS. Indeed, the interstrain difference in the response to saline (< 0.6 °C) was more than three times smaller than that to LPS, and it was not significant. Hence, the most striking phenomenon observed in Expt 1, the robust phase 3 in the CCK-A-receptor-deficient rats, clearly was not an artefact. This phenomenon was further studied in Expts 3 and 5.

Experiment 2: impairment of satiety in CCK-A-receptor-deficient rats

After food deprivation, the OLETF rats started eating somewhat less eagerly and consumed slightly less food during the first 30 min of exposure to food than did the LE rats (2.7 ± 0.1 *vs.* 3.5 ± 0.2 g, respectively; $P = 8.0 \times 10^{-3}$; Fig. 2). However, during the next 150 min, the OLETF rats consumed much more food than the LE rats (9.3 ± 0.4 *vs.* 5.2 ± 1.1 g, respectively; $P = 1.0 \times 10^{-2}$), thus indicating a satiety impairment. These results are consistent with the literature (Moran *et al.* 1998) and confirm a functional

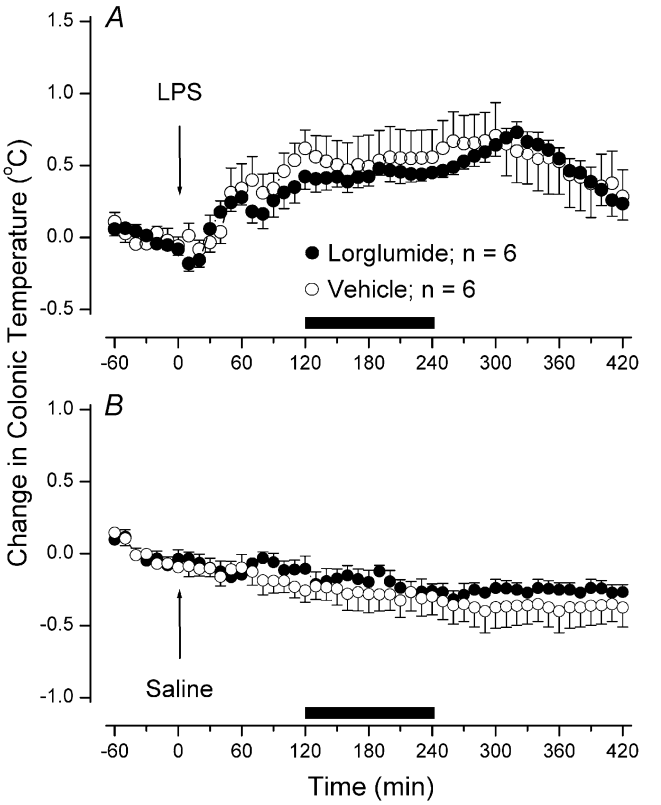

Figure 3. Thermal responses of febrile and afebrile LE rats to sodium lorglumide

The febrile (*A*) and afebrile (*B*) LE rats were infused I.V. (bar along the abscissa axis) with sodium lorglumide (4.3 μg kg^{-1} min^{-1}) or its vehicle (alkalinized saline; pH = 8.0; 17.2 μl kg^{-1} min^{-1}). In *A*, the febrile response was induced by I.V. injection (arrow) of LPS (10 μg kg^{-1}); in *B*, the rats were injected with saline (1.0 ml kg^{-1}). The initial T_c of the LPS-injected rats was 38.2 ± 0.1 °C (sodium lorglumide-infused group) and 38.1 ± 0.1 °C (vehicle-infused group). The initial T_c of the saline-injected rats was 38.2 ± 0.1 °C (sodium lorglumide-infused group) and 38.3 ± 0.2 °C (vehicle-infused group).

deficiency of the CCK-A receptor in the OLETF rats used in the present study.

Experiment 3: effect of sodium lorglumide on phase 3 of LPS-induced fever

As expected, LE rats responded to LPS with a fever characterized by a low phase 3. Infusion of the CCK-A receptor antagonist sodium lorglumide (4.3 μg kg^{-1} min^{-1}, I.V.) 120–240 min after the LPS injection did not affect this response (Fig. 3*A*). Infusion of the same dose of sodium lorglumide also did not affect the normal T_c of the LE rats (Fig. 3*B*).

Experiment 4: effect of sodium lorglumide on the development of satiety

Although ineffective in changing phase 3 of the febrile response to LPS, the same infusion of sodium lorglumide (4.3 μg kg^{-1} min^{-1}, 120 min, I.V.) effectively inhibited the satiety response of the LE rats (Fig. 4). For the first 30 min of their free access to food after food deprivation, the sodium lorglumide-infused rats consumed the same amount as their controls (2.1 ± 0.5 *vs.* 2.5 ± 0.2 g). However, their food intake during the next 150 min significantly exceeded that of the controls (4.5 ± 0.4 *vs.* 2.6 ± 0.2 g; $P = 3.0 \times 10^{-3}$). These data demonstrate the efficacy of the sodium lorglumide infusion used in Expts 3 and 4 to inhibit a known CCK-A-receptor-mediated physiological response, satiety.

Experiment 5: effect of CCK-8 on phase 3 of LPS-induced fever

A 120 min-long I.V. infusion of sulphated CCK-8 in the Wistar rats caused dose-dependent thermal effects (Fig. 5). The smallest dose used (0.03 μg kg^{-1} min^{-1}) induced a short-lasting tail skin vasodilatation (an increase in the HLI from ~0.1 to ~0.4), but did not affect T_c. The intermediate dose (0.17 μg kg^{-1} min^{-1}) caused skin vasodilatation and a moderate decrease in T_c (−0.5 ± 0.1 °C, nadir). The highest dose (0.83 μg kg^{-1} min^{-1}) caused sustained skin vasodilatation and deep hypothermia (−1.4 ± 0.3 °C, nadir). With the increase in the dose, the CCK-8 *vs.* saline differences in the thermal responses became statistically significant. For the highest dose, the P values were 5.5×10^{-3} for the HLI response and 2.3×10^{-3} for the T_c response. The two lower doses of CCK-8 were chosen for the LPS test.

As expected, Wistar rats responded to LPS with a fever characterized by a high phase 3. The infusion of the thermally ineffective dose of CCK-8 (0.03 μg kg^{-1} min^{-1}, I.V.) 120–240 min after the injection of LPS (10 μg kg^{-1}, I.V.) did not affect phase 3, but it tended to accelerate the vasodilatation burst between phases 2 and 3 (Fig. 6). The infusion of the moderately hypothermizing dose of CCK-8

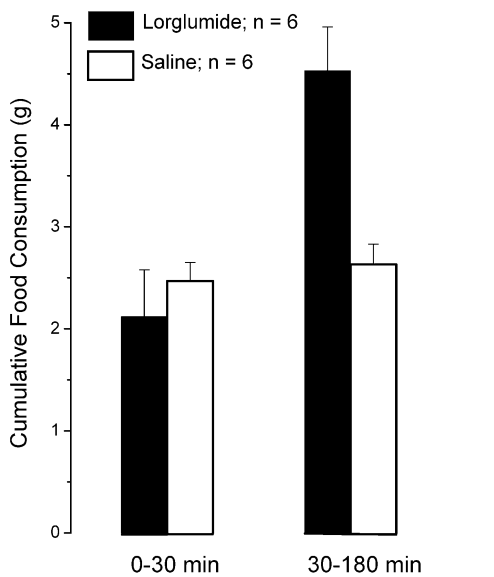

Figure 4. Cumulative food intake of the food-deprived LE rats 0–30 and 30–180 min after food presentation

Food was presented at the end of a 120 min-long I.V. infusion of either sodium lorglumide (4.3 μg kg^{-1} min^{-1}) or alkalinized saline.

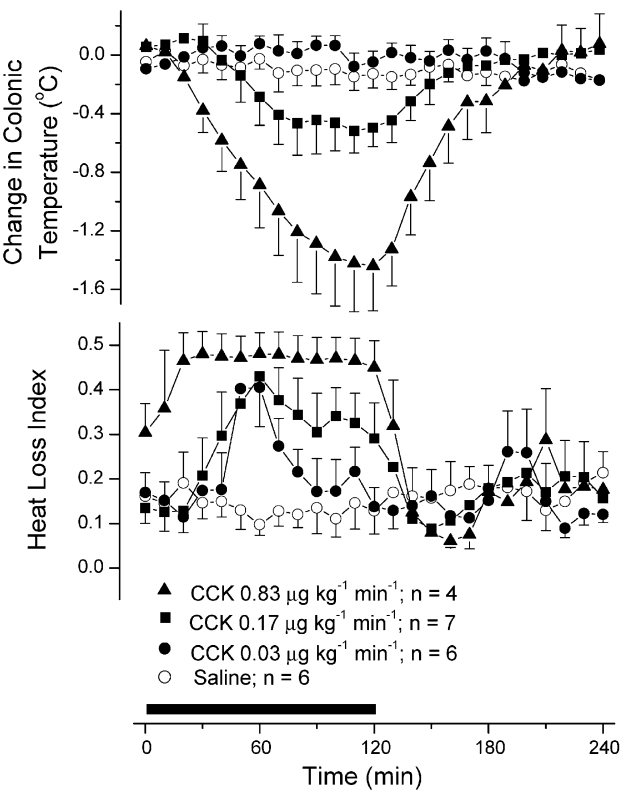

Figure 5. Dose-dependent hypothermic (upper plots) and skin vasodilatory (lower plots) responses of Wistar rats to cholecystokinin (CCK)-8 sulphate

Wistar rats were infused I.V. (bar along the abscissa axis) with CCK-8 sulphate (0.03, 0.17 or 0.83 μg kg^{-1} min^{-1}) or saline (16.7 μl kg^{-1} min^{-1}). The initial T_c in the saline-treated group was 38.1 ± 0.1 °C. The initial T_c in the three CCK-8-treated groups was 38.3 ± 0.1, 38.1 ± 0.1 and 38.0 ± 0.2 °C. The heat loss index is a ratio between two temperature gradients: skin−ambient and deep body−ambient (see Data processing and analysis in Methods); it changes from 0 (full vasoconstriction) to 1 (full vasodilatation).

(0.17 μg kg^{-1} min^{-1}) attenuated the beginning of phase 3 and tended to accelerate the preceding vasodilatation burst. The effects of the latter dose on T_c were then compared under febrile and afebrile conditions and were found to be indistinguishable (Fig. 7). In other words, Expt 5 revealed no antipyretic (specific for the febrile conditions) action of CCK-8.

DISCUSSION

Is the CCK-A receptor involved in triphasic LPS-induced fever? A knockout study

We initiated this study to test the hypothesis that CCK-A receptors on vagal afferents are essential for the development of fever, particularly its phase 1, which is mediated by the afferent vagus (Romanovsky, 2000). To this end, we measured the febrile response to LPS in OLETF rats deficient in the CCK-A receptor subtype. We expected to find a suppressed phase 1 in the OLETF strain compared to its parent strain, LE. However, the data failed to confirm the original hypothesis: it appeared that the febrile response to LPS was initiated normally in the CCK-A-receptor-deficient rats. Serendipitously, we found that the mutant and parent strains differed significantly in the their ability to mount phase 3 of LPS-induced fever.

Phase 3 of LPS-induced fever, which was often misidentified as phase 2 in earlier telemetry studies (for review, see Romanovsky *et al.* 1998*a*), is now confirmed to occur in both restrained (e.g. Romanovsky *et al.* 1998*b*) and freely moving (e.g. Oka *et al.* 2001) animals. This phase is the most prominent hallmark of fever in at least two albino rat strains, Wistar and Sprague-Dawley (Romanovsky *et al.* 1998*a*,*b*), and in some mouse strains (Oka *et al.* 2001). In these strains, it represents the greatest increase in body temperature (both in magnitude and duration) among all three febrile phases identified thus far. In contrast, phase 3 is low or even absent in the febrile response of LE (Romanovsky *et al.* 1998*b*) and LE-derived Zucker obese and Zucker lean (Ivanov & Romanovsky, 2002) rats. Not surprisingly, the LE rats used in the present study exhibited no (Otsuka Pharmaceutical; Fig. 1) or a low (Harlan; Fig. 3) phase 3. Surprisingly, the CCK-A-receptor-deficient OLETF rats, although derived from the LE strain, behaved differently: all developed robust triphasic fevers.

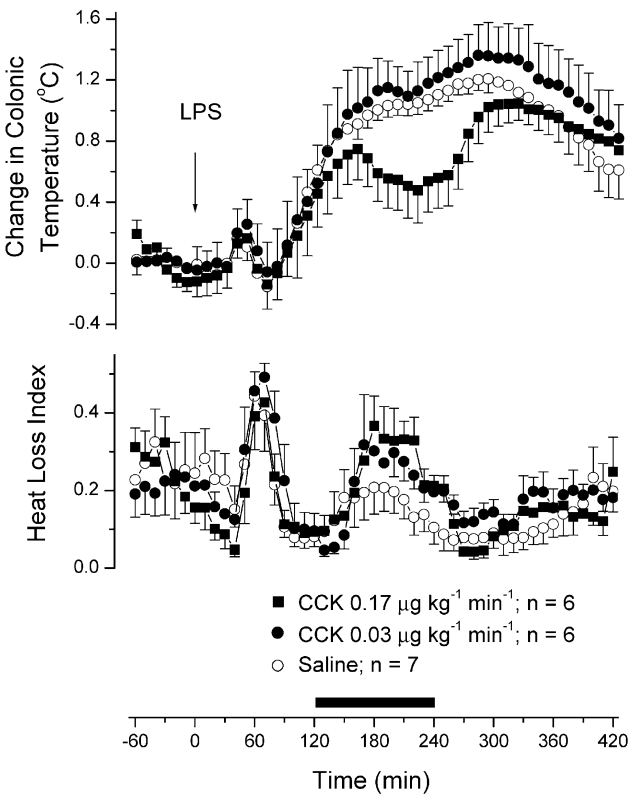

Figure 6. Effect of CCK-8 sulphate on the febrile response of Wistar rats to LPS

Wistar rats were injected I.V. (arrow) with LPS (10 μg kg^{-1}) followed by I.V. infusion (bar along the abscissa axis) of CCK-8 sulphate (0.03 or 0.17 μg kg^{-1} min^{-1}) or saline. The initial T_c was 38.3 ± 0.2 (saline), 38.1 ± 0.4 (smaller dose of CCK-8) and 38.1 ± 0.1 °C (larger dose of CCK-8).

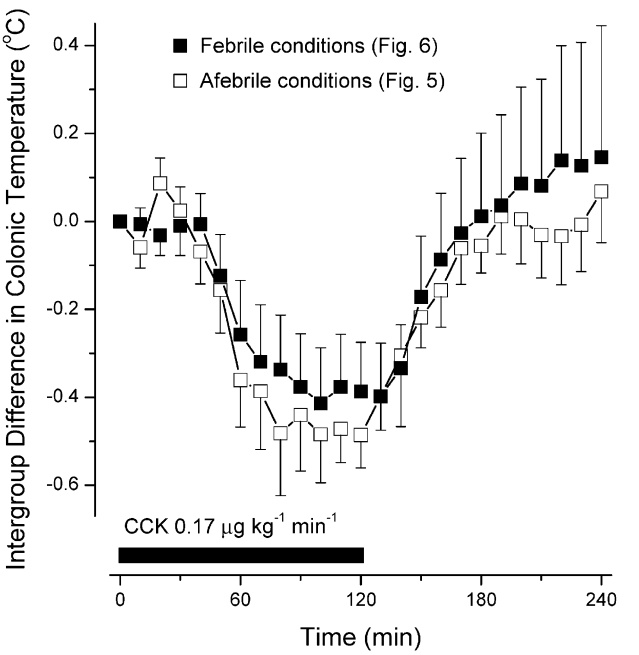

Figure 7. Comparison of the thermal responses to I.V. infusion of CCK-8 sulphate under febrile and afebrile conditions

The thermal responses to I.V. infusion (bar along the abscissa axis) of CCK-8 sulphate (0.17 μg kg^{-1} min^{-1}) under febrile (Fig. 6) and afebrile (Fig. 5) conditions are compared. For both conditions, the thermal response is plotted as the difference in T_c between the CCK-8-infused animals and the corresponding controls. For afebrile conditions, the saline curve was subtracted from the CCK-8 curve. For febrile conditions, the responses to (LPS + saline) and to (LPS + CCK-8) were first aligned at the onset of the I.V. infusion, and then the former curve was subtracted from the latter.

This finding may suggest that CCK-A receptors and their endogenous agonist, CCK, are involved in a natural antipyretic mechanism that limits phase 3. Since the central CCK increases (not decreases) body temperature in the rat, at least at thermoneutral and near-neutral conditions (Shido *et al.* 1989; Székely *et al.* 1994; Szelényi *et al.* 1994; Ghosh *et al.* 1997, 1998; Sugimoto *et al.* 1999), and because the major type of CCK receptor in the brain is the B (not A) type, an antipyretic role for central CCK-A receptors seems unlikely. A more logical proposition is that the putative mechanisms limiting phase 3 of LPS-induced fever involve peripheral CCK-A receptors. Indeed, peripheral CCK always decreases body temperature in rats, regardless of the T_a (Kapás *et al.* 1987). This effect involves both increased heat loss (skin vasodilatation) and decreased heat production (inhibition of non-shivering thermogenesis) and is mediated by the predominant peripheral receptor, CCK-A (South, 1992; Szelényi *et al.* 1994).

The inactivity of the hypothetical antipyretic system involving peripheral CCK-A receptors is not the only plausible explanation of the exaggeration of phase 3 of LPS-induced fever in OLETF rats. Knockout and mutant animals often develop multiple compensatory mechanisms and exhibit undesired biochemical and physiological abnormalities (Kluger *et al.* 1998). *A priori*, such abnormalities may affect the febrile response of OLETF rats. To determine whether the exaggeration of phase 3 reflected the lack of the CCK-A receptor *per se* or a secondary trait of the OLETF strain, a pharmacological analysis was conducted. If the absence of the receptor was responsible, a peripheral administration of antagonists and agonists of the CCK-A receptor would be expected to exaggerate and attenuate, respectively, phase 3 of LPS-induced fever. If, however, pharmacological agents failed to affect phase 3 in the predicted manner, the exaggeration phenomenon could be caused by a secondary trait of OLETF rats.

Role of the CCK-A receptor in phase 3: pharmacological analysis

The experiments with sodium lorglumide, a selective A-receptor antagonist, and sulphated CCK-8, a selective A-receptor agonist, rejected involvement of the A receptor in an antipyretic mechanism. Sodium lorglumide did not affect phase 3 of LPS-induced fever, whereas the same infusion of the antagonist effectively inhibited the CCK-A-receptor-mediated satiety response. Although CCK-8 caused a dose-dependent hypothermia and skin vasodilatation, it failed to exhibit any specific antipyretic activity. Indeed, a small dose of CCK-8 (~1 μg per rat, infused over 120 min), which had no effect on body temperature under afebrile conditions, did not affect phase 3 of LPS-induced fever. When the dose was increased six times and exceeded 10^4–10^5 total amounts of CCK in rat plasma following physiological (feeding) or inflammatory (IL-1β) stimulation of CCK secretion, it did inhibit phase 3. The calculations were based on the plasma CCK concentrations reported by Liddle *et al.* (1984) and Kurosawa *et al.* (1997); the total mass of extracellular fluid was estimated as 20 % of the body mass. Even at this enormous dose, the fever-decreasing effect of CCK-8 was small (0.4 °C) and identical to its hypothermizing effect under afebrile conditions. Such a small and non-specific effect of an agonist cannot explain the robust (1.6 °C) and specific exaggeration of phase 3 of LPS-induced fever in the receptor-deficient rats.

The findings that sodium lorglumide lacks any fever-exaggerating activity and that CCK-8 does not induce antipyresis are consistent with the literature. Several studies have reported that CCK-A receptors are not involved in limiting the febrile or other sickness responses to LPS or pyrogenic cytokines. Selective CCK-A receptor antagonists do not exaggerate (i.e. either inhibit or do not affect) the following responses: LPS-induced fever (Székely, 1995; Martin *et al.* 2000), IL-1β-induced fever (Martin *et al.* 2000), LPS-induced anorexia (Bret-Dibat & Dantzer, 2000), IL-1β-induced anorexia (Daun & McCarthy, 1993; Bret-Dibat & Dantzer, 2000), LPS-induced decrease in social exploration (Bluthé *et al.* 1997), and IL-1β-induced activation of the hypothalamo–pituitary–adrenal axis (Day & Akil, 1999). We therefore conclude that the endogenous antipyretic system does not involve CCK-A receptors.

Hence, the mechanism of the exaggeration of phase 3 in OLETF rats is likely to reflect a secondary trait of this strain. Obesity and hyperlipidaemia are the most obvious candidates (Moran *et al.* 1998). These traits affect the inflammatory response; they can arguably be regarded as symptoms of systemic inflammation (Das, 2001). *A priori*, they may be speculated to enhance febrile responsiveness. However, experimental data do not support such a speculation. Obesity has been reported to have no effect, a highly variable effect or an inhibitory effect – but not an exaggerating one – on fever (Dascombe *et al.* 1989; Rosenthal *et al.* 1996; Plata-Salamán *et al.* 1998; Ivanov & Romanovsky, 2002). Hyperlipidaemia is also likely to inhibit – not exaggerate – the febrile response (Munford *et al.* 1982).

A more plausible explanation is that the enhancement of phase 3 in OLETF rats is associated with the two CCK receptor subtypes that are overexpressed in this strain, the CCK-B receptor (Miyasaka *et al.* 1998) and/or the putative non-A, non-B receptor (Kurosawa *et al.* 1999, 2000). The B receptor is involved in both the hyperthermic response to central CCK (Szelényi *et al.* 1994) and the febrile response to I.V. LPS (Székely *et al.* 1994). The non-A, non-B subtype is involved in mediating the excitation of vagal afferents by CCK (Kurosawa *et al.* 1999); its thermoregulatory role is unknown.

The finding that cytosolic phospholipase A_2 ($cPLA_2$) activity is increased in OLETF rats (Furuya *et al.* 1999) provides another potential clue to the puzzle. Along with other types of phospholipase A_2, $cPLA_2$ is the rate-limiting enzyme in the synthesis of PGE_2 (Murakami *et al.* 1997), and PGE_2 is the key mediator of all three febrile phases (Scammell *et al.* 1998; Oka *et al.* 2001). Recently, we have found that transcription of $cPLA_2\alpha$ in the brain is significantly upregulated during phase 3 of LPS-induced fever (Ivanov *et al.* 2002). Therefore, it is possible that the robust phase 3 in OLETF rats is caused by $cPLA_2$-dependent overproduction of PGE_2. The same mechanism may underlie the reported hypersensitivity of vagal afferents to IL-1β in OLETF rats (Kurosawa *et al.* 2000). Indeed, the excitation of vagal afferents by IL-1 is mediated, at least partially, by PGs (Niijima, 1996; Ek *et al.* 1998).

Conclusions

By using the CCK-A receptor-deficient OLETF rat strain (which has not been previously tested for febrile responsiveness) and 'traditional' pharmacological tools, this study addresses the controversial issue of whether CCK and its A-receptor subtype are involved in fever. The experiment in OLETF rats showed that the CCK-A receptor is not essential for phases 1 and 2 of LPS-induced fever. The same experiment showed that phase 3 of LPS-induced fever is exaggerated in the mutant rat strain. This exaggeration can reflect either the absence of the CCK-A receptor *per se* or a secondary trait of the OLETF strain. By using a selective agonist and antagonist of the CCK-A receptor (CCK-8 sulphate and sodium lorglumide, respectively), we have rejected the former possibility. Hence, the present study shows that the CCK-A receptor and its endogenous ligand, CCK, are not involved in any of the three known phases of the febrile response of rats to a mild dose of LPS.

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