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Opioid Antagonists and Antisera to Endogenous Opioids Increase the Nociceptive Response to Formalin: Demonstration of an Opioid *Kappa* and *Delta* Inhibitory Tone

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ABSTRACT

The present experiments explored the role of endogenous opioids in the behavioral response to a formalin-induced nociceptive stimulus in the rat. Flinching was taken as a measure of the intensity of the nociceptive stimulus after the administration of formalin into the dorsal surface of the paw of control animals, or in animals receiving i.p. administration of receptor-selective doses of opioid antagonists including naloxone, naltrindole (*delta* opioid antagonist), nor-binaltorphimine (*kappa* opioid antagonist) or β-funaltrexamine (*mu* opioid antagonist). Additionally, antisera to [Leu^5]enkephalin, [Met^5]enkephalin and dynorphin A (1–13) (dynorphin) were administered intrathecally before formalin to explore the contribution of endogenous opioids in modulation of the flinching response. Formalin-induced flinching was increased significantly by naloxone, and receptor selective doses of naltrindole and nor-binaltorphimine, but not β-funaltrexamine. Additionally, antisera to [Leu^5]enkephalin and dynorphin also resulted in a significant increase in formalin-induced flinching, whereas antisera to [Met^5]enkephalin had no effect. On the basis of significant increases in formalin-induced flinching produced by 1) receptor-selective doses of *delta* and *kappa*, but not *mu*, opioid antagonists and 2) antisera to [Leu^5]enkephalin and dynorphin A, but not [Met^5]enkephalin, these data suggest the presence of an opioid inhibitory tone which acts to limit the intensity of the pain signal. This tone appears to be mediated *via* activation of *delta* and *kappa* receptors, possibly by a [Leu^5]enkephalin- and dynorphin-like substance, respectively.

The formalin test for nociception has become a well established assay for the evaluation of tonic pain (Dubuisson and Dennis, 1977). The s.c. administration of a formalin solution into the hindpaw of a rat produces a typical syndrome of inflammatory hyperalgesia consisting of favoring of the paw, licking and flinching (Wheeler-Aceto *et al.*, 1990; Wheeler-Aceto and Cowan, 1991). The syndrome appears in two phases, an initial period of acute nociceptive behavior lasting from the time of injection for 10 to 15 min and a second tonic phase occurring 20 min after injection and lasting 50 to 90 min. The acute phase may represent a direct effect on nociceptors, whereas the tonic phase may represent an enhanced response of sensitized dorsal horn neurons resulting from low-level neural input due to peripheral inflammatory insult (Hunskaar and Hole, 1987). This model presents the advantage that a spontaneous, rather than reactive, behavioral response to tonic nociception is observed (Tjolsen *et al.*, 1992). Dickenson and Sullivan (1987) demonstrated in electrophysiological studies that formalin administration to a hindpaw excites primary afferent c-fibers in a biphasic manner and follows a similar time course as observed in behavioral studies, suggesting that formalin-induced nociception is mediated by the small diameter unmyelinated nociceptors.

Peripheral inflammatory processes may elicit changes in spinal levels of endogenous opioids. Noguchi *et al.* (1992) showed a greater than 200% increase in spinal preproenkephalin levels resulting from carrageenan-induced inflammation, whereas Hunter *et al.* (1995) measured no increase in spinal preproenkephalin mRNA after formalin injection. In other studies, Bourgoin and colleagues (1990) demonstrated increased, but transient, spinal [Met^5]enkephalin levels in response to s.c. injections of 10% formalin. Millan and co-workers (1988) demonstrated increased spinal dynorphin levels with no change in either [Met^5]enkephalin or [Leu^5]enkephalin levels after inflammation caused by administration of Mycobacterium butyricum in the right hind-

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ABBREVIATIONS: NTI, naltrindole; nor-BNI, nor-binaltorphimine; β-FNA, β-funaltrexamine; DAMGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; i.t., intrathecal; DPDPE, [D-Pen^5]enkephalin.

paw. Recent studies have also suggested that noxious stimulation induced by s.c. injection of mustard oil induces a nociception that is rekindled by the administration of naloxone (Hu *et al.*, 1993), suggesting a role for released endogenous enkephalins in inflammatory pain. Finally, we have shown recently that carrageenan-induced inflammation increased the potency of morphine in a NTI-sensitive fashion, suggesting an increase in the availability of spinal enkephalins (Ossipov *et al.*, 1995). Moreover, spinal preprodynorphin and preproenkephalin mRNA levels are also increased, as are dynorphin levels. In spite of elevated spinal preproenkephalin mRNA levels, it has been difficult to demonstrate consistently increased enkephalin levels in the spinal cord subsequent to inflammation (Dubner, 1991). The present experiments were therefore designed to investigate the importance of endogenous opioid substances which might be involved in the possible modulation of the nociceptive response.

Methods

All experiments utilized male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN), weighing from 175 to 200 g. All procedures were approved by the institutional Animal Care and Use Committee of the University of Arizona.

Nociceptive testing. Rats received an s.c. injection of 50 μl of 2% formalin solution in the dorsal surface of the right hindpaw. They were placed into observation chambers for the duration of the experiments and were observed for flinching behavior. Flinches were counted in bins of 5 min each, starting with the formalin injection and ending either 50 min or, when antagonists were administered, 90 min later. To quantify the flinch response over the first and second phases, the total number of flinches occurring between 0 and 15 min and between 20 min and termination were summed, giving a cumulative distribution over time. The duration of the observation period for the tonic phase was due to the prolongation of flinching induced by the opioid antagonists. Independent comparisons were performed between groups by Student's *t* test. Multiple comparisons were performed by using analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was established at the 95% level and groups of four to eight animals were used in each study.

Opioid antagonist studies. The opioid antagonist naloxone (5 mg/kg i.p.), the *delta*-opioid-selective antagonist (Sofuoglu *et al.*, 1991) NTI (10 mg/kg i.p.) and the *kappa*-opioid-selective antagonist (Portoghese *et al.*, 1987) nor-BNI (3 mg/kg i.p.) were administered at 0 min, whereas the *mu*-opioid-selective antagonist (Takemori *et al.*, 1981) β-FNA (5 mg/kg i.p.) was administered 24 hr before formalin.

Selectivity confirmation studies. Separate experiments were conducted to confirm the opioid subtype selectivity of the doses of antagonists used. In these studies, a dose of a receptor-selective opioid agonist was administered by direct i.t. injection between L4 and L5, 10 min before formalin in unanesthetized rats; these doses and times were determined in pilot studies to produce antinociceptive actions. In these experiments, the *mu*-opioid-selective agonist DAMGO (0.5 μg i.t.) (Handa *et al.*, 1981; Hirning *et al.*, 1985), the *kappa*-opioid-selective agonist CI-977 (10 μg i.t.) (Hunter *et al.*, 1990) or the *delta*-opioid-selective agonist DPDPE (100 μg i.t.) (Mosberg *et al.*, 1983) were administered 10 min before formalin. The *delta*- or *kappa*-opioid antagonists, NTI or nor-BNI, were administered immediately after formalin, whereas β-FNA was administered 24 hr before formalin. In these experiments, the acute phase was measured from 0 to 15 min and the tonic phase from 20 to 50 min.

Antisera studies. Experiments were designed in an effort to determine the significance of spinal [Leu^5]enkephalin and [Met^5]enkephalin and dynorphin with regard to the possible existence of an opioid tone. Antisera to either of these enkephalins or dynorphin were administered (200 μg in 5 μl) by direct i.t. injection 20 min before formalin. These doses of antisera have been shown previously to have no effect on antinociceptive actions of morphine, DPDPE or [D-Ala^2,Glu^4]deltorphin (Vanderah *et al.*, 1993; T. W. Vanderah and F. Porreca, unpublished observations). Control animals received only i.t. physiological saline. In these experiments, the acute phase was measured from 0 to 15 min and the tonic phase from 20 to 50 min.

Drugs. DAMGO and DPDPE were obtained from Multiple Peptide Systems (San Diego, CA), NTI and nor-BNI were obtained from RBI (Natick, MA) and β-FNA was obtained from the National Institute on Drug Abuse (Rockville, MD). The enkephalin and dynorphin antisera raised against [Met^5]enkephalin and [Leu^5]enkephalin and dynorphin A(1–13) were produced by repeated injection of rabbits with bovine thyroglobulin and were a generous gift of Dr. Leon Tseng (Medical College of Wisconsin, Milwaukee). The specificities of the antisera have been characterized by radioimmunoassay. Anti-[Met^5]enkephalin was obtained as crude rabbit serum. Cross-reactivity was 100% for [Met^5]enkephalin, 29.4% for [Leu^5]enkephalin and < 0.001% for dynorphin A(1–13). The cross-reactivity for [Leu^5]enkephalin antiserum was 100% for [Leu^5]enkephalin, 14% for [Met^5]enkephalin and 2.4% for dynorphin A(1–17) and < 0.14% for dynorphin A(1–8). The antidynorphin A(1–13) serum cross-reactivity profile was dynorphin A(1–13) and A(1–17), 100%; dynorphin A(1–8), < 0.002%; [Leu^5]enkephalin, < 0.001%; and [Met^5]enkephalin < 0.001% (Tseng and Collins, 1993).

Results

The administration of 2% formalin produced a typical biphasic response, with an acute phase determined over 0 to 15 min and a tonic phase determined as 20 to 90 min. Previous work has shown that the number of flinches elicited by this concentration of formalin to be lower than the maximal number of flinches elicited by higher formalin concentrations such as 5% (M. H. Ossipov and F. Porreca, unpublished observations). Naloxone (5 mg/kg i.p.) elicited a significant increase in the number of flinches observed in the tonic, but not the acute, phase of the nociceptive response when given at 0 min (figs. 1 and 2). In one group of controls, an injection of naloxone was made 50 min after formalin (after the peak effect was reached); no significant increase in flinching compared to the control group was observed when naloxone was given at this time (fig. 1). Likewise, the administration of naloxone 90 min after formalin failed to elicit any further increases in the flinch response over the next 15 min (fig. 1) when compared to the control group.

A significant elevation in formalin-induced flinch responses in the tonic, but not the acute, phase was also seen with NTI (10 mg/kg i.p.) (fig. 2) (shown over a 90 min time course). Likewise, nor-BNI (3 mg/kg i.p.) also produced significant elevations in flinch responses in the tonic, but not acute, phase, although not to the same extent as that seen with NTI or naloxone (fig. 2). The increase in flinching observed with nor-BNI was significantly lower than that seen with naloxone or NTI. In contrast, pretreatment with β-FNA (5 mg/kg i.p.) did not produce any appreciable change in flinch response when compared to control values (fig. 2). Groups treated with β-FNA showed a significantly lower number of flinches compared with groups treated with either naloxone, NTI or nor-BNI.

The i.t. administration of DAMGO (0.5 μg), CI-977 (3 μg) or DPDPE (10 μg) significantly attenuated the flinching be-

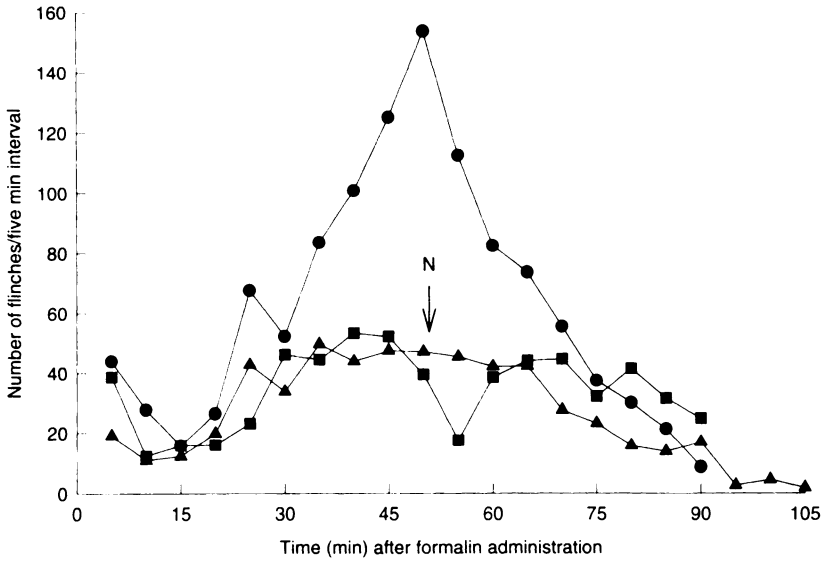

Fig. 1. This figure shows the total number of flinches over 5-min time intervals after 2% formalin administration to the hindpaw. ●, naloxone (N) administered 5 mg/kg i.p. immediately before formalin; ■, N given 50 min after formalin; and ▲, N given 90 min after formalin. S.E.M. error bars are omitted for the sake of legibility; n = 4–8 rats/group.

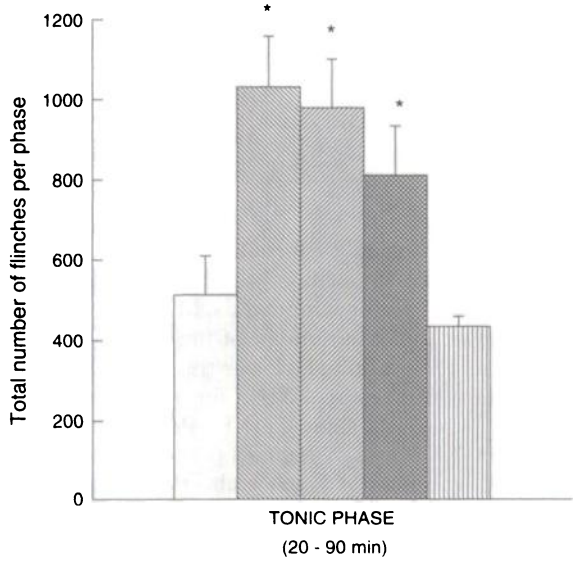

Fig. 2. This figure shows the total number of flinches per phase for each phase after opioid antagonists administered immediately before formalin. The treatment groups are: control (no treatment) (□), naloxone (5 mg/kg i.p.) (▨), NTI (10 mg/kg i.p.) (▨), nor-BNI (3 mg/kg i.p.) (■) and β-FNA (5 mg/kg i.p.) (▥). Error bars indicate S.E.M.; significance is defined as P ≤ .05. *A significant difference from control; n = 4–8.

havior compared to control groups in both the acute and tonic phases. The effects of DAMGO or CI-977 were not antagonized by the subsequent administration of NTI, although this dose of NTI did reverse the antinociceptive effects of DPDPE. Furthermore, the effects of DAMGO or DPDPE were not antagonized by subsequent nor-BNI, although this dose of nor-BNI did reverse the antinociceptive actions of CI-977. Pretreatment with β-FNA antagonized the antinociceptive effects of i.t. DAMGO; β-FNA was not tested against DPDPE or CI-977 as this antagonist did not produce any increases in flinching when given alone. These data are summarized in table 1.

The i.t. administration of [Leu^5]enkephalin or dynorphin antisera both produced a significant increase in tonic phase flinching (fig. 3), whereas [Met^5]enkephalin antiserum produced no change in nociceptive behavior (fig. 3). The flinching

TABLE 1

Antagonism of selective agonists by selective antagonists

These data represent the mean cumulative flinch response observed during the tonic phase (20–50 min). Opioid agonists were administered i.t. 10 min before formalin injection (2%). The opioid antagonists were administered by i.p. injection immediately before formalin injection.

Agonists	Antagonists			
	None	NTI (10 mg/kg)	nor-BNI (5 mg/kg)	β-FNA (5 mg/kg)
None	285 ± 61	450 ± 36*	376 ± 30*	252 ± 34
DAMGO, 0.5 μg	53 ± 31†	127 ± 60	96 ± 64	293 ± 148*
CI-977, 3 μg	144 ± 23†	127 ± 28	205 ± 44*	NT
DPDPE 10 μg	125 ± 27†	338 ± 148*	90 ± 33	NT

* A significant difference from each agonist alone (within row); † a significant difference from control flinching response (no drugs); and NT indicates that β-FNA was not tested against DPDPE or CI-977, as this antagonist did not produce any increases in flinching when given alone.

response observed with [Leu^5]enkephalin or dynorphin antiserum were not significantly different, but both groups were significantly lower than the number of flinches seen with naloxone or NTI alone (fig. 3).

Discussion

The data presented here demonstrate a clear tonic *kappa*- and *delta*-, but not *mu*-, opioid tone after formalin-induced nociception. These observations confirm and extend those of Wheeler-Aceto and Cowan (1993), who showed an increase in formalin-induced flinching after administration of naloxone. In the present studies, pretreatment with naloxone was also shown to increase markedly formalin-induced flinching, indicating that endogenous opioids may well be released as a consequence of the nociceptive stimulus. Although elevated dynorphin levels have been implicated clearly as a consequence of peripheral inflammation produced by a variety of agents (Iadarola *et al.*, 1988a), the role of spinal enkephalins is less clear. Previous work has shown that carrageenan-induced inflammation may elicit a release of endogenous enkephalins, thereby increasing the potency of morphine in this model (Ossipov *et al.*, 1995). Others have shown that there is a differential increase in spinal mRNA expression for preprodynorphin and preproenkephalin, with spinal preprodynorphin mRNA levels increasing 6- to 8-fold and spinal

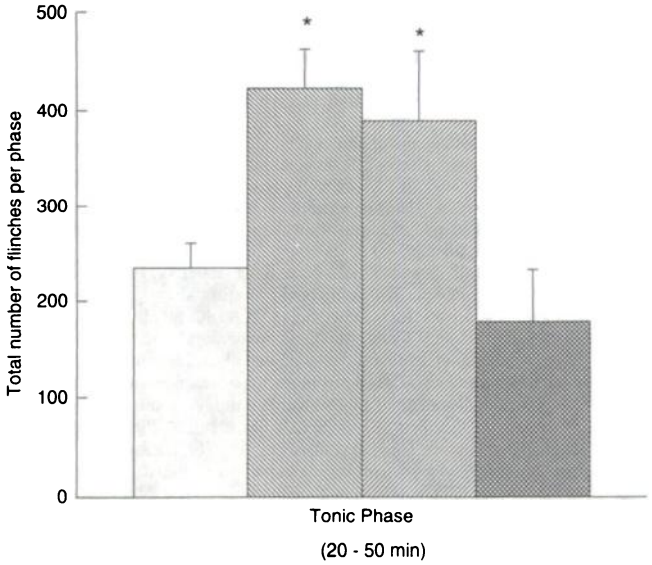

Fig. 3. This figure shows the effect of antisera to endogenous opioids on flinching behavior. The treatments are: control (□), [Leu^5]enkephalin antiserum (▧), dynorphin A(1–13) antiserum (▨) and [Met^5]enkephalin antiserum (■). Error bars indicate S.E.M.; significance is defined as $P \leq .05$. *Significant difference from control; $n = 4–6$ in each group.

preproenkephalin mRNA levels increasing about 80% after inflammation elicited by Mycobacterium butyricum (Iadarola *et al.*, 1988b) or carrageenan (Dubner and Ruda, 1992). In contrast, Hunter *et al.* (1995) showed that, although spinal preprodynorphin mRNA was elevated after formalin administration, preproenkephalin mRNA was not. In contrast, Noguchi and co-workers (1992) showed substantial increases in spinal preproenkephalin mRNA levels subsequent to peripheral inflammation.

In spite of the strong pronociceptive effect of naloxone in this model, β-FNA, at doses sufficient and selective for opioid *mu* receptors, failed to increase significantly the flinching response. However, it was also observed that the selective *mu*-opioid agonist DAMGO inhibited the flinch response to formalin injection. Additionally, it should be emphasized that β-FNA was also shown to antagonize the antinociceptive effects of i.t. DAMGO. These observations strongly suggest that the endogenous opioids released are acting at β-FNA insensitive (*i.e.*, non-*mu*) opioid receptors. Alternatively, one might suggest that endogenous substances may act at a *mu*-opioid receptor subtype that may be activated by DAMGO, but is not sensitive to antagonism by β-FNA. If this were to be true, then DAMGO may be said to act at both β-FNA-sensitive and -insensitive sites.

The concentration of formalin used in these experiments (2%) is submaximal in terms of the greatest number of flinches which can be elicited, an important point in light of the observation of increases in flinching behaviors (*i.e.*, pronociceptive responses). The *kappa*-opioid antagonist nor-BNI, again given at a dose which was sufficient and selective for *kappa*-opioid receptors, produced a significant increase in flinching activity, as would be expected if indeed dynorphin were released and acted as an "analgesic brake" (Iadarola *et al.*, 1988b; Hunter *et al.*, 1995). Nor-BNI was used at a dose sufficient to antagonize the antinociceptive effects of CI-977, but which had no effect on the antinociceptive response to i.t. DPDPE or DAMGO. The most dramatic pronociceptive effect was seen with NTI, which produced a very marked, and significant, increase in tonic flinching behavior. The fact that the dose of NTI used in these studies did not reverse the antinociceptive effect of the *mu*-opioid agonist DAMGO or that of the selective *kappa*-opioid agonist CI-977 (Hunter *et al.*, 1990), whereas antagonizing the antinociceptive response to i.t. DPDPE, strongly indicates that it selectively blocked an endogenous opioid acting at the *delta*-opioid receptor site, possibly [Leu^5]enkephalin or a [Leu^5]enkephalin-like substance, which has been suggested to be an endogenous ligand preferring the *delta*-opioid receptor.

The possibility of involvement of a [Leu^5]enkephalin-like endogenous substance is also supported by the results with antisera (see below). Although it has been difficult to measure increases in enkephalin levels subsequent to inflammation (Dubner and Ruda, 1992), there is a measurable rise in preproenkephalin mRNA expression, which may lead to [Leu^5]enkephalin as well as [Met^5]enkephalin production. However, it may also be argued that [Leu^5]enkephalin may be derived from dynorphins as well. The observation that antisera to [Leu^5]enkephalin and dynorphin greatly increased nociceptive flinching behavior, whereas [Met^5]enkephalin antiserum did not, strongly suggest a tonic opioid action acting at *kappa*- and *delta*-, but not *mu*-opioid receptors.

Interestingly, naloxone administered either 50 or 90 min after formalin injection neither caused a further increase in nociceptive behavior nor did it rekindle it. These data are in contrast to those reported by Hu *et al.* (1993), in which nociception induced by mustard oil was rekindled briefly by naloxone, but are consistent with the electrophysiological findings of Dickenson and Sullivan (1987), in which direct spinal application of DAMGO administered before formalin was still efficacious even when naloxone was administered after formalin. Studies by Kocher (1988) demonstrated that the i.p. administration of naloxone did not alter formalin-induced nociceptive behavior when administered 15 min after formalin. In another study, naloxone administered at doses sufficient to antagonize morphine antinociception failed to increase nociceptive behavior when administered either 30 min or immediately before formalin (North, 1978). However, in both of these studies, a behavioral scale, measuring posture, rearing, biting and licking, rather than quantifying flinching behavior, was used. That the behavior measured is an important determinant was demonstrated by Wheeler-Aceto and Cowan (1993). In that study, naloxone appeared to promote formalin-induced flinching, but reduced formalin-induced licking behavior. The most likely reason that naloxone did not elicit further increases in flinching activity in the present study when administered 50 or 90 min after formalin is that continued afferent nociceptive input initiated by formalin has ended, and there is insufficient nociceptive input to further exacerbate the behavior. The use of mustard oil as a nociceptive agent may elicit nociceptive afferent input of longer duration than that seen with formalin (Hu *et al.*, 1993).

That a tonic opioid influence is elicited by formalin administration is demonstrated further by our results with antisera to endogenous opioids. This technique has been used successfully to demonstrated an involvement of endogenous *delta*- and *kappa*-, but not *mu*-opioids in acute pain (Vanderah *et al.*, 1994). Here, the apparent inactivation of either [Leu^5]enkephalin or of dynorphin by selective antisera was

found to increase significantly nociceptive responses, in parallel with the results of studies with receptor-selective antagonists, whereas [Met^5]enkephalin antiserum did not. These data strongly suggest that dynorphin and [Leu^5]enkephalin production may be induced by the immediate-early genes activated by inflammation.

The data presented in this paper provide strong evidence that formalin-induced nociception elicits the release of endogenous substances acting through *kappa-* and *delta-*, but not *mu-*opioid-mediated mechanisms. This conclusion is supported by the observation that systemic naloxone, NTI, nor-BNI, but not β-FNA, all produced a heightened sensitivity to formalin. Likewise, nociceptive behavior was increased by antisera to [Leu^5]enkephalin or dynorphin but not to [Met^5]enkephalin. Collectively, these data suggest that formalin-induced nociception results in a tonic activation of *kappa-* and *delta*-opioid receptors resulting from increased endogenous dynorphin and [Leu^5]enkephalin production.

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