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Reversal by naloxone of the spinal antinociceptive actions of a systemically-administered NSAID

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1 Possible interactions between non-steroidal anti-inflammatory drugs (NSAIDs) and endogenous opioids were examined in electrophysiological experiments in α-chloralose anaesthetized spinalized rats without or with carrageenan-induced acute inflammation of one hindpaw. Spinal reflex responses, monitored as single motor unit discharges, were elicited by noxious pinch and electrical stimuli.

2 The μ-opioid agonist, fentanyl, was an effective depressant of reflexes under all conditions (ED_{50} 6–14 μg kg^{-1}, i.v.). In rats without peripheral inflammation the NSAID, flunixin, a niflumic acid derivative, had only a small effect that was not dose-dependent. However, in animals with unilateral inflammation, flunixin reduced spinal reflexes evoked both by noxious pinch stimuli (that activate peripheral nociceptors; ID_{50} 4 mg kg^{-1}, i.v.) and by electrical stimuli (that bypass nociceptor endings; ID_{50} 6.5–11 mg kg^{-1}, i.v.), indicating that it has a central site of action at doses comparable to those used clinically.

3 The opioid antagonist, naloxone (1 mg kg^{-1}, i.v.), reversed all actions of fentanyl. It did not reverse the small effects that flunixin had in rats without inflammation, showing that the NSAID is not a direct opioid agonist. In rats with carrageenan-induced inflammation of the hindpaw, however, naloxone fully reversed or prevented the antinociception by flunixin, but not that by the α_2-adrenoceptor agonist, medetomidine.

4 We conclude that under conditions of peripheral inflammation and the resultant central changes, the NSAID, flunixin, has antinociceptive actions that are mediated by endogenous opioids acting within the spinal cord.

Keywords: NSAID; flunixin; inflammation; opioid; fentanyl; naloxone; spinal cord; nociception

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are effective analgesics in situations of hyperalgesia related to tissue inflammation. There is plentiful evidence that they act peripherally (Lim *et al.*, 1964) and that they inhibit the synthesis of prostaglandins released in the inflammatory process (Vane, 1971). There is more recent evidence that they may also have spinal antinociceptive actions (McCormack, 1994). Relevant to the present study is the fact that they can be effective when injected intrathecally, particularly on responses to prolonged nociceptive inputs (Yaksh, 1982; Taiwo & Levine, 1988; Jurna *et al.*, 1992; Malmberg & Yaksh, 1992; but cf. Chapman & Dickenson, 1992). They can also have spinal actions at systemic doses used clinically (Herrero *et al.*, 1996). There have been various suggestions of a link between NSAID and opioid-mediated antinociception (Martini *et al.*, 1984; Taiwo & Levine, 1988; Björkman *et al.*, 1990; Malmberg & Yaksh 1993; Chambers *et al.*, 1995) but the sites and mechanisms of any such connection are not yet clear.

We have now performed electrophysiological experiments to establish whether there is any relationship between spinal opioid and NSAID-mediated antinociception, either in normal animals or in those with acute peripheral inflammation. We selected for study the NSAID, flunixin, a derivative of niflumic acid, because it is widely used by the intravenous route in veterinary practice as an effective analgesic for acute conditions (Booth & McDonald, 1988); it is also a potent agent with a relatively high ratio of antinociception to anti-inflammatory actions (Ciofalo *et al.*, 1977) and is a known inhibitor of prostaglandin synthesis (Lees & Higgins, 1984). We have recently reported that this NSAID has spinal actions at i.v. doses in the range used clinically (Herrero *et al.*, 1995). We now demonstrate that flunixin is not an opioid agonist but that under conditions of peripheral inflammation, naloxone can reverse or prevent the actions of this NSAID, indicating that there is an endogenous opioid peptide link in spinal NSAID antinociception. The data have been presented in abstract form (Herrero & Headley, 1995).

Methods

Details of methods have been described previously (Hartell & Headley, 1991; Herrero & Headley, 1991). Eighteen male Wistar rats (310–380 g) were anaesthetized with halothane (1.5–2% in O_2), and in 12 of them 100 μl of carrageenan (10 mg ml^{-1} in distilled water) was administered by intraplantar injection into one hindpaw. After cannulation of the trachea, carotid artery and both jugular veins, a laminectomy was performed at thoracic segments 8 to 9, the dura opened and the spinal cord transected. The spinalization was performed within 30 min of carrageenan injection, before major inflammation was evident. Halothane was discontinued after the surgery and anaesthesia was maintained with α-chloralose (50 mg kg^{-1}, i.v. initial dose and 20 mg kg^{-1} h^{-1} by i.v. infusion). Core temperature was maintained at 37±0.5°C by means of feedback controlled blanket and lamp systems. Blood pressure was monitored continuously; systolic levels never fell below 100 mmHg other than transiently following i.v. injection of fentanyl.

In all cases the animal was left for at least an hour after the surgery, and 3 h after carrageenan injection, before any drug was injected. The degree of inflammation produced by carrageenan was measured at the end of the experiment (5.5–8 h after the injection) as the volume of water displaced by the paw

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compared to the volume at the start of the experiment. At this stage there was a 75% increase in paw volume (from 1.8 ± 0.1 to 3.2 ± 0.1 ml; mean $\pm$ s.e.mean).

Bipolar Teflon-coated tungsten electrodes were used to record single motor unit activity from hind limb flexor muscles. Responses were elicited in 3 min cycles each of 3 stimuli: noxious pinch (15 s, 2.5 N over 19 mm^2); and two trains of electrical stimuli (2 ms pulse width and 10–20 times threshold for eliciting a reflex, a strength that elicited C- as well as A-fibre latency responses). These trains were delivered via needles inserted percutaneously at the proximal edge of the receptor field at either 'low frequency' (LF; 0.2 Hz, 4 stimuli), or 'high frequency' (HF; 1 Hz, 16 stimuli, during which frequency-dependent facilitation ('wind-up') occurred; Mendell, 1966).

Drugs used were the pro-inflammatory agent carrageenan (Sigma), the μ-opioid agonist, fentanyl citrate (Manorpark Pharmaceuticals), the NSAID, flunixin meglumine (Finadyne, Schering-Plough), the α_2-adrenoceptor agonist, medetomidine HCl (Domitor, Norden Laboratories) and the opioid antagonist, naloxone (Sigma). All drugs were injected intravenously (i.v.) in a volume of 0.3 ml.

Experiments were divided into three groups of six animals each. In two groups ('normal paw' and 'inflamed paw') the protocol of drug administrations was as follows: fentanyl (doses every 6 min to a cumulative total of 4–16 $\mu g\ kg^{-1}$, followed by recovery); flunixin (doses every 12 min to a cumulative total of 8 or 16 mg kg^{-1}); naloxone (1 mg kg^{-1} single dose injected 12 min after the last dose of flunixin); and fentanyl (16 $\mu g\ kg^{-1}$ single dose given 6 min after naloxone). Flunixin was administered at least 30 min after maximum recovery from fentanyl (i.e. 1 h or more after fentanyl injection), and only if responses had recovered above 80% of pre-fentanyl control values. In the third group (also 'inflamed paw') the protocol was similar except that naloxone was injected 15 min before flunixin. In six of the experiments the α_2-adrenoceptor agonist, medetomidine (4 or 8 $\mu g\ kg^{-1}$) was injected at the end of the experiment.

Data are presented as percentages of control ($\pm$ s.e.mean), where control is the mean of the responses recorded over the last 3 cycles prior to the administration of a drug. Statistical significance was assessed by non-parametric analysis of original spike count data. Drug effectiveness was assessed with Friedman's ANOVA with Dunn's post-tests for multiple comparisons (the latter are shown in Figures 1 and 3). Inter-group comparisons were by Mann Whitney U-test. ID_{50} values were estimated by linear regression.

Results

Rats with normal paws

As anticipated, the μ-opioid, fentanyl, dose-dependently depressed reflex responses to all types of noxious stimulus (Figure 1a; $P<0.005$ by Friedman's test). Depression below 20% control was always achieved with 16 $\mu g\ kg^{-1}$ of fentanyl; ID_{50} values for the 3 responses were between 7 and 9 $\mu g\ kg^{-1}$. Complete recovery was observed within the following 30 min (Figure 1a) and flunixin was injected after at least 1 h. The NSAID (2–16 mg kg^{-1} cumulative doses; Figure 1c) caused slight, but nevertheless significant, reductions to between 75 and 83% control values ($P<0.05$ for responses to pinch and low frequency electrical stimuli). These weakly antinociceptive actions were not dose-dependent and were not reversed by 1 mg kg^{-1} of naloxone (Figure 3a). This dose of naloxone completely prevented any action of a subsequent single near-maximal dose of fentanyl (Figure 3b), indicating effective opioid receptor blockade.

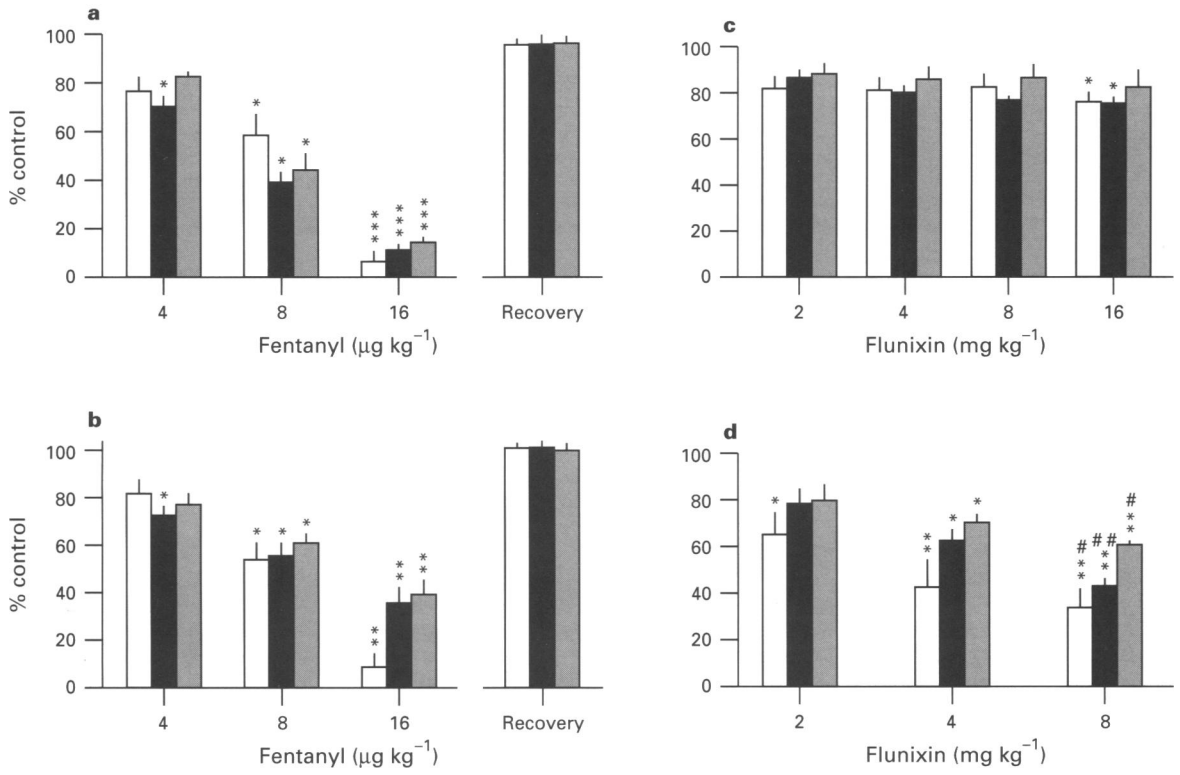

Figure 1 Pooled data of the antinociceptive effects obtained with cumulative i.v. administrations of the μ-opioid agonist, fentanyl, and the NSAID, flunixin. Reflex responses were elicited by noxious pinch (open columns), low frequency electrical (0.2 Hz, 4 shocks; LF, solid columns), and high frequency electrical (1 Hz, 16 shocks; HF, stippled columns) stimulation. Fentanyl was equipotent in reducing responses to all stimuli in rats with normal paws (a) and in those with an inflamed hindpaw (b). Flunixin, however, had little effect under normal conditions (c) but was much more effective following peripheral inflammation (d). See text for ID_{50} values. Statistical symbols: $*P<0.05$, $**P<0.01$, $***P<0.001$ *vs.* pre-drug controls (Dunn's tests post Friedman's; values for the latter are given in the text); $\#P<0.05$, $\#\#P<0.01$ *vs.* non-inflamed values (Mann-Whitney U-test). $n=6$ in all cases.

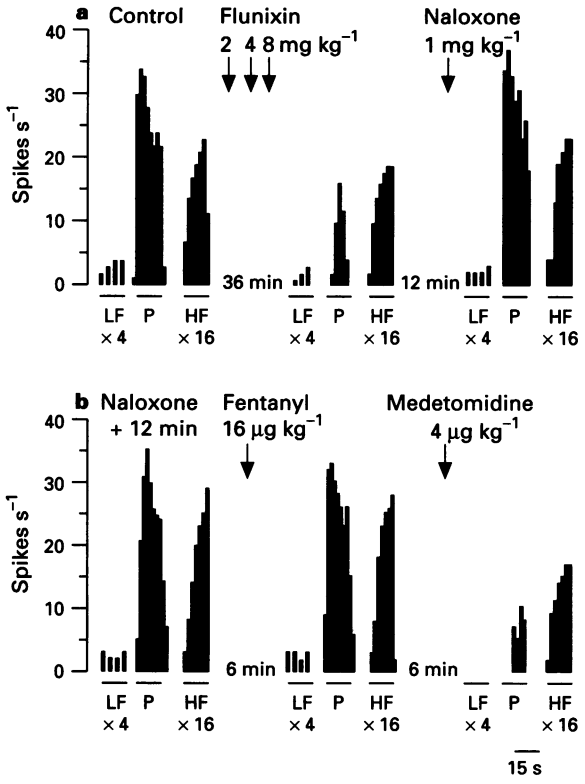

Figure 2 An example of one of the experimental protocols, as performed on a single motor unit in a rat with a carrageenan-inflamed paw. (a) The NSAID, flunixin (cumulative dose 8 mg kg^{-1}, i.v.), depressed all nociceptive responses and naloxone (1 mg kg^{-1}) reversed this action; (b) 15 min after naloxone, a single high dose of the μ-opioid agonist, fentanyl (16 μg kg^{-1}), was without effect whereas a few minutes later the α_2-adrenoceptor agonist, medetomidine (single dose of 4 μg kg^{-1}) reduced all responses. LF = low frequency electrical stimulation; P = pinch; HF = high frequency electrical stimulation.

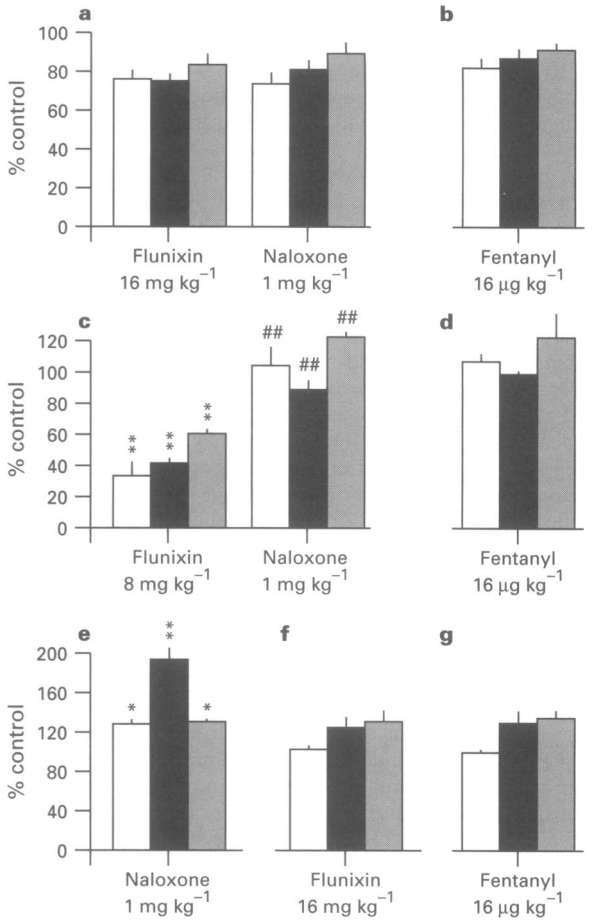

Figure 3 Reversal or prevention by the opioid antagonist naloxone (1 mg kg^{-1}, i.v.) of the effects of the NSAID, flunixin, on spinal reflexes in rats without (a,b) or with (c-g) carrageenan-induced inflammation of one hindpaw. Reflex responses were elicited by noxious pinch (open columns), low frequency electrical (LF, solid columns), and high frequency electrical (HF, stippled columns) stimulation. (a) In animals without hindpaw inflammation, flunixin had only weak effects and these were not reversed by naloxone. (b) The naloxone did, however, effectively prevent any action by a high dose of fentanyl. (c and d) As for (a) and (b) but in animals with an inflamed hindpaw; here the marked antinociceptive effects of flunixin (8 mg kg^{-1}, i.v.) were reversed by naloxone. A subsequent single high dose of fentanyl was again without effect. In (b) and (d) the fentanyl percentages refer to post-naloxone, pre-fentanyl values. (e) When naloxone was administered before flunixin it enhanced responses. In (f) and (g) a subsequent dose of flunixin (16 mg kg^{-1}) and of fentanyl (16 μg kg^{-1}) were without effect. The flunixin and fentanyl percentage values in (f) and (g) relate to post-naloxone responses. Statistical symbols: $*P<0.05$, $**P<0.01$ *vs.* pre-drug control; $\#\#P<0.01$ *vs.* flunixin 8 mg kg^{-1} (Dunn's tests post Friedman's). $n=6$ in all cases.

Rats with inflamed paws

Fentanyl dose-dependently reduced responses to all stimuli (Figure 1b; $P<0.005$ for all responses, Friedman test). Its potency was similar to that obtained in the non-inflamed condition, with ID_{50} values falling between 8 and 14 μg kg^{-1} for the 3 response types. Flunixin too was highly effective in animals with inflamed paws (Friedman test, $P<0.005$ for all stimuli). Figure 2a shows an original trace, and Figure 1d the pooled data. The estimated ID_{50} values for flunixin in rats with inflamed paws were: responses to pinch 4 mg kg^{-1}; to LF electrical stimuli 6.5 mg kg^{-1}; and to HF electrical stimuli 11 mg kg^{-1}. At 8 mg kg^{-1} it was significantly more potent in rats with inflamed as compared with those with normal paws ($P<0.05$ for responses to pinch and HF electrical stimuli; $P<0.01$ for LF electrical stimuli; Mann-Whitney).

These antinociceptive actions of flunixin were fully reversed by the opioid antagonist, naloxone (1 mg kg^{-1}, i.v., administered 12 min after flunixin). An example on a single unit is shown in Figure 2a, and the pooled data are in Figure 3c. A single near-maximal dose of fentanyl (16 μg kg^{-1}, i.v.) injected 12 min after naloxone did not reduce any of the responses (Figures 2b and 3d).

Because naloxone can itself enhance responses in this type of test (Hartell & Headley, 1991), it was important to check whether the apparent antagonism of flunixin was more than simple summation of flunixin depression and naloxone enhancement, and that any naloxone enhancement did not make responses supramaximal and therefore resistant to reduction by antinociceptive agents. Two types of test were performed to check for this.

To check whether enhancement of nociceptive responses by naloxone rendered responses resistant to reduction, the α_2-adrenoceptor agonist, medetomidine (4 μg kg^{-1}, $n=3$) was tested in the presence of naloxone and after fentanyl; it reduced the responses to all stimulus types (Figure 2b; reductions for the three response types were to 18% control for pinch, 2% for LF and 10% for HF electrical stimuli). In a further group of 6 experiments naloxone was administered before flunixin. It significantly enhanced responses to all three stimuli, with peak effects within the first or second stimulus cycle after administration (i.e. 3–6 min after the injection; Figure 3e). There was a slight fading of this effect after 12 min (i.e. by the last cycle before the flunixin injection). A subsequent single dose of 16 μg kg^{-1} flunixin failed to depress any of the responses from the naloxone-enhanced level (Figure 3f). Fentanyl (16 μg kg^{-1}, 12 min after flunixin) did not reduce responses at all (Figure

3g), whereas medetomidine (either 4 or 8 $\mu g\ kg^{-1}$, $n=3$) did depress responses to all stimuli to between 5 and 21% control at the single dose tested (not illustrated).

Discussion

The advantage of the electrophysiological protocol followed here is that the site of drug action could be assessed from the relative effectiveness of drugs on the different responses. The pinch stimuli will have activated nociceptor endings, which is where the peripheral actions of NSAIDs, and also opioids (Joris *et al.*, 1987; Stein *et al.*, 1988) are considered to act; drug actions at these endings could therefore have contributed to the reductions of responses to pinch stimuli. On the other hand the electrical stimuli will have activated afferent fibres proximal to their endings; any drug-induced reduction of the resulting responses must have been mediated proximal to the site of electrical activation, and presumably within the CNS (unless there are active sites along the afferent nerve fibres). Comparison of drug effects on responses to LF and HF electrical stimuli permits an assessment of any drug effects on the process of frequency-dependent facilitation ('wind-up'; Mendell 1966). The combination of stimuli, particularly when alternated, therefore provides a sound basis for assessing the likely site of action for drugs given systemically. The latter route has the great advantage over localized spinal administration of permitting comparison with doses used in clinical situations; the fact that flunixin is used by this route in veterinary practice was one reason for its choice as the NSAID in this study. Intravenous administration also has the advantage of exposing the whole neuronal pathway to similar concentrations of the agent in question.

Two aspects of the results obtained in these experiments indicate that the NSAID flunixin does not act as an opioid agonist. Firstly, in rats with normal paws it had little activity under conditions in which μ-opioid (as here) and κ-opioid agonists are effective (Herrero & Headley, 1991). Secondly, the small effects it had were not reversed by a dose of naloxone high enough to block actions mediated at both μ- and κ-opioid receptors (see Herrero & Headley, 1991) but not α_2-adrenoceptors (as shown here).

As reported recently (Herrero *et al.*, 1996) flunixin was an effective antinociceptive agent in rats with inflamed paws. Since it effectively reduced nociceptive reflexes to electrical as well as pinch stimuli, its antinociceptive effects were not simply due to peripheral anti-inflammatory actions. Unless the NSAID has direct effects on afferent nerves, which seems unlikely, it follows that it has central actions at doses in the range used clinically. Whether these central actions were dependent on prostaglandin synthesis cannot yet be assessed, but NSAIDs can certainly reduce spinal prostaglandin release triggered by nociceptive inputs (Malmberg & Yaksh, 1995; but see also McCormack & Brune, 1991).

Responses to both high and low frequency electrical stimulation were depressed with a similar potency, indicating that neither the μ-opioid agonist, fentanyl (as expected; Fraser *et al.*, 1992; Thorn *et al.*, 1994) nor flunixin had any specific actions on the process of 'wind-up'. The similar potency of fentanyl in rats with normal paws and in those with acutely inflamed paws does not match with the increase of potency observed with systemic morphine in more chronically arthritic rats (Kayser & Guilbaud, 1983).

The principal finding of this study was that the opioid receptor antagonist, naloxone, at a dose of 1 $mg\ kg^{-1}$, reversed the spinal antinociceptive effects of the NSAID, flunixin, but only in those animals with an inflamed paw. High doses of naloxone on its own increased nociceptive reflexes, as expected (Lombard & Besson, 1989; Hartell & Headley, 1991), supporting the suggestion that endogenous opioid systems may exert a tonic control in situations of inflammation (Lombard & Besson, 1989). The 'antagonism' of flunixin cannot, however, be explained by simple summation of separate flunixin and naloxone effects, since flunixin was without effect on the new baseline seen after administration of naloxone. A further possibility was that the enhancement of excitability by naloxone rendered the reflexes supramaximal and therefore resistant to reduction. This seems unlikely since low doses of the selective α_2-adrenoceptor agonist, medetomidine, were still able to reduce responses in the presence of naloxone. The conclusions must therefore be that naloxone acted as a non-competitive antagonist of the NSAID. It follows that the spinal antinociception caused by the NSAID was mediated via release of endogenous opioid peptides, a conclusion consistent with the finding that prostaglandins can block endogenous opioid-mediated analgesia (Taiwo & Levine, 1988). Since only a single, rather high dose of naloxone (1 $mg\ kg^{-1}$, i.v.) was tested in these experiments, it is not possible to deduce whether μ- or κ-opioids are involved. It is, however, known that following peripheral inflammation the level of spinal dynorphin, which is thought to act at κ-opioid receptors, is increased much more than that of enkephalins (Noguchi *et al.*, 1992).

The fact that this NSAID-opioid interaction was detected in spinally-transected animals indicated that the spinal cord is, under these conditions, most likely to be the site of the effect, although an action via systemic release of β-endorphin (Martini *et al.*, 1984) cannot at present be ruled out.

In conclusion, the NSAID, flunixin, is an effective spinal antinociceptive agent in rats with unilateral acute inflammation. Although flunixin does not activate opioid receptors directly, its actions in animals with sectioned spinal cords were reversed by naloxone. This indicates that the antinociceptive effect is dependent on endogenous opioid peptides; presumably the NSAID augments the release that occurs tonically under these conditions of acute inflammation.

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