Evidence that Cholecystokinin Receptors are not Involved in the Hypothalamic-Pituitary-Adrenal Response to Intraperitoneal Administration of Interleukin-1 β

H. E. W. Day and H. Akil

Mental Health Research Institute, University of Michigan, Ann Arbor, MI, USA.

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Abstract

The purpose of this study was to investigate a putative role for cholecystokinin (CCK) in the activation of the hypothalamic-pituitary-adrenal (HPA) axis following intraperitoneal (i.p.) administration of interleukin-1-beta (IL-1 β). Previous studies predict that CCK_A receptors on vagal sensory afferents may be involved in the initiation of the stress response following an acute i.p. injection of IL-1 β . Adult male rats were given an i.p. injection of a specific CCK_A (devazepide, 1 mg/kg) or CCK_B (CI-988, 1 mg/kg) receptor antagonist, 30 min prior to an i.p. injection of rat recombinant IL-1 β (rrIL-1 β), 0.5 μ g/kg in 0.9% sterile saline/0.01% rat serum albumin. Blood samples were obtained via an indwelling jugular vein catheter, and the plasma levels of the stress hormones ACTH (adrenocorticotropin hormone) and corticosterone analysed over time as an indicator of HPA axis activation. This dose of rrIL-1 β resulted in a significant release of ACTH and corticosterone, peaking at 30–60 min, and returning to basal levels by 2 h. Pretreatment with either devazepide or CI-988 had no effect on the rrIL-1 β induced ACTH or corticosterone release. In contrast, the same dose of devazepide completely inhibited the ACTH and corticosterone response to i.p. CCK (octapeptide, sulphated form, CCK-8S), 5 μ g/kg. It is concluded that CCK receptors are not involved in the hormonal stress response to a submaximal i.p. dose of rrIL-1 β .

Systemic administration of the cytokine interleukin-1 β (IL-1 β) results in physiological responses characteristic of an immune challenge, including fever (1), anorexia (2), increased sleep (3) and hypothalamic-pituitary-adrenal (HPA) axis activation (4, 5). Activation of the HPA axis is a defining feature of a stress response, involving activation of the parvocellular neurones of the paraventricular nucleus of the hypothalamus (PVN), which in turn project via the median eminence to the anterior pituitary gland. There, stimulated corticotrophs release ACTH into the general circulation, which stimulates the release of the stress hormone corticosterone (in rats) or cortisol (in humans) from the adrenal gland.

Recently, a great deal of interest has focused on the role of afferent pathways to the hypothalamus in initiating the stress response following an acute immune challenge, such as injection of IL-1 β . Systemic administration of IL-1 β causes a specific pattern of *c-fos* mRNA and Fos protein expression in areas consistent with vagal activation, such as the medial nucleus

tractus solitarius (mNTS), where general visceral vagal sensory afferent fibres terminate (6, 7). Furthermore, knife cuts of the direct pathway from the NTS to the PVN attenuated the PVN Fos protein response to i.v. IL-1 β , suggesting the importance of this pathway in the activation of the PVN (6). Although there are still many questions regarding the mechanisms of action of i.v. IL-1 β , there is increasing evidence that the integrity of vagal sensory afferent fibres is critical for many of the responses observed following i.p. injection of IL-1 β , including HPA axis activation (8, 9). There appears to be some specificity in the type of vagal afferents involved, since a complete subdiaphragmatic vagotomy, which includes disruption of gastric vagal afferents, appears to be effective in inhibiting the effects of i.p. administration of IL-1, whereas a selective hepatic branch vagotomy is not (8, 10). It has been demonstrated that IL-1 can increase vagal afferent discharge (11), and the presence of IL-1 binding sites has been demonstrated on abdominal paranglia in a number of species, including the rat (12). However,

Correspondence to: Heidi E. W. Day, Mental Health Research Institute, University of Michigan, 205 Zina Pitcher Place, Ann Arbor, MI 48109-0720, USA (e-mail: heididay@umich.edu).

their functional role in this model has yet to be demonstrated. Hence in spite of significant progress in demonstrating neural immune–brain interactions, the actual mechanisms whereby immune factors access and mediate changes in the brain remain ill understood.

A peptide which exhibits some similarity to IL-1 β in its behavioural effects is the gastrointestinal hormone cholecystikinin (CCK). For example, i.p. injection of CCK increases sleep (13), anorexia (13) and results in HPA axis activation (14). Although peripheral CCK is unable to cross the blood–brain-barrier, the activation of the HPA axis by systemic CCK is mediated at the level of the hypothalamus, via the release of corticotropin releasing hormone (CRH) from the PVN (15). CCK is a potent stimulator of the vagus nerve (16, 17), and the presence of both receptor subtypes (CCK_A and CCK_B) has been demonstrated on the vagus nerve (18, 19). Evidence suggests that the stimulatory effect of CCK on the HPA axis is mediated by CCK_A receptors and vagal afferent fibres (15, 20). Furthermore, the brain *c-fos* mRNA and Fos protein response to a peripheral injection of CCK shows a striking resemblance to that observed following systemic administration of IL-1 β , with strong expression in areas including the NTS and PVN (6, 7, 21–25). The CCK-induced CNS *c-fos* mRNA and Fos protein expression is also dependent upon intact vagal afferent fibres, and is mediated by CCK_A receptors (22–25). Overall, these data are consistent with the idea that systemic CCK stimulates the HPA axis via activation of CCK_A receptors on vagal afferent fibres which terminate in the NTS, and which project to the hypothalamic PVN.

Beyond the evidence cited above suggesting parallels between the mechanisms of central activation by systemic CCK and IL-1 β , some recent data have suggested that the two systems may be more closely intertwined. For example, i.p. administration of IL-1 α (a peptide very closely related to IL-1 β which acts through the same receptors) increases plasma levels of CCK-like immunoreactivity (CCK-LI) (26). Furthermore, the anorexic effect elicited by IL-1 α i.p. is attenuated by pretreatment with a CCK_A receptor antagonist, suggestive of a role for CCK in this IL-1 stimulated behaviour (26). Of particular interest, is the demonstration that systemic (i.v.) administration of IL-1 β increases plasma levels of CCK-LI, and increases gastric vagal afferent nerve activity partly via stimulation of CCK_A receptors (27). Hence to summarize, the increase in HPA activity following i.p. administration of CCK is dependent on vagal afferent stimulation and is mediated by CCK_A receptors. The increase in HPA axis activity following i.p. injection of IL-1 β is at least partially dependent on vagal afferent stimulation. Systemic IL-1 (IL-1 β i.v. or IL-1 α i.p.) increases plasma levels of CCK, and the gastric vagal afferent nerve activity induced by IL-1 β i.v. is partly mediated by stimulation of CCK_A receptors. Hence this study was undertaken to test the hypothesis that the activation of the HPA axis by i.p. injection of IL-1 β is in part mediated by activation of CCK_A receptors (see Fig. 1).

Materials and methods

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used. All procedures were approved by UCUC (University Committee on Use and Care of Animals, University of Michigan). Rats were

housed three/cage, under conditions of constant temperature and humidity, on a 12-h light:dark cycle (lights on 7.00 a.m.) with access to food and water *ad libitum*. Animals were allowed to acclimatize to the housing conditions for 11–12 days prior to surgery, and were handled daily for 1 week prior to surgery. Following surgery to implant i.p. and i.v. catheters, animals were allowed to recover for 3 days. During this time, catheters were flushed daily with 0.3 ml saline (i.p.) or 0.2 ml 100 U/ml heparin-saline (i.v.).

Surgical procedure

Animals weighed ≈ 300 g at the time of surgery (range 270–324 g). Rats were anaesthetized with sodium pentobarbital, and i.v. and i.p. catheters implanted. Catheters were made from Silastic[®] tubing, 0.02" i.d. and 0.037" o.d. (Storz Medical Supplies, St. Louis, MO, USA). The i.v. catheters were 12 cm long, with 3 cm cuffs at each end. The i.p. catheters were 20 cm long with 3 cm cuffs at each end. The cuffs were made by a double ring of silicon medical adhesive (Dow Corning, Midland, MI, USA). The procedure for i.v. catheterization was adapted from Experimental and Surgical Technique in the Rat (28). Briefly, 1–2 cm of the right external jugular vein was exposed by blunt dissection and tied (anterior) to occlude the vessel. The vein was semi-transected with fine scissors, and the tip of the catheter, which was filled with 100 U/ml heparinized saline and attached to a 1-ml syringe, inserted with the aid of fine forceps. The tip was positioned at the entrance to the right atrium of the heart. A small amount (≈ 0.1 ml) of blood was withdrawn to check positioning of the catheter, and flushed back with an additional 0.2 ml heparinized saline. A stainless steel pin (27 g) was used to plug the catheter, and form a 'heparin lock'. Three ligatures were tied around the vessel and catheter: anterior, between and posterior to the first double cuff, which served to anchor the catheter in the correct position. The catheter was exteriorized subcutaneously with the aid of a 10 G trocar at the dorsal nape of the neck. A ligature was first tied around the second cuff to act as an anchor, and then used to suture the skin incision. The length of catheter between the cuffs provided slack within the body, to allow for the animal's normal movements. The i.p. catheter was inserted into the peritoneal cavity via a small hole made in the peritoneum. A ligature was tied around the first cuff to act as an anchor at the peritoneal cavity, and then used to suture the incision. The catheter was exteriorized via a second incision at the dorsal nape of the neck. Another ligature was tied around the second cuff to act as an anchor at the neck, and then used to suture the incision. Wound clips were used to close the i.p. and i.v. skin incisions. After surgery, animals were housed individually in the sampling cages, with free access to food and water. The sampling cages were circular (floor diameter of 26 cm, height 37 cm, top 30 cm) and constructed of an opaque plastic. On the day of the experiment, food and water was removed, and the two catheters connected to syringes filled with saline (i.p.) or heparin-saline (15 U/ml; i.v.) via a connector (a cut and blunted 21 G needle) and 90 cm of extension tubing (PE 60). This allowed the remote administration of drugs and blood sampling. Animals were connected between 07.00 and 08.00, and were allowed to rest for 2 h prior to the experiment.

Blood sampling and drug administration

Blood was withdrawn via the i.v. catheter without disturbing the animal. The heparin-saline (15 U/ml) in the connecting line was withdrawn slowly via a 1-ml syringe until blood was obtained. A second syringe was used to collect 300 μ l blood, which was then placed on ice in polypropylene tubes containing 30 μ l EDTA (ethylenediamine tetra-acetic acid), 20 mg/ml. The first syringe of blood/heparinized saline was re-injected slowly, and an additional 300–400 μ l heparinized saline (15 U/ml; room temperature) administered. The total time to take a blood sample was ≈ 4 min. Blood samples were spun at 4°C, and plasma aliquots stored at -80°C before analysis of ACTH and corticosterone. It was estimated that $\approx 1/6$ of the total volume of plasma collected (≈ 180 μ l) was the EDTA solution, and this was corrected in the hormone assays. Hence for a 50 μ l plasma sample, 60 μ l of the plasma aliquot was used.

All drugs and their appropriate vehicles were administered via the i.p. catheter, in a 1 ml/kg volume. An additional 0.5 ml saline (room temperature) was administered to allow for the dead space in the connecting line (≈ 0.3 ml). The injection procedure took 60–90 s. rrIL-1 β , CCK-8S and their vehicles were kept on ice prior to injection (see below for a description of controls run to check for the stress effect of cold injections). Devazepide, CI-988 and their vehicles were administered at room temperature.

Experiment 1

A dose response curve to rrIL-1 β was run, to establish a submaximal dose of IL-1 β for future experiments. In addition, the possible stress of injection of

FIG. 1. Schematic diagram to show potential mechanisms (dotted lines) whereby rrIL-1 β activates the HPA axis in the rat. A. Pit: anterior pituitary; CRH: corticotropin releasing hormone; NTS: nucleus tractus solitarius; PVN: paraventricular nucleus of the hypothalamus.

cold solution (rrIL-1 β , CCK-8S or their vehicles) was assessed, together with the effect of addition of rat serum albumin to the saline used to dissolve IL-1 β . In the first part of the experiment, the following groups of animals were used: (1): no injection ($n=6$); (2) 0.9% pyrogen free saline i.p. ($n=5$); (3) rrIL-1 β 0.5 $\mu\text{g/kg}$ i.p. ($n=5$); (4) rrIL-1 β 1 $\mu\text{g/kg}$ i.p. ($n=5$); (5) rrIL-1 β 2 $\mu\text{g/kg}$ i.p. ($n=6$); and (6) rrIL-1 β 4 $\mu\text{g/kg}$ i.p. ($n=4$). All doses of rrIL-1 β for the first part of the experiment were dissolved in pyrogen-free saline. Blood samples were taken immediately prior to the injection ($t=0$), $t=15$, 30, 60, 120 and 240'. Samples were taken between 10.00 and 16.00 h. In the second part of the experiment, the following groups were used: (1) 0.9% pyrogen-free saline with 0.01% rat serum albumin i.p. (saline/rsa; $n=3$); (2) 0.5 $\mu\text{g/kg}$ rrIL-1 β in saline i.p. ($n=2$); (3) 0.5 μg rrIL-1 β in saline/rsa i.p. ($n=3$); (4) rrIL-1 β 1 $\mu\text{g/kg}$ in saline i.p. ($n=3$); (5) rrIL-1 β 1 $\mu\text{g/kg}$ in saline/rsa i.p. ($n=3$); and (6) heat inactivated (boiled for 2 h) rrIL-1 β 0.5 $\mu\text{g/kg}$ in saline/rsa i.p. ($n=3$). Blood samples were taken immediately prior to the injection ($t=0$), $t=10$, 20, 30, 45, 60 and 120. Samples were taken between 10.00 and 14.00 h.

Experiment 2

The effect of pretreatment with the CCK $_A$ or CCK $_B$ receptor antagonists, devazepide or CI-988, respectively, was investigated. Devazepide was dissolved to a final concentration of 1 mg/kg/ml in 1:1:8 dimethylsulphoxide: Tween80: 1% carboxymethylcellulose. CI-988 was dissolved in 0.9% pyrogen-free saline to a final concentration of 1 mg/kg/ml. A baseline blood sample was taken, and the antagonist or appropriate vehicle administered via the i.p. catheter ($t=-30'$). A second blood sample was taken 20 min later. rrIL-1 β 0.5 $\mu\text{g/kg/ml}$ or vehicle (0.9% pyrogen-free saline/0.01% rat serum albumin) was administered 30 min after the antagonist via the i.p. catheter ($t=0'$). In addition, the ability of devazepide to antagonize the stress response to CCK-8S administration was verified. Devazepide (1 mg/kg/ml) or vehicle was administered 30 min prior to CCK-8S (5 $\mu\text{g/kg/ml}$ i.p.) or vehicle (0.9% pyrogen free saline/0.01% rat serum albumin). Blood samples were collected at $t=-40'$, $-10'$, 15', 30', 45', 60' and 120', between 10.00 and 15.00 h. On each day, 7–9 animals were run, and groups were randomized between and within days. The groups were as follows: saline + saline/rsa ($n=5$); DMSO/Tween/CMC + saline/rsa ($n=7$); CI-988 + saline/rsa ($n=5$); Devazepide + saline/rsa ($n=5$); saline + rrIL-1 β ($n=5$); DMSO/Tween/CMC + rrIL-1 β ($n=8$); CI-988 + rrIL-1 β ($n=6$); Devazepide + rrIL-1 β ($n=10$); DMSO/CMC/Tween + CCK-8S ($n=6$); Devazepide + CCK-8S ($n=5$).

ACTH assay

A sandwich radioimmunoassay technique was used to assess plasma levels of ACTH (Nichols Institute Diagnostic Kit, CA, USA). Briefly, the procedure

involved incubation of the plasma sample (50 μl), made up to 200 μl with the zero standard, two antibodies: a radiolabelled (^{125}I) monoclonal antibody, which binds to the N-terminal of ACTH, and a biotinylated polyclonal antibody which binds to the C-terminal region of ACTH. In addition, the reaction mix was incubated with an avidin-coated bead, used to bind the sandwich complex via an avidin biotin reaction. Since the sandwich complex requires both C- and N-terminal regions, the radioactivity was proportional to the amount of intact ACTH in the sample, and was calculated by comparison with a standard curve.

Corticosterone assay

The radioimmunoassay for analysis of plasma corticosterone has been described previously (7). Briefly, 5 μl plasma was incubated overnight with ^3H -corticosterone and an antibody to corticosterone raised in our laboratory, in phosphate buffered saline/0.25% bovine serum albumin. Bound vs free corticosterone was separated by charcoal extraction, and the amount of corticosterone calculated by comparison with a standard curve.

Statistics

Results are expressed as mean $\pm$ SEM throughout. The total area under the curve ($y=0$ baseline) was calculated for ACTH and corticosterone levels across the sampling time period, using trapezoidal integration (GraphPad Prism). Data were analysed by one-way ANOVA followed by the Tukey *post-hoc* comparison test. Linear trend analysis was performed for the initial dose response curve in experiment 1. For analysis of individual time point data, *t*-tests were performed. Significance was set at $P<0.05$.

Results

Experiment 1

Part 1

The procedure of taking blood samples (no injection group) did not result in a significant rise in ACTH or corticosterone release (data not shown). Although not significantly different, there was a slight elevation in corticosterone levels at 240 min (e.g. no injection group rose from 2.0 ± 0.7 at $t=0'$ to 5.4 ± 0.9 $\mu\text{g/dl}$ at $t=240'$, most likely due to the normal circadian rise, since the last samples were taken between

14.00 and 16.00 h. The total volume of blood withdrawn may also have been a factor, despite replacement with saline. Administration of cold fluid i.p. (0.9% pyrogen free saline) did not cause a stress effect, and there were no significant differences between the saline and no injection groups across the time period (Table 1). The administration of rrIL-1 β in saline, 0.5–4 $\mu\text{g/kg}$ i.p. caused a dose-related increase in plasma ACTH (linear contrast, $P < 0.001$; Table 1) and corticosterone levels (linear contrast, $P < 0.001$; Table 1). For ACTH, rrIL-1 β 0.5 $\mu\text{g/kg}$ produced a modest rise, peaking at 30 min (205 ± 82 pg/ml, $n = 5$, compared with saline 8 ± 3.8 pg/ml, $n = 5$; $P < 0.05$), and returning to basal levels

TABLE 1. Experiment 1a: AUC Over 4 h Sampling Period: Effect of IL-1 β i.p. on ACTH and CORT Levels.

Dose IL-1 β $\mu\text{g/kg}$	ACTH pg/ml in 4 h	CORT $\mu\text{g/dl}$ in 4 h	n
No injection	2475 $\pm$ 495	725 $\pm$ 127	6
saline	2829 $\pm$ 674	817 $\pm$ 148	5
0.5	10 268 $\pm$ 1828	1306 $\pm$ 126	5
1	16 358 $\pm$ 5123	1597 $\pm$ 376	5
2	29 644 $\pm$ 10 762*	2094 $\pm$ 285*	6
4	48 377 $\pm$ 19 230†	2341 $\pm$ 468†	4

Values represent the mean total area under the curve (AUC) $\pm$ sem. Data were analysed by ANOVA, followed by Tukey's *post-hoc* comparison test. * $P < 0.05$; † $P < 0.01$ with respect to vehicle control.

TABLE 2. Experiment 1b: AUC Over 2 h Sampling Period: Effect of IL-1 β i.p. $\pm$ 0.01% Rat Serum Albumin (RSA) or Heat-treated IL-1 β i.p. on ACTH and CORT Levels.

Dose IL-1 β $\mu\text{g/kg}$	ACTH pg/ml in 2 h	CORT $\mu\text{g/dl}$ in 2 h	n
Saline + rsa	3209 $\pm$ 274	282 $\pm$ 10	3
0.5 – rsa	10 794 $\pm$ 3606	1104 $\pm$ 240*	2
0.5 + rsa	25 885 $\pm$ 5835*	1986 $\pm$ 69‡§	3
1 – rsa	16 773 $\pm$ 3265*	1554 $\pm$ 162‡	3
1 + rsa	60 743 $\pm$ 12 385‡¶	2241 $\pm$ 202‡	3
heated 0.5 + rsa	3188 $\pm$ 1053	437 $\pm$ 114	3

Values represent the mean total area under the curve (AUC) $\pm$ SEM. Data were analysed by ANOVA, followed by Tukey's *post-hoc* comparison test.

* $P < 0.05$; ‡ $P < 0.001$, with respect to appropriate vehicle control group. § $P < 0.05$ with respect to 0.5 – rsa group. ¶ $P < 0.01$, with respect to 1 – rsa group.

TABLE 3. Experiment 2a: AUC Over 2 h Sampling Period: the Effect of Devazepide (DEV) 1 mg/kg i.p. or Vehicle (DMSO) 30 min Prior to IL-1 β 0.5 $\mu\text{g/kg}$ i.p. or Saline/rsa (Sal+) on ACTH and CORT Levels.

Treatment	ACTH pg/ml in 2 h	CORT $\mu\text{g/dl}$ in 2 h	n
DMSO/Sal +	3175 $\pm$ 490	398 $\pm$ 115	7
DMSO/IL-1 β	30 853 $\pm$ 6265*	1792 $\pm$ 246†	8
DEV/Sal +	6036 $\pm$ 481	603 $\pm$ 121	5
DEV/IL-1 β	31 966 $\pm$ 5148†	1800 $\pm$ 184†	10

Values represent the mean total area under the curve (AUC) $\pm$ SEM. Data were analysed by ANOVA, followed by Tukey's *post-hoc* comparison test. * $P < 0.01$; † $P < 0.001$, with respect to appropriate vehicle control group.

TABLE 4. Experiment 2b: AUC Over 2 h Sampling Period: Effect of CI-988 1 mg/kg i.p. or Saline 30 min Prior to IL-1 β 0.5 $\mu\text{g/kg}$ i.p. or Saline/rsa (Sal+) on ACTH and CORT Levels.

Treatment	ACTH pg/ml in 2 h	CORT $\mu\text{g/dl}$ in 2 h	n
saline/Sal +	4101 $\pm$ 1031	325 $\pm$ 87	5
saline/IL-1 β	25 885 $\pm$ 6038*	1774 $\pm$ 198†	5
CI-988/Sal +	3546 $\pm$ 894	336 $\pm$ 127	5
CI-988/IL-1 β	23 215 $\pm$ 768*	1551 $\pm$ 224†	6

Values represent the mean total area under the curve (AUC) $\pm$ SEM. Data were analysed by ANOVA, followed by Tukey's *post-hoc* comparison test. * $P < 0.01$; † $P < 0.001$, with respect to appropriate vehicle control group.

TABLE 5. Experiment 2c: AUC Over 2-h Sampling Period: the Effect of Devazepide (DEV) 1 mg/kg i.p. or Vehicle (DMSO) 30 min Prior to CCK-8S 5 $\mu\text{g/kg}$ i.p. or Saline/rsa (Sal+), on ACTH and Corticosterone (CORT) Levels.

Treatment	ACTH pg/ml in 2 h	CORT $\mu\text{g/dl}$ in 2 h	n
DMSO/Sal +	3175 $\pm$ 490	398 $\pm$ 115	7
DMSO/CCK-8S	19 762 $\pm$ 4021*	1407 $\pm$ 142*	6
DEV/Sal +	6036 $\pm$ 481	603 $\pm$ 121	5
DEV/CCK-8S	3140 $\pm$ 570†	549 $\pm$ 110†	5

Values represent the mean total area under the curve (AUC) $\pm$ SEM. Data were analysed by ANOVA, followed by Tukey's *post-hoc* comparison test. * $P < 0.001$, with respect to appropriate vehicle control group. † $P < 0.001$ with respect to DMSO/CCK-8S group.

by 120 min (7 ± 3.4 pg/ml). For the 1 $\mu\text{g/kg}$ dose, the time course was similar, but the peak was greater (331 ± 118 pg/ml, $n = 5$; $P < 0.05$ compared with saline at $t = 30'$). For 2 and 4 $\mu\text{g/kg}$ rrIL-1 β , the peak values did not increase (350 ± 129 pg/ml, $n = 6$ and 351 ± 178 pg/ml, $n = 4$, respectively) but the time course was increasingly prolonged (at $t = 60'$ levels for 2 and 4 $\mu\text{g/kg}$ doses were 257 ± 125 and 341 ± 177 pg/ml, respectively, $P < 0.05$ for both doses compared with saline, 22 ± 8.8 pg/ml), and did not return to basal levels until 240 min. For corticosterone, a similar pattern to ACTH was observed, but with a slight delay in response. For example, for the 2 $\mu\text{g/kg}$ dose, the peak for ACTH occurred at 30 min (350 ± 129 pg/ml), and the levels were in decline at 60 min (257 ± 125 pg/ml), whereas corticosterone levels were still maximal at 60 min (15.6 ± 2.8 $\mu\text{g/dl}$ at $t = 30'$, $P < 0.01$ compared with saline 1.5 ± 0.5 $\mu\text{g/dl}$; 16.8 ± 2.5 $\mu\text{g/dl}$ at $t = 60'$, $P < 0.01$ compared with saline 2.3 ± 0.6 $\mu\text{g/dl}$). As was observed for ACTH, the maximal peak for corticosterone was obtained with doses of 1 $\mu\text{g/kg}$ and higher (18.8 ± 2.0 $\mu\text{g/dl}$ at $t = 30'$ for 1 $\mu\text{g/kg}$ dose, $P < 0.01$ compared with saline), while with increasing doses (2 and 4 $\mu\text{g/dl}$), the peak did not increase but the time course was more prolonged (data not shown). For all doses, corticosterone had returned to basal levels by 240 min.

Part 2

The addition of 0.01% rat serum albumin to the saline vehicle had no effect on plasma levels of ACTH and corticosterone when administered alone (Table 2). However, when this was

used as a vehicle for rrIL-1 β , this resulted in at least a doubling of the hormone response, suggesting that the rrIL-1 β was adhering to the tubing (Table 2). For example, a 0.5 μ g/kg dose rrIL-1 β in saline/rsa produced a slightly greater hormonal response than 1 μ g/kg rrIL-1 β in saline. Since 0.5 μ g/kg rrIL-1 β in saline/rsa resulted in a submaximal hormonal response, and gave a sharp peak for both ACTH and corticosterone, returning to basal levels by 120 min, this was chosen as the dose against which to run the antagonists. In order to verify that the stress hormone response was not due to a non-specific pathogen in the rrIL-1 β preparation, three animals were treated with heat inactivated rrIL-1 β , 0.5 μ g/kg in saline/rsa. The ACTH and corticosterone responses in these animals were not significantly different from vehicle controls (Table 2).

Experiment 2

Treatment with either antagonist vehicle (saline or DMSO/carboxymethylcellulose/Tween80), followed by saline/rsa injection did not result in a significant increase in either ACTH or corticosterone secretion (Tables 3–5 and Figs 2, 3). As expected, the administration of antagonist vehicle followed by rrIL-1 β , 0.5 μ g/kg (in saline/rsa) i.p. resulted in a significant rise in ACTH ($P < 0.01$, relative to their appropriate vehicle controls) and corticosterone ($P < 0.001$ relative to their appropriate vehicle controls) (Tables 3, 4; Fig. 2). Levels of ACTH and corticosterone peaked between 30 and 60 min, and were back to (ACTH) or approaching (corticosterone) basal levels by 120 min (Fig. 2). The administration of devazepide 1 mg/kg i.p. (Table 3, Fig. 2) or CI-988 1 mg/kg i.p. (Table 4) 30 min prior to rrIL-1 β , 0.5 μ g/kg i.p., did not prevent the rrIL-1 β -induced rise in ACTH and corticosterone. There were no significant differences across time between the saline/rrIL-1 β and CI-988/rrIL-1 β groups, or DMSO/rrIL-1 β and devazepide/rrIL-1 β groups. In contrast, a 30-min pretreatment with the same dose of devazepide completely inhibited the CCK-8S induced rise in ACTH ($P < 0.01$; Table 5; Fig. 3) and corticosterone ($P < 0.001$; Table 5; Fig. 3).

Discussion

The possibility that systemic IL-1 β activates the HPA axis by a CCK-receptor mechanism was investigated in the present study. No evidence was found to suggest that a submaximal

FIG. 2. Graph to show the effect of the CCK_A receptor antagonist, devazepide (Dev; 1 mg/kg i.p.) or vehicle (DMSO) on ACTH (upper panel) and corticosterone (lower panel) responses to rrIL-1 β , 0.5 μ g/kg i.p. in saline/0.01% rsa (Sal+). Values represent the mean for each group ($n = 5$ –10 per group). Analysis of total area under the curve indicated that ACTH ($P < 0.01$) and corticosterone ($P < 0.001$) levels for DMSO/IL-1 β and Dev/IL-1 β groups were both significantly greater than DMSO/Sal+ group. Levels of ACTH and corticosterone for DMSO/IL-1 β and Dev/IL-1 β groups were significantly greater than DMSO/Sal+ or Dev/Sal+ groups at 30, 45 and 60 min ($P < 0.01$ for all). At $t = 120'$, the DMSO/IL-1 β group had significantly greater corticosterone than DMSO/Sal+ group, $P < 0.05$. There were no significant differences between DMSO/IL-1 β and Dev/IL-1 β groups in either ACTH or corticosterone, for total area under the curve, or at any individual time point.

i.p. injection of IL-1 β releases ACTH or corticosterone via the activation of CCK receptors, since neither pretreatment with the CCK_A receptor antagonist, devazepide, nor CCK_B receptor antagonist, CI-988, inhibited the stress hormone response to this peptide. In contrast, the same dose of devazepide completely inhibited the ACTH and corticosterone response to an i.p. injection of CCK-8S. Although the efficacy of CI-988 was not tested in the present study, this compound has nanomolar affinity for CCK_B receptors, and has been shown to be effective in animal tests of anxiolytic behaviour with a dose as low as 0.001 mg/kg (29). Hence it is highly likely that the 1 mg/kg dose employed in this study inhibits the majority of CCK_B receptors, but was ineffective in inhibiting the IL-1 β -induced stress response. Although our hypothesis did not predict a role for CCK_B receptors in the HPA axis response to i.p. IL-1 β , we cannot rule out the possibility that an additional subtype of CCK_B receptor, at which CI-988 is a less effective antagonist, may be involved in the response (30).

The lack of effect of devazepide in inhibiting the stress response to IL-1 β is in contrast to predicted results. Our original hypothesis was that IL-1 β acts via vagal IL-1 receptors, but also causes release of peripheral CCK, which would act on vagal CCK_A receptors, with both mechanisms involved in HPA axis activation (Fig. 1). Our data raise two fundamental questions. The first is whether or not this dose of IL-1 β is acting via the vagus to elicit HPA axis activation, or whether other mechanisms are more important. Secondly, was CCK in fact released by this dose of IL-1 β ? To address the first issue, current evidence suggests that the dose of IL-1 β is important in determining the relative role of the vagus in mediating its effects, with higher doses likely to engage additional mechanisms of action. For example, with high doses, it is probable that IL-1 β enters the general circulation in sufficient concentrations to access the brain via alternative routes, such as via the circumventricular organs (31), by a direct transport mechanism (32), or by causing an inflammatory reaction at the blood brain barrier, resulting in leakiness (33). There is also evidence that IL-1 β can act directly on components of the HPA axis, such as the median eminence (34). The dose of IL-1 β used in the present experiment was chosen because it was submaximal, and gave a sharp peak of ACTH and corticosterone release, returning

FIG. 3. Graph to show the effect of the CCK_A receptor antagonist devazepide (1 mg/kg i.p.; Dev) or vehicle (DMSO) on ACTH (upper panel) and corticosterone (lower panel) responses to CCK-8S, 5 µg/kg i.p. in saline/0.01% rat serum albumin (Sal+). Values represent the mean for each group (n=5-7 per group). Analysis of total area under the curve indicated that ACTH ($P<0.001$) and corticosterone ($P<0.001$) levels for the DMSO/CCK group were significantly greater than for DMSO/Sal+, Dev/Sal+ and Dev/CCK groups. Levels of ACTH for the DMSO/CCK group were significantly greater than DMSO/Sal+ or Dev/CCK groups at 15 min ($P<0.001$), and at 30 min ($P<0.01$). Levels of corticosterone for the DMSO/CCK group were significantly greater than DMSO/Sal+ or Dev/CCK groups at 15, 30 and 45 min ($P<0.001$), and greater than DMSO/Sal+ group at 60 min ($P<0.05$). There were no significant differences between the Dev/Sal+ and Dev/CCK groups for either ACTH or corticosterone, for total area under the curve, or at any individual time point.

to basal levels by the end of a 2-h test period. Although not tested directly, this dose of IL-1 β (0.5 μ g/kg) is almost certainly low enough to be activating the HPA axis via vagal mechanisms. In published experiments, subdiaphragmatic vagotomies were able to attenuate corticosterone release even after 10 μ g/kg i.p. IL-1 β (8), ACTH release after 20 μ g/kg i.p. IL-1 β (9), and to abolish ACTH secretion after 4 μ g/kg i.p. IL-1 β (9). Hence, evidence to date suggests that for a 0.5- μ g/kg dose of IL-1 β , it is primarily vagal mechanisms that are employed in the stimulation of the HPA axis. With regard to whether CCK was released at this dose of IL-1 β , the data are less clear. Published data indicate that IL-1 α , 4 $\times$ 10.6 D10 units i.p., can increase plasma levels of CCK-LI (26), although the relationship of the dose used in that study, relative to that used in the present study, is not clear. In addition, a 2- μ g/kg i.v. dose of IL-1 β is sufficient to elicit an increase in plasma CCK-LI (27). More experiments will be required to determine more specifically the conditions under which IL-1 is capable of releasing CCK. It is certainly possible that only relatively high, pharmacological doses of IL-1 β are capable of releasing CCK. However, even if lower, more physiologically relevant doses, such as the one used in the present study, are sufficient to release CCK, it is clear from the present study that CCK receptors are not involved in the HPA axis response to a submaximal dose of IL-1 β . Hence, regardless of whether or not CCK is released, we believe that activation of CCK receptors is unlikely to be an important mechanism in physiological activation of the HPA axis by IL-1 β . Interestingly, in keeping with the results reported here, antagonism of CCK receptors was unable to inhibit the decrease in social exploration induced by another type of immune challenge, i.p. injection of lipopolysaccharide (35).

Although in this model, IL-1 β does not appear to activate the HPA axis via a CCK receptor mechanism, there is still the possibility that there are interactions between the two systems. A recent paper by Bucinskaite and colleagues, describes a sensitization of vagal nerve activity to CCK, following IL-1 β pretreatment (36). Hence, it is possible that IL-1 β in combination with CCK may elicit a synergistic effect on HPA axis activity. Interestingly, a functional synergism between CCK and another peptide, leptin, on food intake and Fos protein expression in the brain has been described (37–39). This raises the intriguing possibility of a similar interaction between CCK and IL- β .

Although both CCK and IL-1 β receptors have been demonstrated on the vagus nerve, there is no anatomical evidence to date to show whether the receptors are localized on the same type of vagal afferents. Some evidence suggests that in fact these two receptor populations may be localized on different neuronal populations, since a capsaicin pretreatment to ablate the C-fibre type afferents was not sufficient to inhibit the IL-1 β (4 μ g/kg i.p.)-induced decrease in food motivated behaviour, but was able to reduce CCK-induced anorexia (40). In contrast, capsaicin pretreatment was able to attenuate the ACTH response to an i.v. injection of IL-1 β (2 μ g/kg) (41). The difference between the two studies may be due to differences in route of administration and dose. Alternatively, these data collectively raise the question of whether or not there is more than one type of IL-1 β sensitive afferent pathway. It is possible that different behavioural effects

are mediated by capsaicin sensitive vs non-sensitive sensory fibres. Similarly, it is conceivable that different IL-1 β -mediated responses may be CCK-dependent or -independent. Colocalization studies for the different subtypes of IL-1 and CCK receptors in neurochemically identified neuronal populations of the vagus nerve, will provide further insight into the neural pathways utilized by IL-1 β .

In conclusion, data from the present study provide evidence that CCK receptors are not involved in the release of ACTH and corticosterone in response to intraperitoneal administration of a submaximal dose of IL-1 β .

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References

- 1 Kluger M. Fever: role of endogenous pyrogens and cryogens *Physiol Rev* 1991; **71**: 93–127.
- 2 McCarthy DO, Kluger MJ, Vander AJ. Suppression of food intake during infection: is interleukin-1 involved? *Am J Clin Nutr* 1985; **42**: 1179–1182.
- 3 Krueger J, Walter J, Dinarello C, Wolff S, Chedid L. Sleep promoting effects of endogenous pyrogen (interleukin-1). *Am J Physiol* 1984; **246**: R994–R999.
- 4 Berkenbosch F, Van Oers J, Del Rey A, Tilders F, Besedovsky H. Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science* 1987; **238**: 524–526.
- 5 Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W. Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. *Science* 1987; **238**: 522–524.
- 6 Ericsson A, Kovacs KJ, Sawchenko PE. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. *J Neurosci* 1994; **14**: 897–913.
- 7 Day HEW, Akil H. Differential pattern of *c-fos* mRNA in rat brain following central and systemic administration of interleukin-1-beta: implications for mechanism of action. *Neuroendocrinology* 1996; **63**: 207–218.
- 8 Fleshner M, Goehler L, Hermann J, Relton J, Maier S, Watkins L. Interleukin-1 β induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. *Brain Res Bull* 1995; **37**: 605–610.
- 9 Kapcala LP, He JR, Gao Y, Pieper JO, DeTolla LJ. Subdiaphragmatic vagotomy inhibits intra-abdominal interleukin-1 β stimulation of adrenocorticotropin secretion. *Brain Res* 1996; **728**: 247–254.
- 10 Laviano A, Yang Z-J, Meguid M, Koseki M, Beverly J. Hepatic vagus does not mediate IL-1 α induced anorexia. *Neuroreport* 1995; **6**: 1266–1268.
- 11 Nijima A. The afferent discharges from sensors for interleukin-1 β in the hepato-portal system in the anaesthetized rat. *J Physiol* 1991; **446**: 236P.
- 12 Goehler LE, Relton JK, Dripps D, Kiechle R, Tartaglia N, Maier SF, Watkins LR. Vagal paranglia bind biotinylated interleukin-1 receptor antagonist. *Brain Res Bull* 1997; **43**: 357–364.
- 13 Crawley J. Biological actions of cholecystokinin. *Peptides* 1994; **15**: 731–755.
- 14 Sander L, Porter J. Influence of bombesin, CCK, secretin and CRF on corticosterone concentration in the rat. *Peptides* 1988; **9**: 113–117.
- 15 Kamilaris TC, Johnson EO, Calogero AE, Kalogeras KT, Bernardini R, Chrousos GP, Gold PW. Cholecystokinin-octapeptide stimulates

- hypothalamic-pituitary-adrenal function in rats: Role of corticotropin releasing hormone. *Endocrinology* 1992; **130**: 1764–1774.
- 16 Davison JS. Activation of vagal gastric mechanoreceptors by cholecystokinin. *Proc West Pharmacol Soc* 1986; **29**: 363–366.
 - 17 Blackshaw L, Grundy D. Effects of cholecystokinin (CCK-8) on two classes of gastroduodenal vagal afferent fibre. *J Auton Nerv Syst* 1990; **31**: 191–202.
 - 18 Zarbin MA, Wamsley JK, Innis RB, Kuhar MJ. Cholecystokinin receptors: presence and axonal flow in the rat vagus. *Life Sci* 1981; **29**: 697–705.
 - 19 Mercer JG, Lawrence CB. Selectivity of Cholecystokinin (CCK) receptor antagonists, MK-329 and L-365, 260, for axonally-transported CCK binding sites on the rat vagus nerve. *Neurosci Letts* 1992; **137**: 229–231.
 - 20 Katsuura G, Ibii N, Matsushita A. Activation of CCK-A receptors induces elevation of plasma corticosterone in rats. *Peptides* 1992; **13**: 203–205.
 - 21 Verbalis JG, Stricker EM, Robinson AG, Hoffman GE. Cholecystokinin activates *c-fos* expression in hypothalamic oxytocin and corticotrophin-releasing hormone neurons. *J Neuroendocrinol* 1991; **3**: 205–213.
 - 22 Luckman SM. Fos-like immunoreactivity in the brainstem of the rat following peripheral administration of cholecystokinin. *J Neuroendocrinol* 1992; **4**: 149–152.
 - 23 Fraser KA, Davison JS. Cholecystokinin-induced *c-fos* expression in the rat brain stem is influenced by vagal integrity. *Exp Physiol* 1992; **77**: 225–228.
 - 24 Chen D-Y, Deutsch JA, Gonzalez MF, Gu Y. The induction and suppression of *c-fos* expression in the rat brain by cholecystokinin and its antagonist L364,718. *Neurosci Letts* 1993; **149**: 91–94.
 - 25 Day HEW, McKnight AT, Poat JA, Hughes J. Evidence that cholecystokinin induces immediate early gene expression in the brainstem, hypothalamus and amygdala of the rat by a CCK_A receptor mechanism. *Neuropharmacology* 1994; **33**: 719–727.
 - 26 Daun JM, McCarthy DO. The role of cholecystokinin in interleukin-1-induced anorexia. *Physiol Behav* 1993; **54**: 237–241.
 - 27 Kurosawa M, Uvnäs MK, Miyasaka K, Lundberg T. Interleukin-1 increases activity of the gastric vagal afferent nerve partly via stimulation of type A CCK receptor in anesthetized rats. *J Auton Nerv Syst* 1997; **62**: 72–78.
 - 28 Waynforth H, Flecknell P. *Experimental and Surgical Technique in the Rat*. London: Academic Press, 1992.
 - 29 Singh L, Lewis A, Field M, Hughes J, Woodruff G. Evidence for an involvement of the brain cholecystokinin B receptor in anxiety. *Proc Natl Acad Sci USA* 1991; **88**: 1130–1133.
 - 30 Ladurelle N, Sebret A, Garbay C, Roques B, Dauge V. Opposite effects of CCK_B agonists in grooming behaviour in rats: further evidence for two CCK_B subsites. *Br J Pharmacol* 1998; **124**: 1091–1098.
 - 31 Katsuura G, Arimura A, Koves K, Gottschall P. Involvement of organum vasculosum of lamina terminalis and preoptic area in interleukin 1 β -induced ACTH release. *Am J Physiol* 1990; **258**: E163–E171.
 - 32 Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human interleukin (IL) 1 α , murine IL-1 α and murine IL-1 β are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Ther* 1991; **259**: 988–996.
 - 33 Quagliarello V, Wispelwey B, Long W, Scheld W. Recombinant human interleukin-1 induces meningitis and blood-brain-barrier injury in the rat. *J Clin Invest* 1991; **87**: 1360–1366.
 - 34 Matta S, Singh J, Newton R, Sharp B. The adrenocorticotropin response to interleukin-1 β instilled into the rat median eminence depends on the local release of catecholamines. *Endocrinology* 1990; **127**: 2175–2182.
 - 35 Bluthé RM, Michaud B, Kelley KW, Dantzer R. Cholecystokinin receptors do not mediate the behavioral effects of lipopolysaccharide in mice. *Physiol Behav* 1997; **62**: 385–389.
 - 36 Bucinskaite V, Kurosawa M, Miyasaka K, Funakoshi A, Lundberg T. Interleukin-1 β sensitizes the response of the gastric vagal afferent to cholecystokinin in rat. *Neurosci Letts* 1997; **229**: 33–36.
 - 37 Barrachina MD, Martínez V, Wang LX, Wei JY, Tache Y. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci USA* 1997; **94**: 10455–10460.
 - 38 Matson CA, Wiater MF, Kuijper JL, Weigle DS. Synergy between leptin and cholecystokinin (CCK) to control daily caloric intake. *Peptides* 1997; **18**: 1275–1278.
 - 39 Wang L, Martínez V, Barrachina MD, Tach Y. Fos expression in the brain induced by peripheral injection of CCK or leptin plus CCK in fasted lean mice. *Brain Res* 1998; **791**: 157–166.
 - 40 Bret-Dibat J, Creminon C, Couraud JY, Kelley KW, Dantzer R, Kent S. Systemic capsaicin pretreatment fails to block the decrease in food-motivated behavior induced by lipopolysaccharide and interleukin-1 β . *Brain Res Bull* 1997; **42**: 443–449.
 - 41 Watanabe T, Morimoto A, Tan N, Makisumi T, Shimada S, Nakamori T, Murakami N. ACTH response induced in capsaicin-desensitized rats by intravenous injection of interleukin-1 or prostaglandin E. *J Physiol* 1994; **475**: 139–145.