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Peripheral opioid modulation of pain and inflammation in the formalin test

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Abstract

The effects of local treatment with opioid receptor agonists on the early (0–10 min) and late (20–40 min) behavioural response and extravasation induced by intraplantar injection of 1% formalin in rats were examined. The μ-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) depressed pain behaviour in the late phase, and extravasation in both phases. The κ-opioid receptor agonist *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl] benzeneacetamide methanesulfonate (U50,488H) suppressed the behavioural response in both phases, but extravasation was enhanced in the early phase and not altered in the late phase. The δ-opioid receptor agonist [D-Pen^{2,5}]enkephalin (DPDPE) enhanced the behavioural response in the late phase, but inhibited extravasation in the both early and late phases. Systemic injection of the agonists had no effects, and pretreatment with s.c. naloxone methiodide reversed the effects of locally administered agonists. These data (1) support the notion that different pathophysiological mechanisms underlie the two phases of the formalin test, and (2) indicate that depending on the receptor specificity, opioid receptor agonists have both pro- and antinociceptive effects, as well as pro- and antiinflammatory activity.

Keywords: Formalin test; Inflammation; Pain; Opioid receptor subtype; Peripheral analgesia; Extravasation

1. Introduction

Opioids are believed to produce analgesia primarily through their actions in the central nervous system (for reviews see Pasternak, 1988; Jaffe and Martin, 1990). However, until the late 19th century opium and/or morphine was used topically in conditions involving inflammation (Courtwright, 1978; Golub and Green, 1991, p. 600). Both animal (see Ferreira, 1981; Stein, 1993 for reviews) and, more recently, human (Khoury et al., 1992; Stein et al., 1993; Stein, 1994) studies have demonstrated that peripheral opioid mechanisms play a role in antinociception. The animal studies, with few exceptions, have focused on the effects of opioid agents on hyperalgesia associated with acute or chronic inflammation – that is, increased sensitivity to mechanically or thermally induced pain in inflamed tissue – as a nociceptive model. It has been proposed that μ-opioid receptor agonists act to inhibit activation of adenylate cyclase on peripheral afferent neurons by inflammatory mediators such as serotonin and prostaglandin E_2, while δ- and κ-opioid receptor agonists inhibit secretion of pro-inflammatory substances by sympathetic neurons (Taiwo and Levine, 1991; Levine and Taiwo, 1989; Taiwo et al., 1992). μ-Opioid receptor agonists may also inhibit release of substance P from primary afferent neurons (Belvisi et al., 1989; Yaksh, 1988), an effect that is consistent with data indicating that endogenous opioids suppress the inflammatory process in addition to reducing hyperalgesia (Millan and Colpaert, 1991).

The formalin test is a model of pain associated with tissue injury and inflammation in which the spontaneous behavioural response of a rat to injection of formalin into a paw is assessed. The response to formalin is biphasic, and consists of an initial 5–10 min of licking, elevation, flinching and protection of the injected paw, 5–20 min of relatively normal behaviour, followed by resumption of pain behaviours for 30–60

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min (Abbott et al., 1995). The first phase of the behavioural response is thought to be produced by direct activation of nociceptive neurons by formalin, and the second phase to reflect pain generated in acutely injured tissue (Hunskaar and Hole, 1987; Hunskaar et al., 1986; Dickenson and Sullivan, 1987). Humans report formalin pain to be poorly localised with prominent burning and throbbing qualities (Franklin and Abbott, 1989), and formalin pain shares many characteristic with acute injury-induced pain in humans (see Abbott et al., 1992; Franklin and Abbott, 1989 for discussion of this issue). Although the primary actions of systemic opioids in the formalin test are central (Abbott, 1991; Matthies and Franklin, 1992), there is evidence that peripheral actions may contribute to opioid analgesia in that the effects of systemic ethylketocyclazocine, but not morphine, are attenuated by quaternary naloxone (Abbott, 1988). In addition, systemic morphine has been reported to reduce swelling in the injected paw (Wheeler-Aceto and Cowan, 1991), an effect which may be peripherally mediated.

The present study was undertaken to explore the effects of local treatment with opioid receptor agonists on the behaviour and extravasation produced by formalin. A moderate dose of formalin was used in order to produce maximal sensitivity to weak analgesic effects, and rats were extensively habituated to the test environment to minimize stress-induced attenuation of the pain response (Abbott et al., 1986). μ-, κ- and δ-opioid receptor agonists were used in the dose range previously shown to attenuate hyperalgesia (Stein et al., 1989; Levine and Taiwo, 1989; Taiwo and Levine, 1991). In order to determine if the first and second phases of the behavioural and extravasation responses to formalin are differentially modulated by peripheral opioids, the agonists were injected either before formalin or after the first phase had ended. Some of these data were presented in a preliminary form (Hong and Abbott, 1993).

2. Materials and methods

2.1. Subjects

Male Long-Evans rats weighing 280–380 g (Charles River Quebec) were housed in groups of 2–4 in shoe box cages in the colony room with food and water available ad libitum. A 12:12 h light:dark cycle with lights on at 7:00 h was maintained and testing was done between 9:00 and 17:00 h. Prior to behavioural testing, the rats were acclimatized to the laboratory and habituated to the test boxes for a minimum of 30 min/day for 5 days prior to testing, and for 30 min immediately prior to administration of the agents, to reduce attenuation of nociceptive responses by stress-activated pain suppression mechanisms (Abbott et al., 1986; Franklin and Abbott, 1989). In the behavioural experiments, rats were tested twice, once on each hind paw. After testing on the first paw, they were randomly reassigned to another condition, given a 2-day rest period and then 5 more days of habituation before being tested on the opposite paw. Extravasation was measured in separate groups of rats that were habituated for at least 3 days following arrival.

2.2. Behavioural testing

The formalin test was conducted in clear plastic chambers ($32 \times 32 \times 30$ cm) with a mirror placed at a 45° angle beneath the floor to allow an unobstructed view of the paws. Pain was induced by injecting 50 μl 1% of formalin s.c. into the plantar surface of one rear paw. This formalin concentration produces pain behaviour that remains below the asymptote of continuous pain behaviour during the peak of the second phase (Abbott et al., 1995). The response to formalin was recorded 0–10 min (early phase) and 20–40 min (late phase) after formalin using the following criteria: *lifting* – the injected paw elevated and not in contact with floor; *licking* – the injected paw is licked or bitten. The time spent in each of these categories was recorded using a computer program that allowed simultaneous rating of two rats.

2.3. Extravasation

Extravasation was assessed using the Evans Blue method (Ukada et al., 1970). Rats were anaesthetized with sodium pentobarbital (40 mg/kg i.p.; MTC Pharmaceuticals). Formalin (50 μl, 1%) was injected into the plantar surface of both hind paws, one of which served as a control, and the other as the test paw. The test paw was injected with opioid agonists and the same volume of the vehicles was injected into the control paw. Evans Blue (60 mg/kg, 2.5 ml/kg; Sigma Chemicals) was dissolved in saline and injected into the tail vein. Injections were timed so that animals were killed by exsanguination under anaesthesia or carbon dioxide exposure 30 min after dye injection. This time was either 10 or 50 min after formalin, depending on whether the early or the late phase response was to be measured. Both paws were cut at the distal and proximal ends of the metatarsals. The metatarsal section of the feet were cut into 2 or 3 pieces, placed in 4 ml of formamide, and incubated at 65°C for 48 h or longer until the blue colour of the skin completely disappeared. After filtration and centrifuge, the absorbance at 620 nm of the supernatant was measured. The dye leakage in the treated paw was expressed as a percentage of the vehicle-injected paw.

2.4. Opioid administration

Opioid receptor agonists were injected s.c. into the same region of the hind paw as formalin using a 30 g needle connected to a microsyringe with PE-10 tubing in order to minimize tissue damage during the injection procedure. The injection volume was 40 μl. These injections were done either 5 min before (−5) or 15 min after (+15) formalin to investigate their effects on the first and second phases of the formalin response.

Two methods were used to confirm that the effects of opioids were due to local actions. Firstly, the peripherally acting opioid receptor antagonist, naloxone methiodide, was injected i.p. 5 min before the opioid treatment – that is, 10 min before or 10 min after formalin, depending on whether first or second phase effects had been observed with the agonist. In the experiments on extravasation, a second dose of naloxone methiodide was injected 30 min after the first if the rat was to be killed at 50 min after formalin. Doses of naloxone methiodide were determined in pilot tests using a threshold tracking method in which pairs of rats received logarithmically increasing doses until blockade was observed and then moving half a log step lower. Secondly, the agonist dose applied to the foot, or in the case of DPDPE, the highest dose, was injected i.p. 10 min before formalin.

2.5. Agents

[D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]Enkephalin (DAMGO), *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl] benzeneacetamide methanesulfonate (U50,488H) and [D-Pen^{2,5}]enkephalin (DPDPE) were obtained from Sigma Chemicals and naloxone methiodide from Research Biochemicals Int. DAMGO, DPDPE and naloxone methiodide were dissolved in sterile distilled water and U50,488H was dissolved in sterile saline. 1% formalin solution was prepared 1 part saturated formaldehyde solution (38%) in 99 parts physiological saline.

2.6. Data analysis

Pain was quantified using the sum of the number of minutes of elevation and licking of the injected paw. This measure correlates strongly with formalin concentration ($r = 0.84$) and, using a standard formalin dose, with dose of morphine ($r = -0.75$) and amphetamine ($r = -0.80$); addition of other behaviours such as favouring and flinching to the regression equation does not increase the proportion of variance explained (Abbott et al., 1995). Thus, this measure is sensitive to variations in the pain stimulus and to the effects of centrally acting analgesics with different side effects. The time course of the pain response is shown in the figures, but statistical tests were applied to the area under the curve for the first 10 min after formalin (first phase) and 20–40 min after formalin (late phase). Analysis of variance followed by Turkey's protected *t*-test was used to compare opioid treated groups with control groups.

Extravasation in the treated paw was expressed as a percentage of the control paw for each rat and these values were compared to 100% using *t*-tests with the Bonferroni correction to reduce experiment-wise error.

3. Results

3.1. Effects of opioids on pain behaviour

The upper panel of Fig. 1 shows the effects of local injection of 1 μg DAMGO on the early and late paw

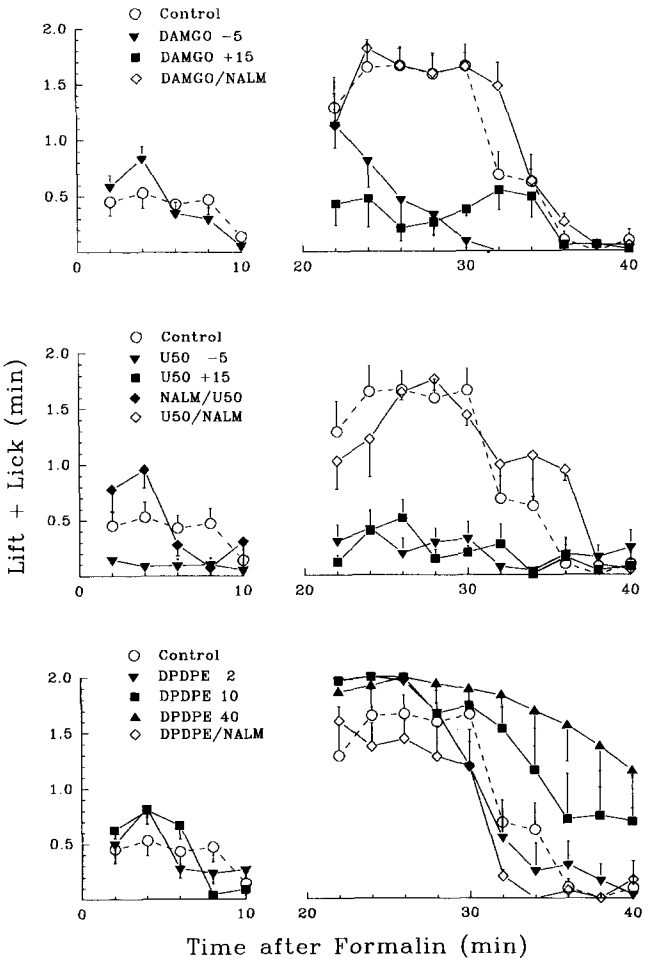

Fig. 1. The effects of local injections of DAMGO (top), U50,488 (middle) and DPDPE (bottom) on the lifting and licking response to formalin, and blockade of these effects by pretreatment with systemic naloxone methiodide (NALM). Agonists were injected either 5 min before (−5), or 15 min after (+15), formalin. Naloxone methiodide was injected 5 min before the opioid when it is written before the agonist in the symbol keys, and after the first phase of formalin pain when written after the agonist. Error bars indicate S.E.M.

lifting and licking response to formalin. The control curve, which is reproduced in each panel of the figure, shows the time course of the response to 50 μl of 1% formalin, preceded by 40 μl of normal saline. In the strain of rats used here, the first phase response to low concentrations of formalin tends to be blunt and the second phase response remains below the behavioural asymptote of continuous pain behaviour (Abbott et al., 1995; Franklin and Abbott, 1993), providing sensitivity to both increases and decreases in pain levels. DAMGO injected 5 min before formalin did not alter the early behavioural response to formalin. However, lifting and licking in the second phase response were markedly reduced by DAMGO, regardless of whether it was injected 5 min before ($t = 8.09$, $P < 0.01$) or 15 min after ($t = 7.7$, $P < 0.01$) formalin. Systemic injection of 1.2 mg/kg naloxone methiodide, a peripherally acting opioid receptor antagonist, after the first phase response was over (i.e., 10 min after formalin) completely blocked the attenuation of the lifting and licking response produced by DAMGO ($t = 0.23$; Fig. 1).

The effects of U50,488 on the lifting and licking response are shown in Fig. 1, middle panel. 20 μg of U50,488, injected 5 min before formalin, almost completely prevented the early ($t = 4.44$, $P < 0.01$) and late ($t = 7.3$, $P < 0.01$) lifting and licking responses. The same dose of U50,488 given 15 min after formalin abolished the lifting and licking response in the second phase ($t = 6.71$, $P < 0.01$) formalin. Systemic treatment with 2.5 mg/kg naloxone methiodide blocked the effects of U50,488H on the lifting and licking response. This was true when the naloxone methiodide was given before formalin to block the first phase effects ($t = 0.93$), and also when naloxone methiodide was given 10 min after formalin to block the second phase effects ($t = 1.14$).

DPDPE administered before formalin (Fig. 1, lower panel) did not alter any formalin-induced behaviour in the first phase ($F(3,34) = 0.55$). The effects of this treatment on the second phase, however, were unexpected. 2 μg of DPDPE had no effect on lifting and licking, but when the dose was increased, the lifting and licking response increased in a dose-dependent manner (10 μg: $t = 3.55$, $P < 0.01$; 40 μg: $t = 5.67$, $P < 0.01$). As indicated in Fig. 1, the increase in the lifting and licking response involved an increase in both the peak effect and in the duration of the response. 3 mg/kg of naloxone methiodide administered 10 min after formalin completely blocked the effects of 40 μg DPDPE on lifting and licking ($t = 0.78$, df = 9,6).

Intraperitoneal injection of 1 μg DAMGO, 20 μg U50,488 or 40 μg DPDPE 10 min before formalin did not alter any formalin-induced pain behaviour (Table 1), confirming that the effects described above are not readily explained by systemic absorption of the agents injected into the paws.

Table 1
Time (min ± S.D.) spent lifting and licking formalin injected paw during the first and second phase reponses after systemic injections of normal saline, DAMGO, DPDPE or U50,488

	First phase [a]	Second phase [b]
Saline	1.37 ± 0.70	7.86 ± 4.39
DAMGO 1 μg	1.48 ± 0.29	8.00 ± 1.82
U50,488 20 μg	0.93 ± 0.10	8.38 ± 1.94
DPDPE 40 μg	1.12 ± 0.56	8.64 ± 6.69

[a] $F(3,19) = 0.74$, NS; [b] $F(3,19) = 0.17$, NS.

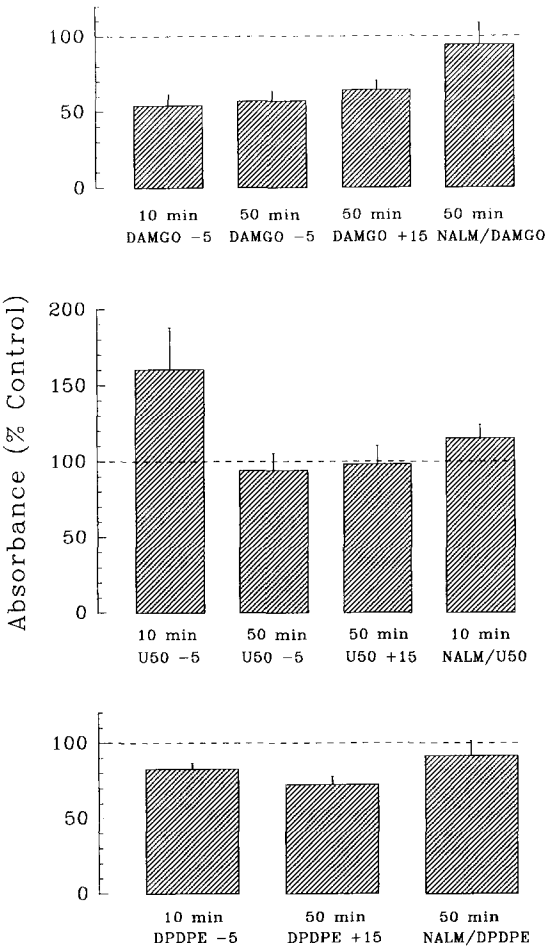

Fig. 2. The effects of local injections of DAMGO (top), U50,488 (middle) and DPDPE (bottom) on extravasation of Evans Blue induced by intraplantar formalin in anaesthetized rats, expressed as a percentage of the opposite paw. Rats were killed either 10 min or 50 min after formalin as indicated, and the time of agonist injection was either 5 min before (−5), or 15 min after (+15), formalin. Naloxone methiodide (NALM) was injected 5 min before the opioid when it is written before the agonist, and after the first phase of formalin pain when written after the agonist. Error bars indicate S.E.M.

3.2. Effects of opioids on extravasation

The effects of opioid treatments on extravasation induced by formalin were examined in anaesthetized

rats. For these experiments, both paws were injected with formalin, and one paw treated with the opioid receptor agonist and the other with the vehicle. Groups of rats were killed 10 or 50 min after formalin to examine the effects in the first and second phases.

As illustrated in the upper panel of Fig. 2, 1 μg DAMGO injected 5 min before formalin reduced extravasation by nearly 50% in the first phase ($P < 0.01$), and also in the second phase, regardless of whether it was given before formalin or after the first phase (P values ≤ 0.01). Treatment with 1.2 mg/kg of naloxone methiodide, 10 min before and 15 min after formalin, blocked the suppressive effect of DAMGO ($P = 0.72$).

U50,488 (Fig. 2, middle) produced a marginally significant increase in extravasation in the first phase ($P = 0.056$) which was antagonized by pretreatment with 2.5 mg/kg naloxone methiodide ($P = 0.16$). Dye leakage in the second phase was not altered by U50,488 (P values ≥ 0.61), and the effects of naloxone methiodide were therefore not examined.

DPDPE produced a small, but highly consistent, suppression of extravasation in both the first and second phases (P values ≤ 0.003). Treatment with 3.0 mg/kg of naloxone methiodide, either 10 min before or 15 min after formalin, blocked this effect ($P = 0.32$).

4. Discussion

The effects of the μ-, κ- and δ-opioid receptor agonists on behaviour and extravasation induced by formalin are summarized in Table 2. Overall, the first phase is less sensitive to peripheral opioids and, at the doses used here, only U50,488 had any effect on lifting and licking behaviour (a decrease). In the second phase, lifting and licking was decreased by the μ- and κ-opioid receptor agonists, DAMGO and U50,488, and increased by the δ-opioid receptor agonist DPDPE. The opioids also had different effects on extravasation in the first and second phases. DAMGO and DPDPE reduced extravasation in both phases of the formalin test, while U50,488 increased extravasation in the first phase and had no effect in the second phase. All of the effects on pain behaviour and extravasation were reversed by a peripherally acting opioid receptor antagonist. These data indicate that the effects of local application of opioid agents on formalin-induced extravasation are not necessarily correlated with their effects on the behavioural pain response: opioids may have both pro- and anti-inflammatory actions, and also both pro- and antinociceptive actions in the periphery.

Table 2
The effects of intraplantar injection of opioid receptor agonists on formalin induced behaviours and on extravasation

Agent	First phase		Second phase	
	Lift + Lick	Dye leakage	Lift + Lick	Dye leakage
DAMGO 1 μg	=	↓↓	↓↓	↓
U50,488 20 μg	↓	↑	↓↓	=
DPDPE 40 μg	=	↓	↑↑	↓

↓, = and ↑ represent decrease, no change and increase, respectively; double arrows indicate large effects.

Selective antagonists for μ-, δ- and κ-opioid receptors were not used to confirm the receptor specificity in the present experiments. It could be argued that local injections produce high concentrations that might be associated with non-specific effects. However, Stein et al. (1989) used local injection of similar doses of the same agonists combined with local treatments with selective antagonists for μ-, δ- and κ-opioid receptors and examined the effects on hyperalgesia associated with chronic inflammation. Only the effects of the corresponding agonist were attenuated by each of the antagonists, indicating that the kinetic parameters of local injections such as those used here do not lead to nonspecific effects.

The suppressive effects of DAMGO on the second phase behavioural response and on extravasation are consistent with the previous literature. μ-Opioid receptor agonists have been reported to attenuate hyperalgesia or pain associated with acute inflammation induced by various inflammatory mediators (Levine and Taiwo, 1989; Taiwo et al., 1992; Ferreira and Nakamura, 1979; Taiwo and Levine, 1991), intraperitoneal irritants (Follenfant et al., 1988; Bentley et al., 1981; Smith et al., 1982), chronic or subacute inflammation induced by Freund's complete adjuvant (Stein et al., 1988a,1989; Maldonado et al., 1994) or carrageenin (Joris et al., 1987), and also to decrease post-surgical pain after instillation into the knee joint in humans (Khoury et al., 1992). They also decrease extravasation induced by nerve stimulation or inflammatory mediators (Lembeck and Donnerer, 1985; Yaksh, 1988; Belvisi et al., 1989). In addition, local treatment with naloxone, which is most potent as a μ-opioid receptor antagonist, increases post-surgical pain in humans (Stein et al., 1993) and blocks endogenous analgesic mechanisms in chronically inflamed tissue (Stein et al., 1990). Furthermore, when opioids are administered systemically, concentrations sufficient to produce peripherally mediated analgesia are probably achieved for some μ-opioid receptor agonists. For example, virtually all of the effects of 2–4 mg/kg morphine on hyperalgesia to pressure in the chronically inflamed paw were blocked by opioid receptor antagonist treatment (Maldonado et al., 1994; Stein et al., 1988b); the volume of formalin injected paws was decreased after 2.5 mg/kg of morphine (Wheeler-Aceto and Cowan, 1991); and systemic methylmorphine attenuated the second phase of the formalin response in mice (Oluyomi et al., 1992). Studies that exclude peripheral effects of

systemic dosing with morphine in the formalin test (i.e., complete blockade by central lesions (Matthies and Franklin, 1992) or central administration of an antagonist (Abbott, 1991), or failure to block morphine analgesia with a peripherally acting antagonist (Abbott, 1988)) have used concentrations of formalin that produce an asymptotic or near asymptotic behavioural response which renders the test insensitive to small decreases (Abbott et al., 1995). Overall, there is strong evidence that peripheral actions play a considerable role in the analgesic effects of μ-opioid receptor agonists in pain and hyperalgesia associated with inflammation.

The previous reports of peripheral actions of κ- and δ-opioid receptors are less consistent. Reduction of hyperalgesia has been reported for agents with varying degrees of specificity for κ-opioid receptors in complex models of inflammation such as adjuvant-induced arthritis (Stein et al., 1989), injection of bradykinin (Taiwo and Levine, 1991) and writhing induced by intraperitoneal injection of irritants (Bentley et al., 1981). On the other hand, U50,488 did not alter hyperalgesia associated with inflammation induced by agents that are believed to act directly on nociceptive afferents such as PGE_2 (Levine and Taiwo, 1989) and $5\text{-}HT_{1A}$ agonists (Taiwo et al., 1992), and it failed to attenuate substance P release evoked by nerve stimulation (Yaksh, 1988). The effects of U50,488 on the first and second phase lifting and licking response are consistent with previous reports of κ-opioid receptor agonists suppressing hyperalgesia associated with complex inflammatory states, and also with the fact that it suppresses the response of dorsal horn cells to injection of formalin into their receptive fields (Haley et al., 1990).

There is less information concerning peripheral actions of δ-opioid receptor agonists, but they also appear to be more effective in inhibiting hyperalgesia in complex models of inflammation (cf. Stein et al., 1989; Taiwo and Levine, 1991 versus Levine and Taiwo, 1989; Taiwo et al., 1992). Our finding that DPDPE exacerbated formalin pain was unexpected. It is possible that the net effect of δ-opioid receptor agonists on inflammatory pain might be positive over a longer time-frame when reduction in discomfort secondary to the anti-inflammatory actions may be more significant.

In a previous study (Abbott, 1988) analgesia in the formalin test induced by systemic morphine was not altered by treatment with a peripherally acting opioid antagonist, but the dose-effect relationship for ethylketocyclazocine was shifted to the right. Those data were interpreted as indicating that peripheral analgesic effects were primarily mediated by κ-opioid receptors. The present data suggests a different interpretation. Abbott (1988) used a considerably higher concentration of formalin to induce pain and observed rats only during the peak of the second phase of the pain response, when pain behaviour is virtually continuous. This renders the formalin test insensitive to weak analgesic agents (Abbott et al., 1995). Because of this, the peripherally mediated componant of ethylketocyclazocine may have been apparent because it activated both μ- and κ-opioid receptors, while the primarly μ-opioid receptor mediated effects of morphine were unable to overcome the strong pain stimulus used unless central mechanisms were active.

It has been proposed that the initial activation of nociceptive afferents by formalin in the first phase induces sensitization of nociceptive systems which facilitate pain transmission in the second phase (Dickenson and Sullivan, 1987; Woolf and Walters, 1991). Stein (1994) has suggested that the long duration of action (> 12 h) of opioids instilled into the intra-articular space may be due to suppression of the sensitizing process in the early hours after the injury. In the present experiments, even though U50,488 produced virtually complete suppression of the lifting and licking response in the first phase, naloxone methiodide given after the first phase led to complete reinstatement of the second phase response. Suppression of the inflammatory response in the first phase by DAMGO or DPDPE also did not affect ability to reinstate the behavioural response or extravasation in the second phase with an antagonist given immediately before the second phase. These observations argue against opioids acting peripherally to inhibit sensitization, although these mechanisms may be involved in spinal effects of opioids. Rather, they support the notion that, in the time scale during which the response to formalin evolves, opioids alter an ongoing local pain generating process. If anti-inflammatory actions or suppression of sensitization play a role in the effects of intra-articular opioids observed in humans, a much longer time-frame is probably involved.

The data imply that the peripheral transduction of pain is different in the two phases, since if similar mechanisms were involved, it would be expected that opioids would modulate them in a similar fashion. Alternative explanations are not convincing. The insensitivity of the first phase to DAMGO and U50,488 is not due to the first phase involving higher pain scores, because the pain scores are lower in the first phase than in the second. There is also little evidence for differences related to drug kinetics, because both DAMGO and U50,488 produced very similar effects on the second phase, regardless of whether they were injected before the first phase, or just before the second phase. Similarly, DPDPE effects on the second phase occurred in the absence of any effect on the first phase, despite the fact that it was injected before the formalin. The notion that the first and second phases depend on distinct afferent mechanisms is consistent

with the two phases being differentially sensitive to non-steroidal anti-inflammatory agents (Hunskaar and Hole, 1987; Hunskaar et al., 1986).

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