Contribution of Endogenous Enkephalins to the Enhanced Analgesic Effects of Supraspinal μ Opioid Receptor Agonists after Inflammatory Injury

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This study examined a mechanism responsible for the enhanced antihyperalgesic and antinociceptive effects of the μ opioid receptor agonist (ORA) [D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin (DAMGO) microinjected in the rostroventromedial medulla (RVM) of rats with inflammatory injury induced by injection of complete Freund's adjuvant (CFA) in one hindpaw. In rats injected with CFA 4 hr earlier, microinjection of the μ opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$ (CTAP) in the RVM antagonized both the marginal enhancement of the potency of DAMGO and its antinociceptive effect. The δ opioid receptor antagonist naltriben (NTB) was without effect. In rats injected with CFA 2 weeks earlier, CTAP antagonized the effects of DAMGO to a lesser extent. However, NTB completely prevented the enhancement of the potency of DAMGO, whereas it did not antagonize DAMGO's antinociceptive effects. Microinjection of NTB alone, but not CTAP in the RVM of CFA-treated rats, enhanced the hyperalgesia present in the ipsilateral hindpaw and induced hyperalgesia in the contralateral, uninjured hindpaw. These results suggest that persistent inflammatory injury increased the release in the RVM of opioid peptides with preferential affinity for the δ opioid receptor, which can interact in a synergistic or additive manner with an exogenously administered μ opioid receptor agonist. Indeed, the levels of [Met^5]enkephalin and [Leu^5]enkephalin were increased in the RVM and in other brainstem nuclei in CFA-treated rats. This increase most likely presents a compensatory neuronal response of the CNS of the injured animal to mitigate the full expression of inflammatory pain and to enhance the antinociceptive and antihyperalgesic effects of exogenously administered μ opioid receptor analgesics.

Key words: μ opioid receptor; δ opioid receptor; antinociception; complete Freund's adjuvant; hyperalgesia; nucleus raphe magnus

Peripheral inflammatory injury alters the pharmacology and physiology of primary afferent fibers that convey tactile and nociceptive information (Noguchi et al., 1988; Schaible and Schmidt, 1988; Donaldson et al., 1992; Ji et al., 1995; Leslie et al., 1995; Neumann et al., 1996; Tanaka et al., 1998), as well as the pharmacology and physiology of the dorsal horn neurons on which they synapse (Hylden et al., 1989; Noguchi et al., 1989; Weihe et al., 1989; Noguchi and Ruda, 1992; McCarson and Krause, 1994; Goff et al., 1998). More recently, peripheral inflammation has been recognized to also affect the efferent pain modulatory pathways. For example, persistent inflammatory injury can enhance either the inhibition or facilitation of spinal nociceptive transmission by medullary or pontine neurons (Schaible et al., 1991; Herrero and Cervero, 1996; Ren and Dubner, 1996; Tsuruoka and Willis, 1996; Urban et al., 1996, 1999; Kauppila et al., 1998; Wei et al., 1998, 1999; MacArthur et al., 1999; Terayama et al., 2000). Alterations in the activity of supraspinal neurons were inferred in most of these studies from lesion-induced changes in response latency or in the responses of the dorsal horn neurons to which these neurons project. However, insights into the response of supraspinal neurons to persistent inflammatory

nociception can also be gained from changes in the potency or efficacy of drugs administered directly into these sites. We recently reported that the potency of a μ or δ opioid receptor agonist (ORA) microinjected in the rostroventromedial medulla (RVM) is enhanced in a time-dependent manner after the injection of complete Freund's adjuvant (CFA) in one hindpaw (Hurley and Hammond, 2000). This enhancement is evident as an increase in the antihyperalgesic potency of these agonists as determined by their ability to alleviate hyperalgesia on the ipsilateral, inflamed hindpaw, and also as an increase in antinociceptive potency as determined by their ability to suppress nociceptive responses of the contralateral, uninjured hindpaw. Although numerous mechanisms may be considered, there is substantial evidence that μ and δ ORAs can interact in an additive or synergistic manner to produce antinociception (Roerig and Fujimoto, 1989; Porreca et al., 1990, 1992; Malmberg and Yaksh, 1992; Adams et al., 1993; Rossi et al., 1994; He and Lee, 1998). This study therefore investigated whether the enhanced antinociceptive and antihyperalgesic potency of [D-Ala^2-NMePhe^4-Gly^5-ol]enkephalin (DAMGO) results from the interaction of this exogenously administered μ ORA with endogenous enkephalins, which preferentially act at δ opioid receptors and the synthesis of which is increased in brainstem nuclei as a consequence of persistent nociception. Specifically, initial experiments characterized the ability of μ and δ opioid receptor selective antagonists to attenuate the enhanced antihyperalgesic and antinociceptive effects of DAMGO microinjected in the RVM of rats that had received an injection of CFA in one hindpaw either 4 hr or 2

weeks earlier. Subsequent experiments examined whether micro-injection of these antagonists by themselves enhanced CFA-induced thermal hyperalgesia. Finally, this study measured the levels of [Met^5]enkephalin and [Leu^5]enkephalin in brainstem nuclei at various times after the injection of CFA.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Sasco, Kingston, NY) weighing 275–350 gm were prepared with an intracerebral guide cannula aimed at the RVM and allowed to recover for 6–7 d as described previously (Hurley et al., 1999). Baseline measurements of paw withdrawal latency (PWL) to noxious radiant heat were then made for each hindpaw (Hargreaves et al., 1988; Dirig et al., 1997), after which 150 μl of either CFA [100 μg *Mycobacterium butyricum*, 85% Marcol 52, and 15% Aracel A mannide monoemulsifier (Calbiochem, LaJolla, CA)] or saline (0.9%) was injected into the plantar surface of one hindpaw under brief halo-thane anesthesia. The rats were then returned to their home cage for a period of either 4 hr or 2 weeks. These time points were chosen to represent the acute and chronic phases of inflammatory injury. Longer periods of inflammation were not examined to avoid the possible systemic spread of CFA and induction of a polyarthritic state. Animals were tested only once.

Experimental design. The first series of experiments verified the specificity of the agonists and antagonists at the doses injected in this study and confirmed that the antinociceptive effect of DAMGO microinjected in the RVM of saline-treated rats was mediated by a μ opioid receptor. For these experiments, baseline PWL was redetermined 4 hr or 2 weeks after the intraplantar injection of saline. DAMGO was then coadminis-tered with 0.33 μg of the μ opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$ (CTAP), and PWL was redetermined 15, 30, and 60 min later. In other experiments, either saline or 80 ng of naltriben (NTB), a δ_2 opioid receptor antagonist, was microinjected in the RVM 15 min before microinjection of DAMGO at the same site. Paw withdrawal latency was redetermined 15, 30, and 60 min later. This study was restricted to rats that received an intraplantar injection of saline 4 hr earlier. Other experiments confirmed the specificity of the antagonists and were largely restricted to rats that had received an intraplantar injection of saline 4 hr earlier to minimize animal usage. After redeter-mination of baseline PWL, 0.33 μg of CTAP was coadministered with the δ_2 ORA [D-Ala^2, Glu^4]enkephalin (DELT). In other rats, 8 or 80 ng of NTB was administered in the RVM 5 min before microinjection of DELT at the same site. Paw withdrawal latency in both experiments was redetermined 15, 30, and 60 min after the injection of DELT. The order in which the antagonists and agonists were administered in all studies ensured that their peak effects would coincide.

The second series of experiments examined the extent to which μ or δ opioid receptors mediate the enhanced antihyperalgesic and antinocicep-tive effects of DAMGO microinjected in the RVM of CFA-treated rats. These experiments were conducted in rats that had received an in-traplantar injection of CFA either 4 hr or 2 weeks earlier, because these times corresponded to the times at which the potency of DAMGO was minimally and maximally enhanced, respectively (Hurley and Hammond, 2000). In these experiments, baseline PWL was redetermined 4 hr or 2 weeks after the injection of CFA. DAMGO was then coadministered in the RVM with 0.33 μg of CTAP or was administered in the RVM 15 min after microinjection of either 8 or 80 ng of NTB. Paw withdrawal latency was redetermined 15, 30, and 60 min later. Doses of DAMGO in excess of 100 ng were not tested because of the occurrence of profound cata-tonia and other behaviors that interfered with the measurement of PWL.

The third series of experiments examined whether microinjection of the opioid receptor antagonists alone altered PWL. In these studies, either saline, 8 or 80 ng of NTB, or 0.33 μg of CTAP was microinjected into the RVM of rats that had received an intraplantar injection of either saline or CFA 4 hr or 2 weeks earlier. To ensure that antagonist-induced alterations in body temperature did not confound the measurements of thermal nociceptive threshold (Berge et al., 1988), paw temperature and paw diameter were also measured as described previously (Hurley and Hammond, 2000). The assessment of antagonist effects on paw tempera-ture and diameter was restricted to rats that had received an injection of CFA 4 hr earlier to minimize the number of rats used. At the conclusion of testing, the rats were killed by CO$_2$ inhalation, and the brains were removed for histological localization of the microinjection sites (Hurley and Hammond, 2000).

The final series of experiments examined whether induction of inflam-matory nociception altered the levels of [Met^5]enkephalin or [Leu^5]enkephalin in the brainstem. After intraplantar injection of 150 μl of either CFA or saline into the plantar surface of one hindpaw, the rats were returned to their home cage for a period of either 4 hrs, 4 d, or 2 weeks. These time points were chosen to represent the acute, subacute, and chronic phases of inflammatory injury. To obtain tissue, the rats were deeply anesthetized with ketamine (85 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.), supplemented by sodium pentobarbital, and then exsangui-nated. The midbrain, pons, and medulla were cut into transverse blocks 2–3 mm in depth and rapidly frozen for removal of the nuclei of interest using a standardized array of tissue micropunches (Stoelting, Chicago, IL). These regions included the nucleus raphe magnus (NRM), ipsilat-eral and contralateral nucleus reticularis gigantocellularis pars α (NGCα), ventrolateral periaqueductal gray (PAG) at the levels of the pons, and the midbrain, ipsilateral, and contralateral parabrachial nuclei, and ipsilateral and contralateral microcellular tegmentum.

Radioimmunoassay. Tissue samples were homogenized in 1N acetic acid and centrifuged at $10,000 \times g$ for 20 min at 4°C, and the pellet was extracted again in 1N acetic acid and centrifuged. Supernatants were combined, lyophilized, and stored at $-80°C$ until analysis. Protein was determined by the bicinchoninic acid method (Smith et al., 1985). Levels of [Leu^5]enkephalin and [Met^5]enkephalin in each sample were deter-mined by radioimmunoassay according to the manufacturer's protocol (Peninsula Laboratories, San Carlos, CA). Tissue samples from three to four CFA-treated rats at each time point were processed concurrently with tissue samples from two to three saline-treated rats. Levels were expressed as picomole per milligram of protein. Because the recovery of a known amount of [Met^5]enkephalin added at the outset of the exper-iment ranged from 95 to 100%, values were not corrected for any potential loss of analyte during the assay procedure.

Statistical analyses: behavioral experiments. Two-way ANOVAs for repeated measures were used to compare the effect of DAMGO in the presence of antagonist with that in the absence of antagonist, or to compare the effects of the antagonists alone on response latency, paw temperature, or paw diameter with those of saline. The Newman–Keuls test was used for *post hoc* comparisons among the individual group means. Dose–response relationships for DAMGO were determined by linear regression analysis using the individual PWLs measured at the time of peak effect, which was determined previously to be 15 min (Hurley and Hammond, 2000). Fieller's theorem as applied by Finney (1964) was used to determine the 95% confidence limits. Analysis of covariance was used to compare ED$_{50}$ values for DAMGO in the pres-ence and absence of the antagonists. Calculation of the ED$_{50}$ values for the antihyperalgesic and antinociceptive effects of DAMGO were based on the entire dose–effect relationship. The ED$_{50}$ value was defined as the dose of agonist that produced 50% of the maximum possible increase in PWL. In the noninflamed hindpaw, the average baseline PWL was ~10 sec, and the maximum response latency was 20 sec. Therefore, the criterion latency for calculation of the ED$_{50}$ value for production of antinociception on the noninflamed hindpaw was set to 15 sec. In the inflamed hindpaw, the mean baseline PWL was 3.6 ± 0.1 ($n = 154$) and 5.7 ± 0.1 sec ($n = 190$) at 4 hr and 2 weeks after injection of CFA, respectively. To examine the antihyperalgesic (as opposed to the antino-ciceptive) potency of the agonists, the maximum response latency was set to 10 sec, i.e., a return of PWL to normal threshold. The criterion latency for calculation of the ED$_{50}$ for the production of antihyperalgesia on the inflamed hindpaw 4 hr or 2 weeks after injection of CFA was therefore set to 6.8 and 7.9 sec, respectively.

Statistical analyses: radioimmunoassay. Values for individual samples that were greater than or less than 1.5 times the interquartile range for the group plus the third quartile or minus the first quartile, respectively, were excluded from analysis. With one exception, no significant differ-ences were observed in the levels of either peptide in any region as a function of time after injection of saline. The data for these three time points were pooled for each region of interest to yield a single saline-treated control group for comparisons with CFA-treated rats. One-way ANOVAs followed by a *post hoc* Bonferroni *t* test were used to compare levels of the peptides in the three CFA-treatment groups with that in the saline-treatment group. Where assumptions of normality and equal vari-ance were not met, a Kruskal–Wallis one-way ANOVA on ranks and Dunn's test were used for these comparisons.

Drugs. DAMGO [lot no. 121H58153; molecular weight (MW) = 513.6] and DELT (lot no. 88H13351; MW = 782.5) were purchased from Sigma (St. Louis, MO). CTAP (lot no. MPSP-13-02; MW = 1103) and NTB
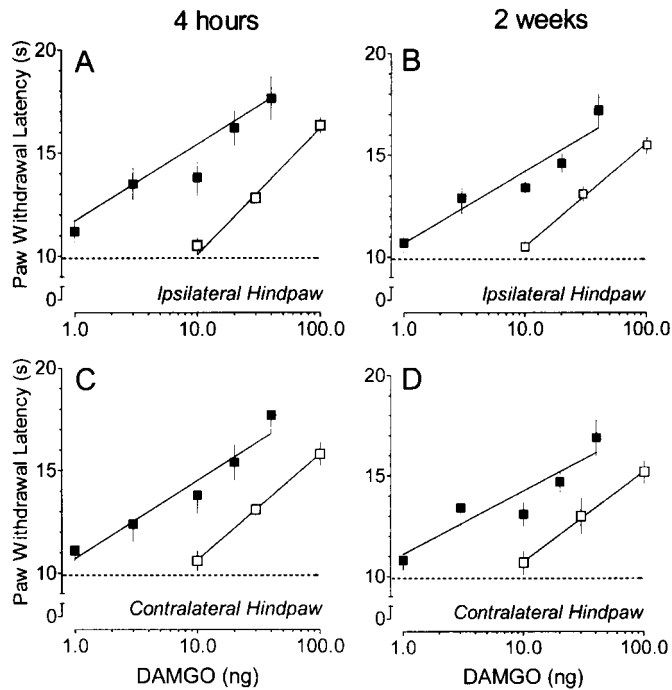
Figure 1. Dose–response relationships for DAMGO alone (■) and in combination with 0.33 µg of CTAP (□) in rats that received an intraplantar injection of saline either 4 hr (*A*, *C*)) or 2 weeks (*B*, *D*) earlier. *A*, *B*, Rightward shift for the ipsilateral hindpaw. *C*, *D*, Rightward shift for the contralateral hindpaw. Solid lines represent the least square linear regression of the individual data at 15 min, the time of peak effect. Each symbol is the mean ± SEM of determinations in 6–11 rats. The dashed horizontal line in each panel represents the average baseline PWL of rats after the intraplantar injection of saline and before the microinjection of any drug. Data for DAMGO alone are taken from Hurley and Hammond (2000).

(lot no. PG-111-83; MW = 511.6) were obtained from the Research Technology Branch of the National Institute of Drug Abuse (Bethesda, MD). All drugs were dissolved in saline, pH 7.4, and delivered in a 0.25 µl volume via a 33 gauge stainless steel injector that extended 3 mm beyond the tip of the guide cannula.

RESULTS

Distribution of microinjection sites

Microinjection sites in this study were predominantly distributed throughout the rostrocaudal extent of the NRM, with a smaller percentage of sites within the adjacent NGCpα. The distribution of sites in the NRM and NGCpα did not differ systematically among the various treatment groups and was comparable to that depicted in previous studies from this laboratory (Thomas et al., 1995; Hurley et al., 1999, 2000) and is therefore not illustrated here. Very few microinjection sites were located outside the NRM or NGCpα. Microinjection of DAMGO alone (Hurley and Hammond, 2000) or in combination with the antagonists (data not shown) was ineffective at these sites. In view of the limited number of sites and the lack of efficacy of the agonist, these sites were excluded from further analysis.

The antinociceptive effect of DAMGO is mediated by a µ opioid receptor in saline-treated rats

Coadministration of 0.33 µg of CTAP with DAMGO produced a parallel, rightward shift in the dose–response relationships of DAMGO for both the ipsilateral and contralateral hindpaw of

saline-treated rats (Fig. 1). The magnitude of the shift averaged five-fold and was consistent among rats that had received an intraplantar injection of saline 4 hr or 2 weeks earlier (Table 1). In contrast, this dose of CTAP did not antagonize the increase in PWL produced by microinjection of the δ_2 ORA DELT in the RVM of rats that received an intraplantar injection of saline 4 hr earlier (Fig. 2). Furthermore, the increase in PWL produced by either 3 or 20 ng of DAMGO was not antagonized by previous microinjection of 80 ng of NTB, a δ_2 opioid receptor antagonist, in the RVM. This dose of NTB completely antagonized an equiantinociceptive dose of DELT (Fig. 2). Similar specificity of the antagonists was demonstrated in rats that had received an intraplantar injection of saline 2 weeks earlier (data not shown).

µ and δ opioid receptors mediate the enhanced antihyperalgesic effects of DAMGO in the CFA-treated rat in a time-dependent manner: findings for the ipsilateral hindpaw

In rats injected 4 hr earlier with CFA, coadministration of CTAP with DAMGO shifted the dose–response relationship for the antihyperalgesic effect of DAMGO to the right by 10-fold with a slight decrease in slope (Fig. 3*A*, Table 1). In contrast, in rats that had received an injection of CFA 2 weeks earlier, coadministration of CTAP with DAMGO shifted the dose–response relationship for DAMGO for the ipsilateral hindpaw to the right in a parallel manner, but only by 3.5-fold (Fig. 3*B*, Table 1).

To examine the role of the δ_2 opioid receptor, it was necessary to reduce the dose of NTB from 80 to 8 ng (see below). In rats that had received an intraplantar injection of CFA 4 hr earlier, 8 ng NTB did not significantly shift the dose–response relationship of DAMGO to the right (Fig. 3*A*, Table 1). However, in rats that had received an intraplantar injection of CFA 2 weeks earlier, this dose of NTB shifted the dose–response relationship of DAMGO to the right by 12.5-fold in a competitive manner (Fig. 3*B*, Table 1).

µ and δ opioid receptors mediate the enhanced antinociceptive effects of DAMGO in the CFA-treated rat in a time-dependent manner: findings for the contralateral hindpaw

In rats that had received an injection of CFA 4 hr earlier, coadministration of CTAP with DAMGO shifted the dose–response relationship for the antinociceptive effect of DAMGO in the contralateral hindpaw to the right by 17-fold (Fig. 3*C*, Table 1). This large shift was anticipated on the basis that CTAP should not only antagonize the antinociceptive effects of DAMGO to the same extent as in saline-treated rats, but should also antagonize the enhancement of the antinociceptive effect of DAMGO if this latter effect involved µ opioid receptors. Indeed, the 17.1-fold shift produced by CTAP in CFA-treated rats at 4 hr closely approximated the product of the antagonism of the enhancement (2.4-fold rightward shift) and antagonism of the antinociceptive effects of DAMGO (5.2-fold rightward shift). Furthermore, the ED_{50} of DAMGO in the presence of CTAP in CFA-treated rats (94.1 ng) did not differ from that in saline-treated rats (70.1 ng; $p > 0.1$).

In rats that had received an injection of CFA 2 weeks earlier, coadministration of CTAP with DAMGO shifted the dose–response relationship for DAMGO for the contralateral hindpaw to the right by 20-fold. This rightward shift included the "dog-leg" portion of the DAMGO dose–response relationship wherein the

Table 1. ED$_{50}$ values in nanograms (and 95% confidence limits) for DAMGO administered alone and in combination with antagonists in the RVM of rats that received an intraplantar injection of saline or CFA

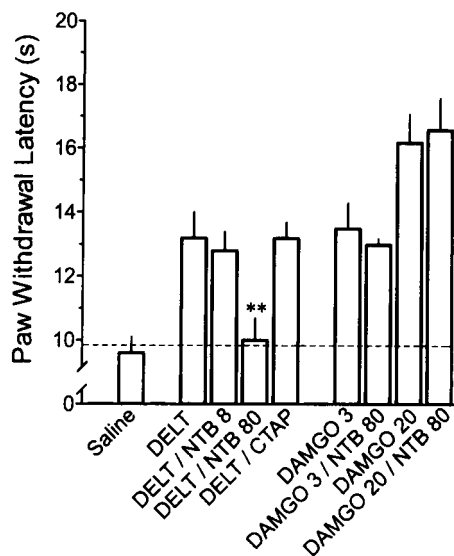| Intraplantar treat-ment interval	Intraplantar saline				Intraplantar CFA			
	Ipsilateral	Dose ratio	Contralateral	Dose ratio	Ipsilateral	Dose ratio	Contralateral	Dose ratio
Four hours								
DAMGO	10.8		13.4		0.75		5.5	
	(6.7–20.0)		(8.1–28.1)		(0.4–1.1)		(3.3–8.5)	
DAMGO/CTAP	62.8**	5.8	70.1**	5.2	7.8**	10.4	94.1**	17.1
	(52.1–79.1)		(52.0–108.6)		(2.9–18.9)		(29.4–n.d.)	
DAMGO/NTB	n.a.		n.a.		1.5	2.0	7.1	1.3
					(0.3–3.5)		(4.0–12.0)	
Two weeks								
DAMGO	16.7		17.8		0.02		1.9	
	(10.4–34.0)		(9.9–48.4)		(0.009–0.04)		(1.1–3.1)	
DAMGO/CTAP	75.9**	4.5	89.0**	5.0	0.07**	3.5	38.9	20.5
	(55.6–120.8)		(54.2–247.7)		(0.02–0.2)		(23.1–66.4)	
DAMGO/NTB	n.d.		n.d.		0.25**	12.5	19.4**	10.2
					(0.09–0.6)		(10.0–45.1)	

Asterisks indicate ED$_{50}$ values for DAMGO in the presence of an antagonist that differ from that of DAMGO alone for the corresponding treatment group; **$p < 0.01$. Dose ratio is the ED$_{50}$ value of DAMGO in the presence of the antagonist divided by the ED$_{50}$ of DAMGO alone. n.d., Not determined or indeterminate; n.t., not tested; n.a., no antagonism and indicates that NTB did not antagonize the antinociceptive effects of DAMGO in saline-treated rats as illustrated in Figure 2.

effects of very low doses were enhanced. However, this shift was less than the 47-fold that was predicted by the product of the antagonism by CTAP of the enhancement of antinociceptive effects of DAMGO (9.4-fold rightward shift) and its antagonism of the antinociceptive effects of DAMGO (5.0-fold rightward shift). Indeed, the ED$_{50}$ of DAMGO in the presence of CTAP in

Figure 2. Specificity of the opioid receptor agonists and antagonists microinjected in the RVM is illustrated for rats that received an intraplantar injection of saline 4 hr earlier. From the *left* to *right*, the *bars* depict the effects of saline, 1.25 μg of DELT alone, 1.25 μg of DELT with 8 ng of NTB, 1.25 μg of DELT with 80 ng of NTB, 1.25 μg of DELT with 0.33 μg of CTAP, 3 ng of DAMGO alone, 3 ng of DAMGO with 80 ng of NTB, 20 ng of DAMGO alone, and 20 ng of DAMGO with 80 ng of NTB. Paw withdrawal values are expressed as the mean ± SEM of determinations in six to eight rats. The PWL shown is that of the ipsilateral hindpaw 15 or 30 min after the microinjection of DAMGO or DELT in the RVM, which corresponds to their respective times of peak effect. The *dashed horizontal line* represents the average PWL determined after the intraplantar injection of saline and before the microinjection of any drug. *$p < 0.05$, **$p < 0.01$ compared with the effect of agonist alone.
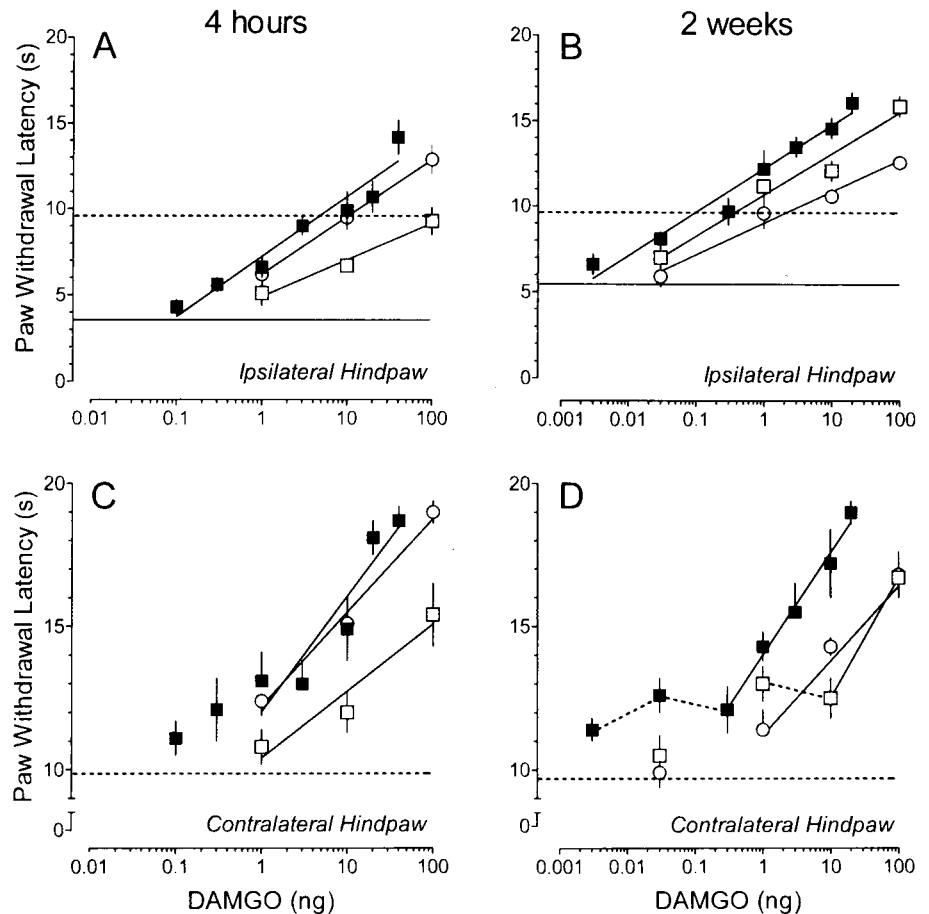
CFA-treated rats at 2 weeks (38.9 ng) was significantly less than that in saline-treated rats (89.0 ng; $p < 0.01$).

Pretreatment with 8 ng NTB did not shift the dose–response relationship of DAMGO for the contralateral hindpaw of rats injected 4 hr earlier with CFA (Fig. 3C, Table 1). It did not antagonize either the marginal enhancement of the potency of DAMGO or its antinociceptive effects (Fig. 4A). In contrast, in rats that had received an intraplantar injection of CFA 2 weeks earlier, this same dose of NTB shifted the dose–response relationship of DAMGO to the right by 10-fold in a competitive manner (Fig. 3D, Table 1). Figure 4B further illustrates that this latter effect was restricted to an antagonism of the enhancement of the potency of DAMGO and did not extend to antagonism of its antinociceptive potency. Thus, the ED$_{50}$ value for DAMGO in rats that were injected 2 weeks earlier with CFA, and in which NTB was microinjected in the RVM, did not differ from the ED$_{50}$ value of DAMGO in saline-treated rats (Table 1).

The dose–effect relationship of DAMGO in rats treated 2 weeks earlier with CFA was characterized by a dog-leg wherein very low doses produced antinociception; this effect was not dose-dependent over a 100-fold dose range (0.003–0.3 ng). It was noted that CTAP only marginally antagonized the antinociceptive effect of 1 ng of DAMGO yet paradoxically produced a greater antagonism of 10 ng of DAMGO (Fig. 3D). Microinjection of NTB also produced a partial antagonism of the antinociceptive effect of 1 ng of DAMGO (Figs. 3D, 4B). However, when CTAP and NTB were coadministered, the antinociceptive effect of 1 ng of DAMGO was completely antagonized (9.2 ± 0.8 sec). That neither dose alone completely antagonized the effects of DAMGO, yet the two did so when coadministered, suggests that 1 ng of DAMGO interacts in an additive manner with an endogenous δ ORA. Finally, the increase in PWL produced by a lower dose of DAMGO, 0.03 ng, was completely antagonized by microinjection of either CTAP or NTB (Fig. 3D). That either antagonist alone was sufficient to completely antagonize the enhanced antinociceptive effect of this low dose of DAMGO is consistent with a possible synergistic activation of μ and δ opioid receptors, although additional studies would be required to verify this.
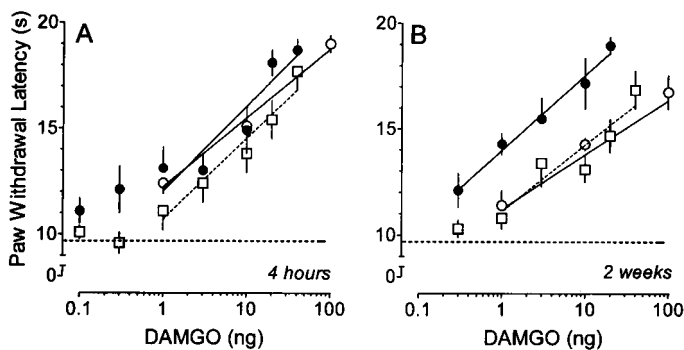
Figure 3. Dose–response relationships for DAMGO alone (■) and in combination with either 0.33 µg of CTAP (□) or 8 ng of NTB (○) in rats that received an intraplantar injection of CFA either 4 hr (*A, C*) or 2 weeks (*B, D*) earlier. *A, B,* Effects of the antagonists on the antihyperalgesic effect of DAMGO as determined by the ipsilateral inflamed hindpaw. *C, D,* Effects of the antagonists on the antinociceptive effect of DAMGO as determined by the contralateral, uninflamed hindpaw. The *horizontal dashed* and *solid lines* represent the average baseline PWL of rats before and after the intraplantar injection of CFA, respectively. *Solid lines* represent the least squares linear regression of individual values 15 min after microinjection of DAMGO, the time of peak effect. Each *symbol* is the mean ± SEM of determinations in 6–11 rats. Data for DAMGO alone are taken from Hurley and Hammond (2000).

RVM neurons in saline-treated rats are not subject to a tonic, opioid-mediated input

Microinjection of 0.33 µg of CTAP in the RVM of rats that had received an intraplantar injection of saline 4 hr or 2 weeks earlier did not alter PWL of either the ipsilateral or contralateral hind-


Figure 4. Dose–response relationships for DAMGO microinjected alone (●, *solid lines*) or in combination with 8 ng of NTB (○, *solid lines*) in the RVM of rats that received an intraplantar injection of CFA in one hindpaw either 4 hr (*A*) or 2 weeks (*B*) earlier, and the dose–response relationship of DAMGO in rats that received an intraplantar injection of saline (□, *dashed lines*) at the corresponding time. The values are the PWLs of the contralateral, uninjected hindpaw. *Solid* and *dashed lines* represent the least squares linear regression of the individual data at 15 min, the time of peak effect. Each *symbol* represents the mean ± SEM of determinations in 6–11 rats. The *horizontal dashed lines* represent the average baseline PWL of rats after the intraplantar injection of CFA or saline, and before microinjection of the drugs.

paw as compared with the effects of saline in the RVM (Fig. 5*A,B*). This dose of CTAP did not produce any adverse motor effects. However, a 10-fold higher dose of CTAP produced severe motor impairment and abnormal behavior, including circling, hyperventilation, and abnormal postures in five of five rats. Microinjection of 80 ng of NTB in the RVM of saline-treated rats also did not alter the PWL of either hindpaw as compared with the effects of saline (Fig. 5*A,B*). This dose of NTB did not produce any adverse behavioral effects.

RVM neurons in CFA-treated rats receive a tonic, opioid input mediated by δ_2 opioid receptors

Microinjection of 0.33 µg of CTAP in the RVM of rats that had received an intraplantar injection of CFA 4 hr or 2 weeks earlier did not alter PWL of either the ipsilateral or contralateral hindpaw (Fig. 5*C–F*). In contrast, microinjection of 80 ng of NTB in five CFA-treated rats produced behaviors indicative of nociception, including flinching of the inflamed hindpaw, excessive grooming, and freezing behavior. Mechanical allodynia was also present bilaterally on the hindpaws, flank, and abdomen. Neither ipsilateral nor contralateral PWL could be reliably measured in three of these rats because they simply would not place the inflamed hindpaw on the glass surface. This state was maintained for at least 60 min. Microinjection of 8 ng of NTB in the RVM of CFA-treated rats did not produce this constellation of behaviors. However, this dose of NTB decreased the withdrawal latency of the ipsilateral hindpaw in both the 4 hr (Fig. 5*C*) and 2 week (Fig. 5*D*) treatment groups compared with the effect of saline in the RVM. Thus, NTB enhanced the CFA-induced hyperalgesia in
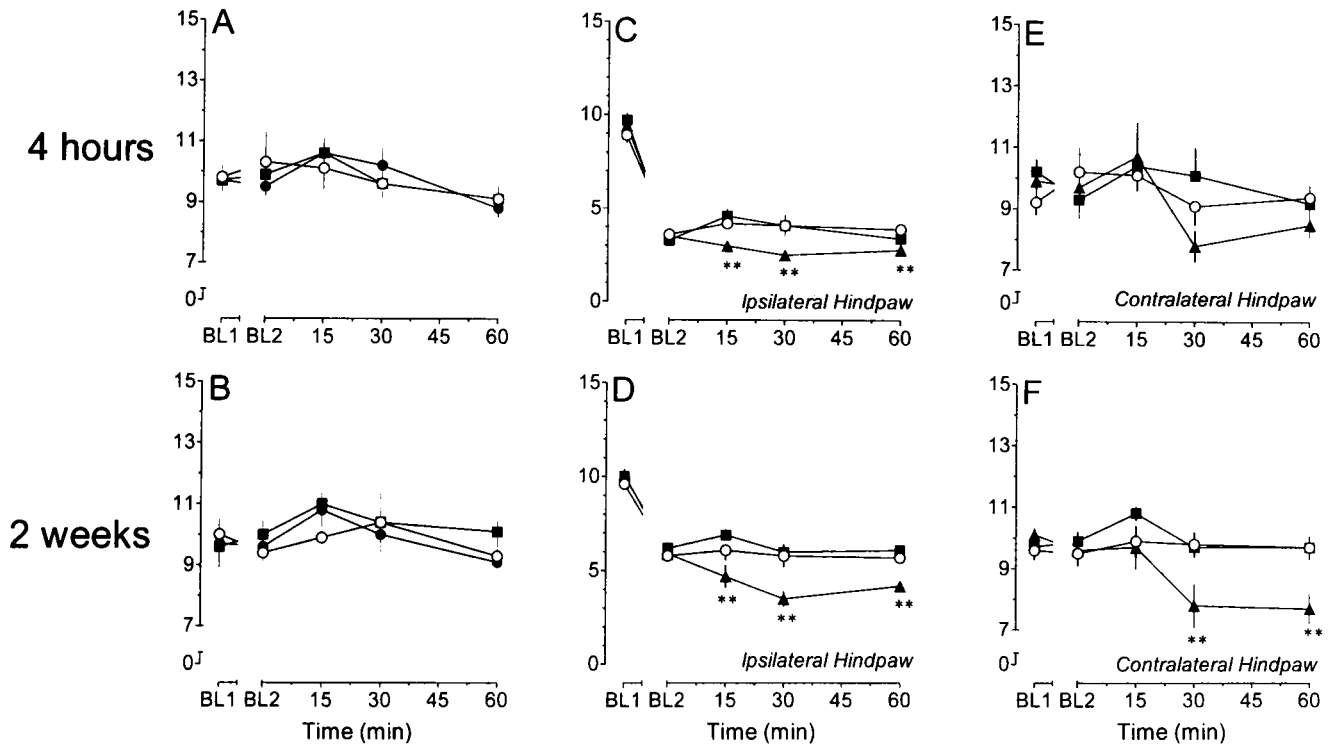
Figure 5. Time course of PWL after microinjection of either saline (○), 0.33 μg of CTAP (■), 80 ng of NTB (●), or 8 ng of NTB (▲) in the RVM of saline (*A*, *B*)- or CFA-treated *C–F*) rats. Only the withdrawal latency of the ipsilateral hindpaw is depicted for rats that received an intraplantar injection of saline 4 hr or 2 weeks earlier. The 80 ng dose of NTB could not be tested in CFA-treated rats because it caused allodynia and hyperesthesia. *BL1* represents the baseline PWL determined before intraplantar injection of saline or CFA. *BL2* represents the baseline PWL after the intraplantar injection of saline or CFA, and before the microinjection of the antagonists. Each *symbol* represents the mean ± SEM of determinations in six to eight rats, with the exception of CTAP in saline-treated rats at 2 weeks where $n = 3$. *$p < 0.05$, **$p < 0.01$ compared with saline at the corresponding time point.

these animals. This dose of NTB also significantly decreased the withdrawal latency of the contralateral hindpaw of CFA-treated rats. This effect was best observed in the 2 week treatment group (Fig. 5*F*); the decrease in the 4 hr treatment group did not achieve statistical significance (Fig. 5*E*). Thus, microinjection of NTB in the RVM effectively induced hyperalgesia in the uninjured, contralateral hindpaw of CFA-treated rats.

The hyperalgesia induced by NTB was not secondary to an increase in either paw diameter or paw temperature. Microinjection of 8 ng of NTB in the RVM of rats that received an intraplantar injection of CFA 4 hr earlier did not alter either the temperature or the diameter of the ipsilateral (33.7 ± 0.3°C; 9.5 ± 0.3 mm) or contralateral (28.6 ± 0.4°C; 6.0 ± 0.4 mm) hindpaws compared with the effect of saline (ipsilateral: 34.0 ± 0.2°C; 9.5 ± 0.2 mm; contralateral: 29.6 ± 0.6°C, 6.1 ± 0.1 mm; $p > 0.3$, both paws). The effects of CTAP on paw temperature or diameter were not assessed because it did not affect PWL.

Persistent inflammatory nociception is associated with an increase in the levels of endogenous enkephalins in brainstem nuclei

Figure 6 illustrates tissue levels of [Met⁵]enkephalin in nine different regions of the brainstem of saline- and CFA-treated rats. The location and relative size of the tissue punches are also illustrated in this Figure. Intraplantar injection of CFA was associated with a time-dependent increase in the levels of [Met⁵]enkephalin in some but not all nuclei. Levels of [Met⁵]enkephalin in the NRM were increased 4 d and 2 weeks but not 4 hr after the injection of CFA as compared with levels in saline-treated rats.

Levels of [Met⁵]enkephalin were similarly increased by three- to fourfold within the adjacent NGCpα both ipsilateral and contralateral to the injury 4 hr and 4 d after injection of CFA. Levels of [Met⁵]enkephalin were also increased at 2 weeks in the contralateral NGCpα, but the increase in the ipsilateral NGCpα failed to achieve statistical significance ($p = 0.1$). In the caudal aspect of the ventrolateral PAG, the levels of [Met⁵]enkephalin were uniformly increased by 2.5-fold regardless of time after injury. A similar but smaller increase occurred in the rostral aspect of the ventrolateral PAG. Levels of [Met⁵]enkephalin were also increased in both the ipsilateral and contralateral parabrachial region as a consequence of injury. However, the largest increase occurred in the parabrachial contralateral to the injury. Levels of [Met⁵]enkephalin in this region increased progressively as a function of time after the injection of CFA, with a nearly 10-fold increase evident by 2 weeks. Within the microcellular tegmental nucleus, levels of [Met⁵]enkephalin were increased 4 d and 2 weeks after the injection of CFA both ipsilateral and contralateral to the injury, as well as by 4 hr on the side ipsilateral to the injury.

Basal tissue levels of [Leu⁵]enkephalin in saline-treated rats ranged from one-half to one-third those of [Met⁵]enkephalin in the corresponding nucleus. Intraplantar injection of CFA was also associated with an increase in the tissue levels of [Leu⁵]enkephalin, although the increase rarely exceeded twofold and was more restricted in its distribution. For example, levels of [Leu⁵]enkephalin were significantly increased in the NRM of rats 2 weeks after the injection of CFA (1.02 ± 0.2 pmol/mg protein) as compared with
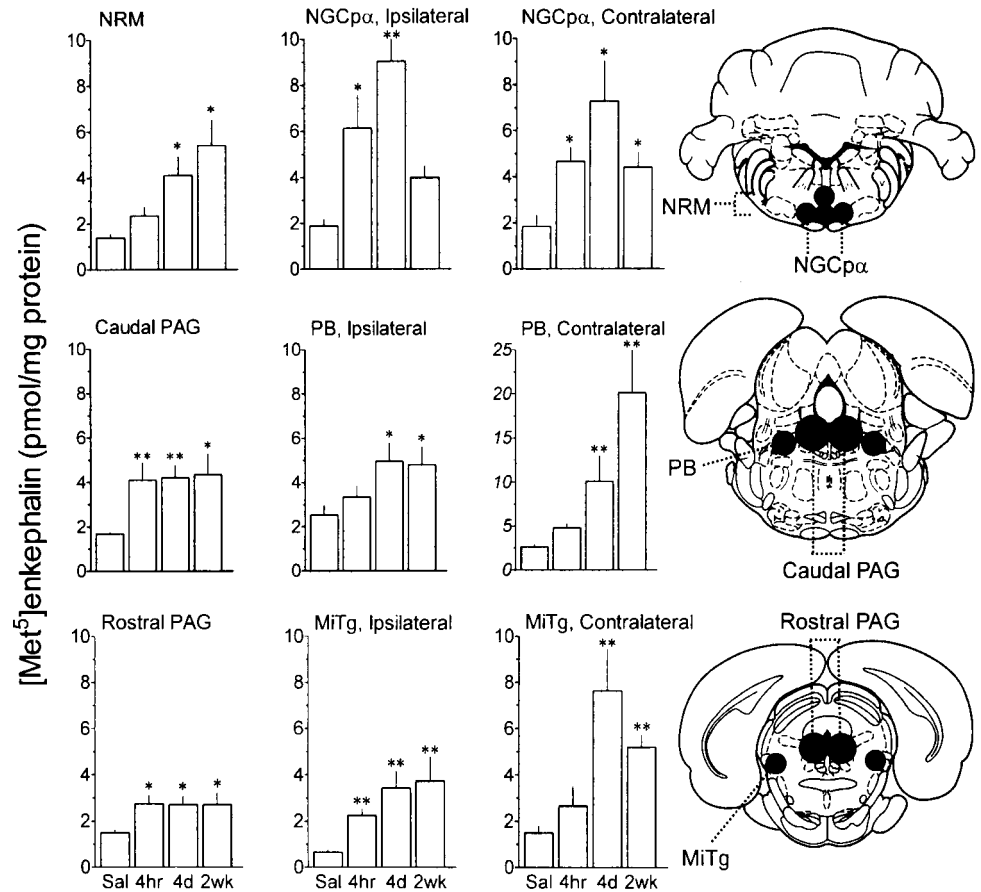
Figure 6. Levels of [Met⁵]enkephalin (picomole per milligram of protein) in brainstem nuclei of rats that received an intraplantar injection of saline or of CFA either 4 hr, 4 d, or 2 weeks earlier. The location and relative size of each punch is depicted on the adjacent sections from the atlas of Paxinos and Watson (1997). For the NRM, caudal PAG, and rostral PAG, two punches were combined. *NRM*, Nucleus raphe magnus; *NGCpα*, nucleus reticularis gigantocellularis pars α; *PAG*, periaqueductal gray; *MiTg*, microcellular tegmental nucleus; *PB*, parabrachial nucleus. Each *bar* represents the mean ± SEM of determinations in 6–11 rats. Note the ordinate for the contralateral parabrachial nucleus differs from those in the other panels by a factor of 2.5. *$p < 0.05$, **$p < 0.01$ compared with values in saline-treated rats for that region.

saline-treated rats (0.49 ± 0.04 pmol/mg protein; $p < 0.01$). Tissue levels of [Leu⁵]enkephalin were uniformly increased in the caudal ventrolateral PAG 4 hr (1.15 ± 0.25 pmol/mg protein), 4 d (1.16 ± 0.18 pmol/mg protein), and 2 weeks (1.18 ± 0.17 pmol/mg protein) after the injection of CFA as compared with saline-treated rats (0.55 ± 0.03 pmol/mg protein; $p < 0.05$, all times). A smaller increase in the levels of [Leu⁵]enkephalin occurred in the rostral aspect of the ventrolateral PAG at all time points. Finally, levels of [Leu⁵]enkephalin were also increased in the contralateral microcellular tegmental nucleus 4 d after the injection of CFA (0.53 ± 0.04 pmol/mg protein) compared with levels in saline-treated rats (0.38 ± 0.02 pmol/mg protein; $p < 0.05$).

DISCUSSION

The present findings provide direct support for our proposal (Hurley and Hammond, 2000) that enhancement of the antinociceptive and antihyperalgesic effects of DAMGO in CFA-treated rats is attributable to increased levels of endogenous opioid peptides within the RVM that have preferential affinity for the δ opioid receptor. They also provide provocative evidence that additional mechanisms may contribute to the upregulation of activity in pain modulatory pathways after the induction of persistent inflammatory nociception.

Neither CTAP nor NTB altered PWL when microinjected in the RVM of saline-treated rats, suggesting that RVM neurons do not normally receive tonically active opioid inputs (Kiefel et al., 1993; Roychowdhury and Fields, 1996; Thorat and Hammond, 1997; Hirakawa et al., 1999). Yet, in CFA-treated rats, microinjection of NTB in the RVM decreased PWL in both the ipsilat-

eral and contralateral hindpaws. Because an antagonist exerts no effect in the absence of agonist, this finding suggested that inflammatory nociception evoked the release of opioid peptides in the RVM that act preferentially at δ opioid receptors. [Met⁵]enkephalin and [Leu⁵]enkephalin were considered likely candidates because they are 10-fold selective for the δ opioid receptor *in vitro* (Corbett et al., 1993) and act preferentially at this receptor when released *in vivo* (Takemori and Portoghese, 1993; Tseng et al., 1995). Indeed, levels of [Met⁵]enkephalin and [Leu⁵]enkephalin were increased in the RVM of CFA-treated rats. They were also increased in the parabrachial and microcellular tegmental nuclei, which contain enkephalinergic neurons that project to the RVM (Beitz, 1982; Williams and Klobuchar, 1998). These increases occurred most consistently 4 d and 2 weeks after the induction of inflammation, when the antinociceptive and antihyperalgesic effects of DAMGO were progressively enhanced. An increase in enkephalin release would disinhibit or activate OFF cells in the RVM (Harasawa et al., 2000) and be consistent with an activation of bulbospinal pain inhibitory pathways under conditions of inflammatory nociception (Ren and Dubner, 1996; Wei et al., 1999).

Although PWLs of the contralateral hindpaws of CFA-treated rats were not increased relative to those of saline-treated rats as initially expected (Hurley and Hammond, 2000; this study), δ ORAs are less efficacious than μ ORAs in producing antinociception after microinjection in the RVM (Rossi et al., 1994; Hurley and Hammond, 2000) or stimulating GTPγS binding in the medulla (Hurley et al., 2000). Thus, although the increase in enkephalin in the RVM was not sufficient to produce a frank

antinociception, it was "unmasked" by NTB. The increase in enkephalin synthesis in the RVM therefore represents a compensatory response to mitigate the full expression of inflammatory nociception. Although such an increase was demonstrated in the spinal cord (Noguchi et al., 1992; Ossipov et al., 1996), this report is the first to demonstrate its occurrence supraspinally.

Substantial evidence indicates that δ ORAs can potentiate the antinociceptive effects of μ ORAs (Roerig and Fujimoto, 1989; Malmberg and Yaksh, 1992; Porreca et al., 1992; Adams et al., 1993; Ossipov et al., 1995; He and Lee, 1998). Naltriben blocked the enhancement of the antinociceptive effects of DAMGO but not its antinociceptive effects when microinjected at the same site in the RVM. This finding provides the first evidence that the RVM is an important site for the synergistic or additive interaction of δ and μ ORAs. The interaction may result from coincident activation of μ and δ opioid receptors colocalized to the same neurons (Wang and Wessendorf, 1999) or the activation of neurons of complementary function (Harasawa et al., 2000) within the RVM.

The increase in enkephalin in nuclei other than the RVM suggests that the mechanisms that subserve the enhancement of the effects of DAMGO are not necessarily restricted to the RVM. Additive or synergistic interactions can occur between μ and δ ORAs administered at disparate sites, such as DAMGO in the RVM and DELT in the PAG (Kiefel et al., 1993; Rossi et al., 1994). Enkephalin levels were also increased in the PAG of CFA-treated rats. The enhancement of the antinociceptive and antihyperalgesic potency of DAMGO in the RVM therefore may also be mediated by a synergistic or additive interaction of the exogenously administered μ ORA with enkephalins the release of which is increased in the PAG. The widespread increase in enkephalin suggests that similar mechanisms may exist for an enhancement of the endocrine, autonomic, or addictive effects of μ ORAs under conditions of inflammatory nociception.

The study design did not permit a definitive conclusion regarding whether the interaction that subserves the enhancement of the effects of DAMGO was synergistic or additive. The manner in which μ and δ ORAs interact is dependent on the dose ratio of the agonists (Adams et al., 1993; He and Lee, 1998). In this study, the amount of enkephalin released was probably constant, whereas increasing doses of DAMGO were microinjected to construct a dose–response relationship. This feature increased the likelihood that the nature of the interaction would differ along the continuum of doses, because the ratio of exogenous μ ORA to endogenous δ ORA was changing continually. Nonetheless, the ability of NTB to completely, rather than partially, antagonize the enhancement of DAMGO throughout the linear portion of its dose–response relationship 2 weeks after the injection of CFA suggested that the interaction was largely synergistic. Different conclusions were reached for the extremely low doses of DAMGO that comprised the dog-leg in the dose–response relationship. The enhancement of 1 ng of DAMGO appeared to be additive, whereas that of 0.03 ng appeared synergistic. Studies in which the agonists are administered in fixed dose ratios will be required to define the nature of the interaction.

These data do not exclude a contribution by other endogenous opioid peptides. β-Endorphin binds with equal potency to δ and μ opioid receptors (Corbett et al., 1993). However, β-endorphin does not appear to act preferentially at δ opioid receptors *in vivo*. When administered intracerebroventricularly, its antinociceptive effects are not antagonized by intracerebroventricular administration of δ opioid receptor antagonists or antisense oligode-

oxynucleotides (Heyman et al., 1989; Wang et al., 1996; but see Shook et al., 1988). Also, molar quantities of β-endorphin in the RVM are $\frac{1}{10}$ those of [Met^5]enkephalin (Rossier et al., 1977; Palkovits and Eskay, 1987; this study), and β-endorphin-immunoreactive fibers are sparse (Bloom et al., 1978; Finley et al., 1981). The greater density of β-endorphin-immunoreactive fibers in the PAG and parabrachial nuclei (Finley et al., 1981; Palkovits and Eskay, 1987), however, suggests that further investigation may be warranted.

The increase in apparent affinity of NTB in CFA-treated rats was most intriguing. The affinity of a competitive antagonist is characteristic of the receptor at which it binds (Kenakin, 1997). The dose of NTB that induced hyperalgesia and antagonized the enhanced effects of DAMGO in CFA-treated rats was 10-fold less than the dose that antagonized DELT in the saline-treated rat. It also antagonized DELT in CFA but not saline-treated rats (our unpublished observations). This decrease in dose suggested that δ opioid receptors in CFA-treated rats bound NTB with higher affinity. The potency of CTAP also differed. Four hours after the induction of inflammation, CTAP antagonized the effects of DAMGO to the predicted extent. Yet 2 weeks after the injection of CFA, CTAP was less effective than predicted, suggesting that μ opioid receptors in CFA-treated rats bound CTAP with lower affinity. The basis for these divergent changes in receptor affinity is unclear. However, the change in apparent affinity is provocative evidence that persistent inflammatory nociception may alter the properties of μ and δ opioid receptors in the RVM. *In vitro* evidence indicates that δ opioid receptors can heterodimerize with δ opioid receptors (George et al., 2000; Gomes et al., 2000), supporting an earlier proposal that these receptors can associate as a functional complex (Vaught and Takemori, 1979; Rothman and Westfall, 1982; Schoffelmeer et al., 1990). The μ–δ dimer exhibited 10-fold lower affinity for prototypic μ and δ ORAs, yet a two- to three-fold higher affinity for [Leu^5]enkephalin (George et al., 2000). Extremely low doses of δ opioid receptor agonist or antagonist greatly increased the binding of DAMGO, as well as its potency and efficacy in stimulating phosphorylation of MAP kinase (Gomes et al., 2000). The affinity of antagonists for the μ–δ dimer has not been determined. However, one could speculate that inflammation leads to changes in the proportion of μ and δ opioid receptors that associate as a functional complex in the RVM, which is evident here as a change in the apparent affinity of the antagonists.

To conclude, persistent inflammatory nociception increases the synthesis and presumably the release of enkephalins in many brainstem nuclei implicated in pain modulation. This increase represents a compensatory response by the CNS to mitigate the full magnitude of nociception but also serves to enhance the effects of exogenously administered μ ORAs through either an additive or synergistic interaction. Inflammatory nociception may also lead to changes in the association of μ and δ opioid receptors, resulting in a complex that not only binds endogenously released enkephalins with greater affinity, but in so doing enhances the actions of exogenously administered μ opioid receptor agonists. This latter possibility remains to be confirmed.

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