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Peripheral opioid receptors mediating antinociception in inflammation. Activation by endogenous opioids and role of the pituitary-adrenal axis

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Summary This study investigated the involvement of endogenous opioid peptides in mediating cold water swim (CWS) stress-induced antinociception (SIA) in rats with unilateral hind paw inflammation induced by Freund's complete adjuvant (FCA). Following 0.5, 1 and 2 min of CWS, there was a duration-dependent elevation of paw pressure threshold (PPT) in both inflamed and non-inflamed paws which was maximal immediately after CWS and returned to control values within 15 min. The antinociception elicited in the inflamed paw was significantly greater than that elicited in the non-inflamed paw. The antinociception induced by a 1 min CWS was dose dependently antagonized by tertiary naloxone (0.125-1 mg/kg s.c.) and completely reversed by tertiary naltrexone (0.5 mg/kg). Quaternary naltrexone (5-40 mg/kg s.c.) was similarly effective in reversing the elevation of inflamed PPT induced by a 1 min CWS stress. In contrast, similar doses of quaternary naltrexone had no effect against centrally mediated morphine antinociception in non-inoculated rats. Adrenalectomy was without effect on the pattern of SIA seen in FCA-treated rats. Surgical hypophysectomy completely abolished the differential antinociception induced by 0.5 and 1 min durations of CWS but had little effect on that following 2 min of CWS stress. Inhibition of hypophysial corticotrophic cell secretion with dexamethasone (300 µg/kg) injected s.c. 120 min prior to CWS completely abolished the differential SIA at all durations of CWS tested. β-Endorphin 12.5 µg/kg administered i.v. in non-stressed rats also caused a greater elevation of PPT in inflamed than in non-inflamed paws. This effect was not reversed by concomitant i.v. administration of (-) tertiary naloxone 5 mg/kg or quaternary naltrexone 20 mg/kg.

Key words: Inflammation; Stress-induced antinociception; Peripheral opioid receptors; Endogenous opioid peptides; Adreno-hypophysial axis; (Rat)

Introduction

In recent studies on inflammatory pain, we and others have presented data that are contrary to the classically held view that the antinociceptive ac-

tions of opioids are mediated exclusively by activation of opioid receptors within the central nervous system (CNS) [3,9,22,38,55]. Thus, exogenous opioids can have antinociceptive effects outside the CNS through activation of peripheral opioid receptors in inflamed tissues of Freund's complete adjuvant (FCA)- or carrageenan-inoculated rats [4a,4b,16,35,40-42]. It therefore seems likely that endogenous opioid peptides released following a variety of environmental stimuli [7,13,20,21,28,29,31,32,47,49,50] could produce

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very different patterns of antinociception in rats with adjuvant-induced inflammation.

Although the phenomenon of stress-induced antinociception (SIA) in normal rats is well documented in the literature, the involvement of endogenous opioid systems therein is still a matter of controversy [see 17,23–25,30,44,45,51]. Thus, whilst naloxone has been reported to reverse SIA in mice, the efficacy of this antagonist in reversing presumptive antinociceptive actions of endogenous opioids following stress in rats is poor and highly dependent on the modality, intensity and temporal patterning of the stress paradigms and nociceptive models utilized.

Therefore, whilst the integrity of the anterior hypophysis seems to be a prerequisite for the antinociception induced by several forms of stress [1,27,48], it seems unlikely that the stress-induced secretion of β -endorphin from the anterior and neurointermediate hypophysis into plasma can fully account for the SIA [7,20,27,31,32,47]. This is perhaps not surprising as large, hydrophilic opioid peptides such as β -endorphin would have considerable difficulty in crossing the blood–brain barrier in sufficient concentrations to mediate antinociceptive actions at central opioid receptors [14]. However, the peripheral opioid receptors revealed in inflamed tissues of adjuvant-inoculated rats would seem to be an ideal target for opioid peptides such as β -endorphin released into the plasma following stress.

We therefore felt it to be important to evaluate the involvement of endogenous opioid systems in mediating SIA in FCA-inoculated rats. A model of mono-inflammatory pain developed in our laboratory was chosen for these studies as it has considerable scientific advantages over poly-inflammatory models and moreover is, in our opinion, more ethically acceptable [see 26,39]. Cold water swim (CWS) was chosen as the stress stimulus. We have previously presented preliminary data showing that these peripheral opioid receptors are indeed activated by endogenous ligands following CWS stress [43]. This study was therefore undertaken to: (1) further characterize the antinociception elicited by CWS stress in rats with unilateral hind paw inflammation; (2) investigate the effects of various surgical and

pharmacological manipulations of the adreno-hypophysial axis on CWS SIA in rats with unilateral inflammation; (3) test whether the effects of CWS can be mimicked by intravenous administration of opioid peptides.

Methods

Animals

Male Wistar rats (180–200 g; Savo Ivanovas, Kisslegg, F.R.G.) were housed individually within the experimental laboratory in cages lined with sawdust and maintained under a 12 h light/dark cycle with a room temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Rats had free access to standard laboratory chow and either water, 0.9% NaCl or 5% sucrose solution (see below). All testing was performed during the light phase, employing separate groups of animals. The guidelines on ethical standards for investigation of experimental pain in animals [57] were followed.

Induction of unilateral inflammation

Rats were briefly anaesthetized with ether and injected in the right hind paw with 0.15 ml of killed *Mycobacterium butyricum* (Freund's Complete Adjuvant FCA; Calbiochem). The animals rapidly developed a unilateral inflammation such that by day 4 post-FCA treatment the inoculated paw was approximately twice the volume of the control paw; all algometric tests were performed at this time point. Control animals were anaesthetized as above and injected in the right paw with 0.15 ml NaCl. Further details on the development of inflammation are given in Millan et al. [26].

Paw pressure threshold

The algometric test chosen for this study was paw pressure threshold (PPT). All rats were adapted to handling, gentle restraint and the test apparatus (Ugo, Basile, Comerio, VA, Italy) on days 2 and 3 post inoculation. To evaluate nociceptive thresholds, rats were gently restrained under paper wadding and incremental pressure (16 g/sec) was applied to the dorsal aspect of the hind paw via a blunt, wedge-shaped piston (tip diame-

ter 1.75 mm²). The PPT was taken as the pressure necessary to cause the animal to remove, or attempt to remove its paw from the test apparatus. Cut-off was 250 g. A mean PPT from 3 tests separated by 10 sec intervals was evaluated for both hind paws. The order of inflamed versus non-inflamed paw testing was alternated with successive rats in order to preclude order effects.

Cold water swim stress

Rats were removed from their home cages and baseline thresholds for both hind paws were evaluated 5 or 10 min prior to a forced swim for 0.5, 1, or 2 min in cold water (approx. 1°C). PPTs were then re-evaluated 1, 5 and 15 min following the CWS. Each rat was stressed only once and was used as its own control. Stress-induced changes in PPT were normalized to a percentage maximal possible effect (% MPE) using the following formula.

$$\% \text{ MPE} = (\text{post-CWS PPT} - \text{control PPT}) / (\text{cut-off PPT} - \text{control PPT}).$$

Drugs

Where appropriate, drug doses quoted refer to the salts of the compounds used. Sources were as follows: naloxone HCl (DuPont, U.S.A.), naltrexone HCl (Sigma), naltrexone methobromide (Boehringer, Ingelheim, F.R.G.), morphine HCl (Merck, F.R.G.), dexamethasone (Sigma), β -endorphin(1-31) (Novabiochem A.G., Läufelfingen, Switzerland).

Pharmacological and surgical manipulations of the adreno-hypophysial axis

All surgical manipulations were performed under sodium hexobarbital anaesthesia (200–250 mg/kg i.p.; Evipan, Bayer).

Adrenalectomy

To test the involvement of the adrenal glands in SIA in rats with unilateral inflammation both glands were removed 4 days prior to inoculation with FCA. Total adrenalectomy (TA) was performed by a dorso-lateral approach according to

the procedure given by Ingle and Griffith [15]. In sham animals (SA) the adrenal glands were located but were not removed. Adrenalectomized animals were provided with free access to 0.9% NaCl solution.

Hypophysectomy

Total hypophysectomy (TH) was performed 5 days prior to inoculation with FCA. A ventral approach similar to that detailed in Ingle and Griffith [15] was followed. Briefly, following incision of the skin of the neck, the bony ridges of the occipito-sphenoid synchondrosis and the crista occipitalis were exposed by blunt surgery whilst retracting the trachea for short periods of time. The hypophysis was exposed by drilling through the bone with a no. 9 dental drill. The dura was gently removed and the whole hypophysis was then evacuated. The skin wound was closed with catgut sutures. In sham hypophysectomized animals (SH), the hypophysis was exposed as above but was not removed. Both TH and SH rats were then injected with a single dose of penicillin (2000 I.U. i.m.). Hypophysectomized animals were provided with free access to 5% sucrose solution.

Intravenous cannulations for studies with β -endorphin

Sodium hexobarbital-anaesthetized rats were implanted with a single jugular cannula which was externalised through a small incision in the dorsal skin overlying the scapulae. Neck and dorsal incisions were then closed with surgical clips. Rats were inoculated with FCA in the right hind paw before being returned to their home cages for recovery from the anaesthetic. Intravenous (i.v.) cannulae were kept patent by flushing each day with sterile isotonic saline. On day 4 post inoculation, baseline PPTs were measured, peptides dissolved in saline were injected i.v. and rats were returned to their home cages. PPTs were re-determined over the next 30 min at 5 or 10 min intervals.

Dexamethasone

Dexamethasone was first dissolved in 100% ethanol and diluted with 0.9% NaCl to a final concentration of 100 μ g/ml in 2% ethanol. A

single dose of dexamethasone 300 $\mu\text{g}/\text{kg}$ was given 2 h prior to CWS. Control animals were injected with 0.2 ml vehicle subcutaneously (s.c.).

Verification of surgical efficacy and control studies

The efficacy of adrenalectomy and hypophysectomy were assessed by estimation of serum levels of corticosterone and prolactin respectively. Animals were removed from their home cages and immediately decapitated with minimal stress. Trunk blood was collected and serum was then separated by centrifugation at 4000 rpm for 15 min at 4°C. Serum samples were then stored at -30°C before radio-immunoassay (RIA). RIA estimation of serum corticosterone levels was performed using an [¹²⁵I]corticosterone kit purchased from DRG Instruments (Marburg, F.R.G.). Estimation of serum prolactin concentrations was made using a [¹²⁵I]prolactin RIA developed by Dr. O. Almeida within this department.

Verification of hypophysectomy was further assessed by (1) binocular microscopic examination of the brains and skulls of operated rats and (2) testicular weight.

The influence of adrenalectomy and hypophysectomy on (1) body weight, (2) core temperature, (3) food consumption, and (4) solute consumption was assessed every day for 9 days following surgery in separate groups of operated and sham-operated animals. These same animals were used to study the influence of manipulations of the adreno-hypophysial axis on (1) the development of inflammation and (2) the drop in core temperature (recorded every 30 sec for 3 min) following various durations of CWS. Paw volume was measured by immersing the paw to the tibio-tarsal joint into the solute filled Perspex cell of a plethysmometer (Ugo Basile, Comerio, VA, Italy).

Analysis and statistics

Rats were divided into groups of 6 for testing at each duration of CWS or drug dose. All tests were performed blind to the experimenter and under similar laboratory conditions. Frequently, results from two or more experiments addressing the same question were pooled after first testing the validity of this procedure by analysis of vari-

ance (ANOVA) at each duration of CWS or drug dose.

Parametric ANOVA was used to test for the statistical significance of dose and duration dependency of CWS responses; multiple ANOVA 2-factor interaction analysis was used to test for differences between (1) inflamed and non-inflamed paws and (2) different experimental groups. Multiple ANOVA was further utilised to assess trends in metabolic measures, development of inflammation and differences between experimental groups in this regard. The non-parametric 2-tailed Wilcoxon matched-pairs signed-ranks test was used to test for (1) differences between elevations in PPT for inflamed and non-inflamed paws at each duration of CWS or drug dose, and (2) differences in paw volume and metabolic measures at different time points within groups. The Mann-Whitney U test was used to test for differences in paw volume and metabolic measures at set time points between experimental groups.

Results

The elevation of PPT induced by various durations of forced CWS was evaluated in 42 control and 42 FCA-treated rats. In agreement with our previous studies on unilateral inflammation [26,39] the mean control PPT of inflamed paws (53.2 ± 3.0 g) was significantly lower than the mean control PPT of non-inflamed paws (78.8 ± 3.5 g; Wilcoxon $P < 0.0001$).

As can be seen from the results depicted in Fig. 1, when tested immediately following the stress, there was a duration-dependent elevation of PPT in left and right hind paws of both control and FCA-treated rats. This antinociceptive effect was maximal immediately following the stress, much diminished 5 min later and fully recovered to control levels after 15 min (data not shown). Due to the similarity in the time course for recovery of PPT for all stress experiments, the results detailed below represent the antinociceptive peak effects evident immediately following CWS.

In FCA-treated rats the antinociception elicited in the inflamed paw was significantly greater than that elicited in the non-inflamed paw (multiple

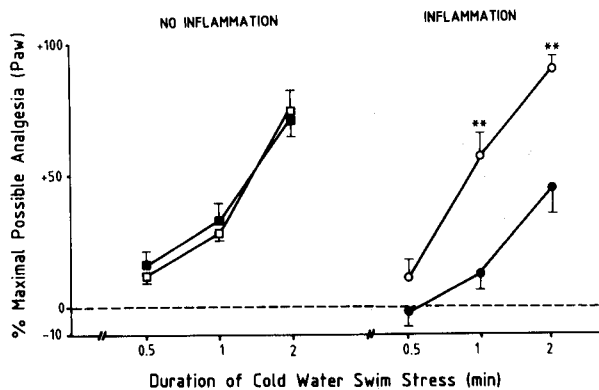


Fig. 1. Elevation of paw pressure threshold (PPT) induced by various durations of cold water swim (CWS) in control and FCA-treated rats. The mean elevation of PPT measured immediately after the CWS was normalized to a % maximal possible effect (% MPE) and plotted against the duration of CWS. A duration of CWS-dependent elevation of PPT was seen in both hind paws of control rats (left panel): ANOVA (2, 39) was as follows: left (closed squares) $F=18.902$, $P < 0.0001$, right (open squares) $F=44.446$, $P < 0.0001$. In FCA-treated rats (right panel) there was also a significant elevation of PPT in both inflamed (open circles) and non-inflamed (closed circles) paws (ANOVA (2, 39): inflamed $F=26.457$, $P < 0.0001$; non-inflamed $F=9.221$, $P < 0.0001$). Error bars depict the standard error of the means (S.E.M.). The significance of differences between inflamed and non-inflamed paws is shown for each duration of CWS; $**P < 0.01$ (Wilcoxon test). The number of animals tested at 0.5, 1 and 2 min durations of CWS was 12, 18 and 12 respectively.

ANOVA (1, 78) $F=32.138$, P of $F < 0.0001$). When FCA-inoculated rats and normal rats are compared, it is apparent that this differential SIA seen in rats with unilateral inflammation results from both a potentiation of SIA seen in the inflamed right paw (ANOVA (1, 78) $F=4.900$, $P=0.029$) and a decrease in SIA seen in the non-inflamed left paw (ANOVA (1, 78) $F=9.446$, $P=0.003$).

As can be seen in Fig. 2, when injected subcutaneously 5 min prior to a 1 min CWS, the opioid antagonist naloxone, dose dependently antagonized the differential SIA seen in FCA-treated rats by reducing the antinociception evident in the inflamed paw; ANOVA (4, 43), inflamed $F=3.456$, $P=0.016$, non-inflamed $F=0.197$, $P=0.939$. Tertiary naltrexone 0.5 mg/kg injected subcutaneously 5 min prior to a 1 min CWS stress was also able to completely antagonise

the differential SIA (mean % MPE were 25.7 ± 8.3 and 26.9 ± 9.5 for inflamed and non-inflamed paws respectively, $n=12$; Wilcoxon $P=0.724$).

Similarly, quaternary naltrexone (5–40 mg/kg s.c.) dose dependently reversed the elevation of PPT seen in the inflamed paw following a 1 min CWS (Fig. 3). This effect was significant (ANOVA (4, 49) $F=4.404$, $P=0.004$) whereas the slight elevation of PPT seen in the non-inflamed paw was not (ANOVA (4, 49) $F=0.592$, $P=0.386$). In contrast, quaternary naltrexone (10 and 20 mg/kg) was completely ineffective in antagonising the centrally mediated antinociceptive actions of morphine (1–4 mg/kg s.c.) in non-inoculated rats (data not shown). None of the antagonists tested had any direct effect on paw volume.

Taken together, these results provide strong evidence that CWS stress induces a release of endogenous opioids which act at least partly out-

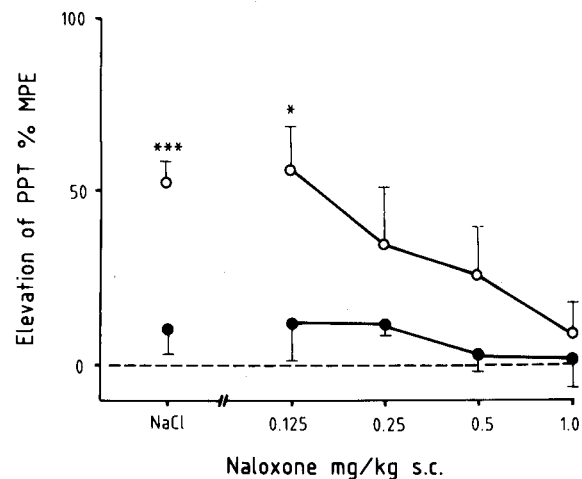


Fig. 2. Reversal of SIA in monoarthritic rats by the opioid antagonist naloxone. The normalized elevation of PPT produced by a 1 min CWS has been plotted against the dose of naloxone. Isotonic saline (NaCl) or naloxone (0.125–1 mg/kg) was administered subcutaneously 5 min prior to the stress. Error bars depict the standard error of the means (S.E.M.). The significance of differences between inflamed (open circles) and non-inflamed paws (closed circles) is shown for each dose of naloxone; $*P < 0.05$, $***P < 0.001$ (Wilcoxon test). Baseline PPTs in grams were as follows: inflamed 50.6 ± 3.5 , non-inflamed 77.2 ± 4.8 . The number of animals tested at each dose was as follows: saline and naloxone 0.5 and 1 mg/kg, $n=12$, naloxone 0.125 and 0.25 mg/kg, $n=6$.

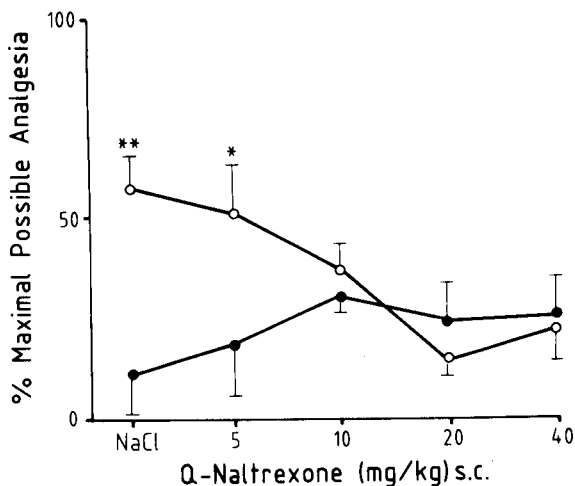


Fig. 3. Evidence for the involvement of peripheral opioid receptors in mediating SIA in inflamed tissues. The normalized elevation of PPT produced by a 1 min CWS has been plotted against the dose of quaternary naltrexone (Q-naltrexone). Isotonic saline (NaCl) or Q-naltrexone (5–40 mg/kg) was administered subcutaneously 5 min prior to the stress. Error bars depict the standard error of the means (S.E.M.). The significance of differences between inflamed and non-inflamed paws is shown for each duration of CWS; * $P < 0.05$, ** $P < 0.01$ (Wilcoxon test). Baseline PPTs in grams were as follows: inflamed 47.3 ± 3.6 , non-inflamed 75.2 ± 4.7 . The number of animals tested at each dose was as follows: saline and Q-naltrexone 5, 10 and 40 mg/kg, $n = 12$, Q-naltrexone 20 mg/kg, $n = 6$.

side the CNS to produce a greater antinociception in inflamed than in non-inflamed tissues. We therefore set out to investigate the possible sources of endogenous opioids presumed to mediate this SIA.

Manipulations of the adreno-hypophysial axis

Adrenalectomy. The efficacy of adrenalectomy was confirmed by a drastic fall in circulating corticosterone levels. Thus, in 48 of the 50 rats tested (2 excluded from further analysis), serum levels of corticosterone were below the detection limit of 20 ng/ml. In contrast, the mean serum corticosterone level in a subset of sham-operated animals was 161 ± 27 ng/ml ($n = 16$ from total $n = 47$). Importantly, TA and SA rats showed no differences in the development of inflammation (see Table I). Inflamed and non-inflamed paw

volume were unaffected by CWS in all experimental groups (data not shown).

As assessed by basal PPT, both inflamed ($39.5 \text{ g} \pm 2.0$) and non-inflamed paws ($65.8 \text{ g} \pm 2.2$) of TA rats were hyperalgesic when compared to SA rats (inflamed $47.1 \text{ g} \pm 2.1$, non-inflamed $75.5 \text{ g} \pm 2.1$; Mann-Whitney TA versus SA, inflamed $P = 0.016$, non-inflamed $P < 0.001$).

In contrast, the differential SIA produced by various durations of CWS was almost identical in TA ($n = 45$) and SA ($n = 47$) rats (Fig. 4). Thus whilst in both TA and SA rats, the elevation of PPT induced by various durations of CWS was higher in the inflamed than in the non-inflamed paw (ANOVA TA rats (1, 84) $F = 16.307$, $P = 0.0001$; ANOVA SA rats (1, 88) $F = 57.39$, $P < 0.0001$) when TA and SA rats were compared there was no significant difference in the anti-

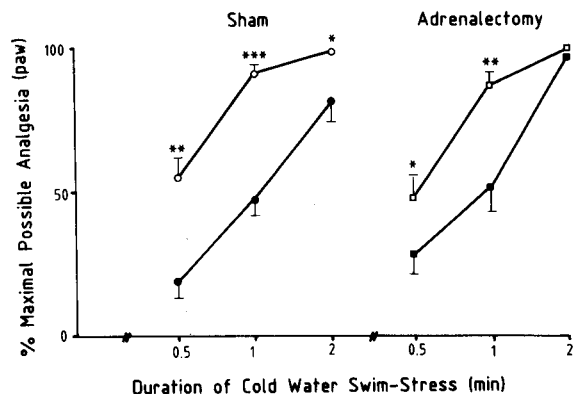


Fig. 4. Comparison of elevations of PPT produced by various durations of CWS in TA and SA rats. The mean elevation of PPT measured immediately after the CWS was normalized to a % maximal possible effect (% MPE) and plotted against the duration of CWS. In SA rats there was a significant CWS duration-dependent elevation of PPT in both inflamed (open circles) and non-inflamed (closed circles) paws (ANOVA (2, 44): inflamed $F = 24.98$, $P < 0.0001$; non-inflamed $F = 22.24$, $P < 0.0001$). This pattern of SIA was almost identical in TA rats (ANOVA (2, 42): inflamed (open squares) $F = 21.01$, $P < 0.0001$; non-inflamed (closed squares) $F = 17.34$, $P < 0.0001$). Error bars depict the standard error of the means (S.E.M.). The significance of differences between inflamed and non-inflamed paws for both SA and TA rats is shown for each duration of CWS; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Wilcoxon test). The number of animals at 0.5, 1 and 2 min durations of CWS was as follows: SA 18, 18 and 11 respectively; TA 18, 17 and 10 respectively.

nociception seen in either the inflamed (ANOVA (1, 86) $F = 0.961$, $P = 0.340$) or in the non-inflamed paws (ANOVA (1, 86) $F = 2.058$, $P = 0.155$).

Hypophysectomy. Total and sham hypophysectomies were performed to assess the potential role of hypophysial pools of endogenous opioids in mediating SIA in FCA-inoculated rats. The efficacy of TH was confirmed by a drastic fall in circulating levels of prolactin. Thus, in 42 of 50 rats tested, serum levels of prolactin were below the detection limit of 0.25 ng/ml. In contrast, in sham-operated animals ($n = 59$) the mean serum concentration of prolactin was 3.63 ± 0.66 ng/ml. Similarly, by day 10 post surgery, the testicles of effectively hypophysectomized rats had rapidly regressed in size (TH $n = 42$, mean 572 ± 23 mg, SH $n = 59$, mean 1486 ± 59 mg). Microscopic examination of the brain/skull of operated rats further confirmed the effectiveness of TH in all but 8 rats. These 8 rats were precisely those showing near 'normal' serum prolactin levels, weight gain (data not shown) and testicular size and as such they were excluded from further analysis. Im-

portantly there was no difference in the development of inflammation between TH and SH rats (see Table I).

Following hypophysectomy the pattern of hyperalgesia seen in both inflamed and non-inflamed paws was almost identical to that seen following adrenalectomy. Thus baseline PPTs in grams were as follows: TH, inflamed 40.6 ± 2.9 , non-inflamed 59.0 ± 2.7 ; SH, inflamed 48.3 ± 2.7 , non-inflamed 78.2 ± 3.6 (Mann-Whitney SH versus TH values were $P = 0.044$ and $P < 0.001$ for inflamed and non-inflamed paws respectively).

In contrast to the results following adrenalectomy, the pattern of SIA produced by various durations of CWS was also very different between TH ($n = 35$) and SH ($n = 42$) rats. Thus, whilst both experimental groups showed significant differences in the CWS duration-dependent elevation of PPT induced in inflamed and non-inflamed paws (TH ANOVA (1, 64) $F = 7.516$, $P = 0.008$, SH ANOVA (1, 78) $F = 27.67$, $P < 0.0001$), when TH and SH rats were compared there was a significant difference in the antinociception seen in the inflamed (ANOVA (1, 71) $F = 17.36$, $P =$

TABLE I

INFLUENCE OF MANIPULATIONS OF THE ADRENO-HYPOPHYSIAL AXIS ON THE DEVELOPMENT OF INFLAMMATION FOLLOWING UNILATERAL HIND PAW INOCULATION WITH FREUND'S COMPLETE ADJUVANT (FCA) IN RATS

See Methods for details of inoculation and measurement of paw volume. Abbreviations used: TA = total adrenalectomy; SA = sham adrenalectomy; TH = total hypophysectomy; SH = sham hypophysectomy; Pre-FCA = mean paw volumes (ml \pm S.E.M.) prior to inoculation with FCA; FCA day 4 and FCA day 5 = mean paw volumes (ml \pm S.E.M.) 4 and 5 days post inoculation with FCA respectively. In the case of studies with dexamethasone (300 μ g/kg) and dexamethasone vehicle (0.2 ml, Dex-vehicle) paw volumes were measured 3 h (FCA day 4) and 24 h (FCA day 5) after the subcutaneous injection of these drugs.

Manipulation	n	Paw volume (ml)			
		Non-inoculated paw		Inoculated paw	
		Pre-FCA	FCA day 4	Pre-FCA	FCA day 4
TA ^{a,b}	18	1.28 \pm 0.02	1.25 \pm 0.03	1.29 \pm 0.03	2.53 \pm 0.10
SA ^{a,b}	18	1.25 \pm 0.03	1.20 \pm 0.02	1.22 \pm 0.03	2.38 \pm 0.06
TH ^{a,b}	18	1.22 \pm 0.03	1.11 \pm 0.02	1.26 \pm 0.03	1.95 \pm 0.04
SH ^{a,b}	18	1.19 \pm 0.02	1.13 \pm 0.02	1.22 \pm 0.03	2.10 \pm 0.08
		FCA day 4	FCA day 5	FCA day 4	FCA day 5
Dexamethasone ^a	18	1.13 \pm 0.02	1.16 \pm 0.02	2.18 \pm 0.05	1.93 \pm 0.06 ^c
Dex-vehicle ^a	18	1.16 \pm 0.02	1.16 \pm 0.02	2.27 \pm 0.04	2.44 \pm 0.05 ^c

Superscripts denote statistical significances of differences as follows: ^a ANOVA (3, 68) within experimental group (e.g., TA), $F > 5$ and $P < 0.0001$; ^b Wilcoxon test inoculated versus non-inoculated paw size on FCA day 4, $P < 0.001$; ^c Mann-Whitney U test between dexamethasone and vehicle-treated rats, $P < 0.001$.

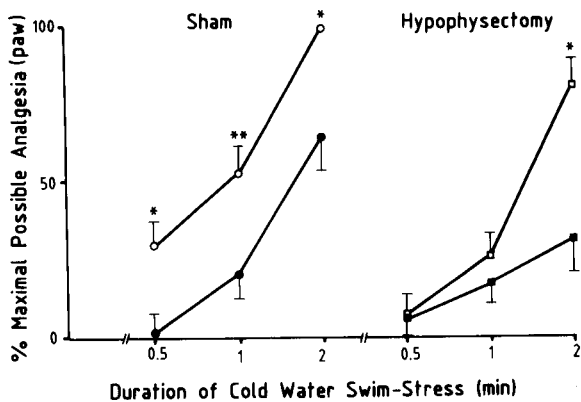


Fig. 5. Comparison of elevations of PPT produced by various durations of CWS in TH and SH rats. The mean elevation of PPT measured immediately after the CWS was normalized to a % maximal possible effect (% MPE) and plotted against the duration of CWS. In SH rats there was a significant duration-dependent elevation of PPT in both inflamed (open circles) and non-inflamed (closed circles) paws (ANOVA (2, 39): inflamed $F = 26.32$, $P < 0.0001$; non-inflamed $F = 13.72$, $P < 0.0001$). In contrast, in TH rats the duration dependence of elevations in PPT was significant in the inflamed paw (open squares, ANOVA (2, 32) $F = 20.37$, $P < 0.0001$) but not in the non-inflamed paw (closed squares, ANOVA (2, 32) $F = 1.964$, $P = 0.157$). Error bars depict the standard error of the means (S.E.M.). The significance of differences between inflamed and non-inflamed paws for both SH and TH rats is shown for each duration of CWS: * $P < 0.05$, ** $P < 0.01$ (Wilcoxon test). The number of animals at 0.5, 1 and 2 min durations of CWS was as follows: SH 12, 18 and 12 respectively; TH 8, 19 and 8 respectively.

0.0001) but not in the non-inflamed paw (ANOVA (1, 71) $F = 1.737$, $P = 0.192$). As can be seen in Fig. 5, TH resulted in a complete abolition of the differential antinociception evoked by 0.5 and 1 min durations of CWS stress. In contrast, following a 2 min CWS, the differential SIA was of similar magnitude in both TH and SH rats.

Dexamethasone. The role of adreno-hypophysial corticotrophic cell secretory activity in mediating CWS SIA in FCA-inoculated rats was tested by dexamethasone pretreatment of 36 animals. Importantly, this steroid had no effect on the degree of inflammation seen in the inoculated paw when measured immediately after evaluation of SIA (i.e., 180 min post s.c. injection, see Table I).

As can be seen from the results in Fig. 6, dexamethasone 300 $\mu\text{g}/\text{kg}$ given 120 min prior to CWS resulted in a complete abolition of the differential PPT elevation evident in vehicle controls ($n = 36$) following 0.5, 1 and 2 min CWS stress. Thus, whilst vehicle-treated animals showed significant differences in the antinociception induced in inflamed and non-inflamed paws (ANOVA (1, 70) $F = 9.596$, $P = 0.0028$) the antinociception in both hind paws of dexamethasone-treated animals was nearly identical (ANOVA (1, 70) $F = 0.026$, $P = 0.873$).

It should be noted that the pattern of CWS SIA seen in dexamethasone-treated FCA-inoculated rats resembles closely that seen in both hind paws of control rats without unilateral inflammation

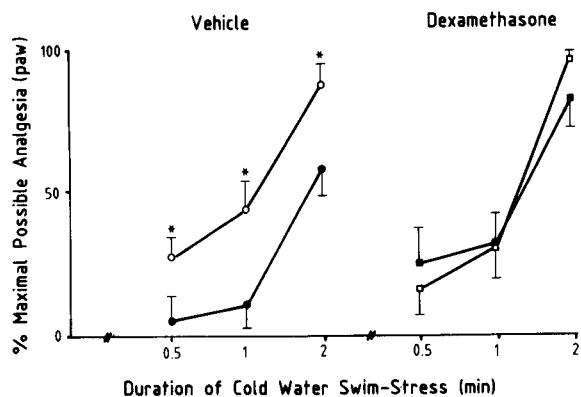


Fig. 6. Comparison of elevations of PPT produced by various durations of CWS in 36 dexamethasone- (300 $\mu\text{g}/\text{kg}$ s.c.) and 36 vehicle (0.2 ml 2% ethanol/isotonic saline s.c.)-treated rats ($n = 12$ at each duration of CWS). The mean elevation of PPT measured immediately after the CWS was normalized to a % maximal possible effect (% MPE) and plotted against the duration of CWS. In both dexamethasone- (squares) and vehicle (circles)-treated rats there was a significant CWS duration-dependent elevation of PPT in both inflamed (open symbols) and non-inflamed (filled symbols) paws. ANOVA (2, 33) values were as follows (dexamethasone: inflamed $F = 26.43$, $P < 0.0001$, non-inflamed $F = 8.268$, $P = 0.001$; vehicle: inflamed $F = 17.295$, $P < 0.0001$, non-inflamed $F = 9.777$, $P = 0.0005$). However, multiple ANOVA (see text) and Wilcoxon analysis revealed that the differential antinociception seen between inflamed and non-inflamed paws at all durations of CWS in vehicle-treated rats was completely abolished by dexamethasone; * $P < 0.05$. Error bars represent S.E.M. Baseline PPTs in grams were as follows: ethanol, inflamed 51.3 ± 3.4 , non-inflamed 77.5 ± 3.6 ; dexamethasone, inflamed 57.6 ± 3.4 , non-inflamed 74.2 ± 2.9 .

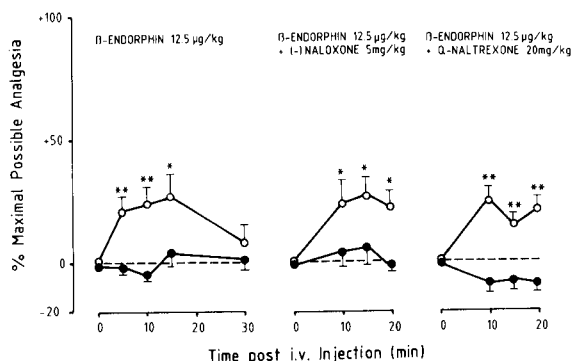


Fig. 7. Non-opioidergic antinociceptive actions of intravenous β -endorphin in monoarthritic rats. The mean elevation of PPT produced by the β -endorphin 12.5 $\mu\text{g}/\text{kg}$ i.v. was normalized to a % maximal possible effect (% MPE) and plotted against time post injection. The first panel illustrates the time course for the differential antinociception produced by i.v. β -endorphin alone ($n = 11$). The second and third panels illustrate the lack of antagonism of this effect by the co-administration with β -endorphin of (-)naloxone 5 mg/kg i.v. ($n = 6$) and quaternary naltrexone 20 mg/kg i.v. ($n = 9$) respectively. Error bars represent S.E.M.s. Wilcoxon probabilities for differences between inflamed and non-inflamed paws are given for each time point, * $P < 0.05$, ** $P < 0.01$. Baseline PPTs in grams were as follows: inflamed 48.9 ± 1.6 , non-inflamed 67.6 ± 2.9 .

(cf., left panel Fig. 1). Thus when paws of dexamethasone- and vehicle-treated rats are compared, it is apparent that the abolition of the differential SIA results from both a decrease of SIA in the inflamed paw (ANOVA (1, 70) $F = 3.425$, $P = 0.049$) and an increase in SIA in the non-inflamed paw (ANOVA (1, 70) $F = 4.488$, $P = 0.038$).

Intravenous β -endorphin. β -Endorphin(1-31) was tested over a wide dose range (pg to $\mu\text{g}/\text{kg}$) in preliminary studies and the results with the maximally effective dose of 12.5 $\mu\text{g}/\text{kg}$ are presented in Fig. 7. β -Endorphin did indeed cause a greater elevation of PPT in inflamed as compared to non-inflamed tissues (ANOVA over 30 min period (4, 100) $F = 2.857$, $P = 0.028$). However, the actions of β -endorphin were not reversed by high doses of opioid antagonists. Thus neither (-)naloxone 5 mg/kg (panel 2) nor quaternary naltrexone 20 mg/kg (panel 3) were able to antagonize this differential antinociception when co-administered with β -endorphin (ANOVA results were (3,

40) $F = 2.988$, $P = 0.043$ and (3, 64) $F = 8.868$, $P = 0.0001$ respectively).

Discussion

Whilst in 'normal' rats the antinociceptive actions of exogenous opioid agonists are generally agreed to be mediated exclusively within the CNS [3,9,22,38,55], in rats with adjuvant or carrageenan-induced inflammation, at least a component of the analgesic actions of opioids appears to be mediated by activation of receptors within inflamed peripheral tissues [4a,4b,16,35,40-42].

Thus, we have previously shown that local intra-plantar or s.c. injection of opioid agonists elicits a preferential elevation of PPT in inflamed over non-inflamed paws of rats unilaterally inoculated with FCA [40-42]. Furthermore, we were able to differentiate the types of peripheral opioid receptors involved [41,42]. These 'novel' peripheral opioid receptors would seem to be an ideal target for opioid peptides released into the plasma following stress [see 23-25]. This study investigated the possible involvement of endogenous opioid systems in mediating SIA in rats with unilateral hind limb inflammation.

CWS stress caused a duration-dependent, preferential elevation of inflamed over non-inflamed PPT and interestingly, the magnitude of the differential antinociception following a 1 min CWS was almost identical to that seen with exogenous opioid agonists tested in rats with unilateral inflammation [40-42].

The antinociceptive effects of a 1 min CWS were completely antagonized by tertiary naloxone and naltrexone, providing strong evidence for the involvement of endogenous opioid systems in mediating SIA in this model. This is in fact not surprising as CWS is one of the few stress paradigms relatively consistently reported to induce a naloxone-reversible antinociception in normal rats [2,44,47].

However, in contrast to studies in normal rats, it seems likely that peripheral opioid receptors are important in mediating the potentiated CWS SIA evident in the inflamed tissues of FCA-treated rats as s.c. quaternary naltrexone selectively an-

tagonized the elevation of PPT manifested in the inoculated paw. Importantly, the doses of quaternary naltrexone used did not antagonize the centrally mediated elevation of PPT produced by s.c. morphine in rats without unilateral inflammation (data not shown) [3].

It is well documented that both the adrenal glands and the hypophysis release opioid peptides into the systemic circulation following a variety of stressful stimuli [7,13,20,21,28,29,31,32,47,49,50] and that an adreno-hypophysial mechanism is implicated for an analgesic response to various forms of stress in normal animals [1,23,24,27,48,52]. The abolition of 0.5 and 1 min CWS stress-induced differential antinociception by surgical hypophysectomy in this study provides strong evidence that the integrity of the hypophysis is necessary for this effect. The importance of adreno-hypophysial corticotrophic cell secretory activity is further supported by the effects of the steroid dexamethasone, which is known to inhibit the secretion of ACTH and β -endorphin by inhibition of secretion from these cells [5,20]. Importantly, the effects of surgical and chemical hypophysectomy on CWS SIA were not due to possible anti-inflammatory actions of the two procedures (see Table I) nor due to changes in the drop in core temperature induced by CWS (data not shown).

Although the results from these experiments implicate a physiological role for the hypophysis and endogenous opioids in mediating antinociception in inflamed peripheral tissues, it is important to emphasize several points at this juncture.

Thus, in dexamethasone-pretreated rats, CWS was still able to produce a duration-dependent antinociception of intermediate magnitude which was similar in both inflamed and non-inflamed paws. Similarly, foot-shock stress-induced elevations of tail flick latency have been reported to be resistant to dexamethasone pretreatment [27]. This implies that a non-adreno-hypophysial corticotrophic mechanism may be important in permitting the SIA manifested in non-inflamed tissues. Furthermore, at least a component of the differential antinociception produced by greater levels of stress (i.e., 2 min CWS) was not abolished by total hypophysectomy and was insensitive to antagonism by naloxone (data not shown). Thus, higher

levels of stress seem to activate non-hypophysial, non-opioid mechanisms to mediate greater elevations of PPT in inflamed than in non-inflamed paws (see also Terman et al. [44]).

As stress has been shown to cause a release of hypophysial β -endorphin into the plasma [7,13,20,28,29,31,32,47] and alters hypophysial levels of mRNA for pro-opiomelanocortin [12], this endogenous opioid seemed, to us, to be a likely candidate as a blood-borne factor acting directly in the inflamed paw to manifest the differential SIA produced by a 1 min CWS. We therefore tested this peptide intravenously at various doses in non-stressed rats with unilateral inflammation to try and mimic CWS SIA. Although intravenous β -endorphin also produced a differential antinociception in our model a role for this pool of β -endorphin in mediating CWS SIA in either normal or in inflamed tissues seems unlikely for several reasons. Firstly, the rapid time course of CWS SIA in our model is not in keeping with either the known plasma half-life of β -endorphin in rodents [14] or with the time to peak effect of intravenous β -endorphin seen in this study [see also 46]. Secondly, whilst the SIA seen in this study was reversed by naloxone and naltrexone, the differential antinociception produced by intravenous β -endorphin was not.

It therefore seems likely that a hypophysial factor other than, or in addition to, β -endorphin is of relevance. Whilst dexamethasone has somewhat more selective effects on the release of hypophysial factors than does total hypophysectomy, its actions are by no means restricted to the secretion of β -endorphin. Thus, dexamethasone also inhibits the release of ACTH and other pro-opiomelanocortin-derived peptides from corticotrophic cells [7] and indirectly inhibits lactotrophic secretion of prolactin [33]. Although neither prolactin nor ACTH would be expected to have direct agonist actions at central or peripheral opioid receptors, this does not rule out their signalling the mobilization/release of opioids from other peripheral, non-hypophysial pools.

The adrenal glands are known to contain enkephalins and release them in response to stressful stimuli [18,19,21,36,37,49,50,56]. Moreover, the degree of pre-stress hyperalgesia produced by

adrenalectomy and hypophysectomy was very similar [see also 48]. However, it seems unlikely that adrenal pools of enkephalins could be involved in mediating the differential SIA as the pattern of PPT elevation, seen in TA and SA animals, was nearly identical. Adrenalectomy has previously been shown to cause a 3-fold elevation in basal plasma levels of β -endorphin [20]. Thus, the inefficacy of TA in modifying the SIA seen in our model seems to provide further evidence against a role of hypophysial β -endorphin therein as any elevation of plasma β -endorphin produced by TA might, in turn, be expected to increase basal and stress-elevated nociceptive thresholds in the inflamed paw. However, this argument only holds true in the absence of the development of tolerance to the effects of elevated levels of β -endorphin following adrenalectomy.

A direct role for hypophysial pools of prodynorphin-derived peptides [6,10] also seems unlikely as stress induces selective changes in anterior lobe levels of dynorphin of questionable physiological significance [29], whereas dexamethasone has previously been shown to selectively increase neurointermediate lobe levels of dynorphin-like peptides [8,25]. The involvement of hypophysial enkephalins [4] also seems unlikely as this pool is unresponsive to stress [28] and plasma levels of met-enkephalin are unaffected by dexamethasone pretreatment [25].

As a final point it should be noted that adjuvant-induced inflammation results in rapid, profound changes in opioid peptide neuroanatomy at several loci within the brain and spinal cord [11,26,34,53,54]. Whilst the results of this study implicate an important role for endogenous opioids acting outside the CNS, the possibility of a functional significance of asymmetrical changes in central opioid peptide levels in rats with unilateral inflammation should not be forgotten. This is particularly important in view of the lower level of CWS SIA seen in the non-inflamed paws of FCA-inoculated rats than in both hind paws of (1) control rats without unilateral inflammation and (2) FCA-inoculated, acutely dexamethasone-pretreated rats.

In summary, the results presented in this paper indicate that, in rats with unilateral hind limb

inflammation, the stress produced by various durations of CWS induces a greater degree of antinociception in inflamed than in non-inflamed tissues. Furthermore, following 1 min CWS stress this differential SIA is mediated, at least in part, by activation of peripheral opioid receptors by endogenous ligands, the activity of which is dependent on the integrity of the hypophysis but not that of the adrenal glands. We have further investigated the nature of the endogenous opioid systems mediating SIA in inflamed tissues of FCA-treated rats using selective antibodies, 'enkephalinase' inhibitors and opioid receptor antagonists. The results of these studies have been submitted for publication and some are now in press [39a].

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