The Journal of Neuroscience, February 1, 2000, *20*(3):1249–1259

The Analgesic Effects of Supraspinal μ and δ Opioid Receptor Agonists Are Potentiated during Persistent Inflammation

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This study examined the antihyperalgesic and antinociceptive effects of opioid receptor agonists microinjected in the rostral ventromedial medulla (RVM) of rats 4 hr, 4 d, and 2 weeks after the induction of an inflammatory injury by injection of complete Freund's adjuvant (CFA) in one hindpaw. Nociceptive sensitivity of the ipsilateral, inflamed and the contralateral, uninflamed hindpaws was determined by the radiant-heat paw withdrawal test. The antihyperalgesic potency of the μ opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), determined for the inflamed hindpaw, was enhanced 4 d and 2 weeks after injury. The antinociceptive potency of DAMGO, determined for the contralateral, uninflamed hindpaw, was also progressively enhanced 4 hr, 4 d, and 2 weeks after injury. The magnitude of enhancement paralleled the chronicity of the injury. The greatest potentiation occurred 2 weeks after injury when the ED_{50} value of DAMGO in CFA-treated rats was one-tenth that in saline-treated rats. The antihyperalgesic and antinociceptive effects of the δ opioid receptor agonist [D-Ala^2,Glu^4]deltorphin were also increased 2 weeks after injury. These results indicate that peripheral inflammatory injury alters the pharmacology of excitatory and inhibitory inputs that modulate the activity of RVM neurons in such a manner as to enhance the effects of opioid agonists in this region. These changes have ramifications not only for the alleviation of hyperalgesia at the site of injury but also for opioid-induced antinociception at sites remote to the injury as revealed by increases in the potency of opioid agonists to suppress nociceptive responses of the contralateral, uninflamed hindpaw.

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

Previous studies have asserted that the antihyperalgesic and antinociceptive effects of systemically administered μ opioid receptor agonists are enhanced under conditions of persistent, inflammatory nociception (Kayser and Guilbaud, 1983; Stein et al., 1988). Both peripheral and spinal mechanisms are implicated. In the periphery, the enhancement is attributed in part to an increased transport and accessibility of opioid receptors at the site of injury (Stein, 1995). In the spinal cord, the complexity of the inflammation-induced changes in neurotransmitter synthesis, receptor number, and responses of dorsal horn neurons (Dubner and Ruda, 1992) makes it difficult to ascribe a single mechanism. However, Ossipov and colleagues (1995) proposed that the enhancement results from an additive or synergistic interaction of the exogenous opioid with endogenous opioid peptides whose levels in the spinal cord are increased as a consequence of peripheral inflammation (Iadarola et al., 1988b; Przewlocka et al., 1992).

Comparatively little is understood about the changes that occur supraspinally after inflammatory injury. Peripheral inflammation is known to increase levels of [Met^5]enkephalin in the periaqueductal gray (PAG) and the microcellular tegmentum (Williams et al., 1995; Bellavance et al., 1996) and to increase κ opioid receptor binding in the PAG (Millan et al., 1987). In the polyarthritic rat, the spontaneous discharge and responsiveness to peripheral stimulation of ON-like cells and the number of OFF-like cells in the rostral ventromedial medulla (RVM) are increased (Montagne-Clavel and Oliveras, 1994). Polyarthritic rats not only have higher levels of serotonin in the spinal cord (Weil-Fugazza et al., 1979; Godefroy et al., 1987), which derives exclusively from medullary nuclei, but systemic administration of morphine in these rats increases serotoninergic metabolites in the spinal cord to a greater extent than in uninjured rats (Weil-Fugazza et al., 1979). Finally, recent studies indicate that the inhibitory and facilitatory modulation of spinal nociceptive transmission by supraspinal nuclei is enhanced after peripheral inflammation (Schaible et al., 1991; Herrero and Cervero, 1996; Ren and Dubner, 1996; Wei et al., 1998; MacArthur et al., 1999). Collectively, these data suggest that peripheral inflammation alters the physiology and pharmacology of supraspinal neurons that modulate nociception.

Despite the importance of the PAG and RVM as sites at which opioids act to produce antinociception, the functional consequences of these changes for opioid-mediated antinociception are not known. This study investigated the effects of μ and δ opioid receptor agonists microinjected in the RVM of rats as a function of time after the induction of inflammatory injury by intraplantar injection of complete Freund's adjuvant (CFA) in one hindpaw. It not only characterized the antihyperalgesic effects of these opioids on the ipsilateral, inflamed hindpaw, but also examined their antinociceptive effects on the contralateral, uninflamed hindpaw. Many studies of persistent nociception have focused predominantly on changes in the ipsilateral hindpaw or spinal cord, often using the contralateral hindpaw or spinal cord as a control.

Received Sept. 9, 1999; revised Nov. 11, 1999; accepted Nov. 12, 1999.

This work was supported by U.S. Public Health Service Grants DA06736 to D.L.H. and DA05784 to R.W.H. We thank William (Brad) Ruzicka for his excellent technical assistance.

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Table 1. Time-dependent effects of intraplantar injection of CFA or saline on paw withdrawal latency, paw diameter, and body weight

Intraplantar treatment	Paw withdrawal latency (sec)		Paw diameter (mm)		
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Weight (% baseline)
4 hr					
CFA	3.7 ± 0.1[a,b]	10.2 ± 0.2	9.6 ± 0.07[a,b]	6.2 ± 0.04	98.4 ± 0.8
Saline	9.7 ± 0.2	9.7 ± 0.2	6.2 ± 0.04	6.1 ± 0.03	100.8 ± 1.0
4 d					
CFA	4.9 ± 0.1[a,b]	9.8 ± 0.1	9.5 ± 0.08[a,b]	6.2 ± 0.04	104.0 ± 1.2
Saline	9.7 ± 0.1	9.7 ± 0.1	6.1 ± 0.04	6.2 ± 0.04	105.9 ± 0.9
2 weeks					
CFA	5.7 ± 0.1[a,b]	9.8 ± 0.1	8.9 ± 0.06[a,b]	6.3 ± 0.03	109.6 ± 1.5
Saline	9.7 ± 0.1	9.8 ± 0.1	6.3 ± 0.04	6.3 ± 0.03	112.5 ± 1.5

Values represent the mean ± SEM of determinations made in 61–99 CFA-treated and 46–71 saline-treated rats.
[a]Indicates a significant difference from the corresponding hindpaw of saline-treated rats, $p < 0.01$.
[b]Indicates a significant difference from the contralateral hindpaw, $p < 0.01$.

However, such an approach can obscure the presence and/or magnitude of the induced alterations because neuroanatomical and behavioral alterations can also occur contralateral to a unilateral injury (Donaldson, 1999; Koltzenburg et al., 1999).

MATERIALS AND METHODS

Experimental design. Male Sprague Dawley rats (Sasco, Kingston, NY) weighing 275–350 gm were anesthetized and prepared with an intracerebral guide cannula that terminated 3 mm dorsal to the RVM as previously described (Hurley et al., 1999). Six to seven d later, the rats were weighed, and measurements of thermal nociceptive threshold were made for both hindpaws using a radiant heat device (Hargreaves et al., 1988; Dirig et al., 1997). Briefly, the rat was placed in a clear Plexiglas box resting on an elevated glass plate that was maintained at 25°C. A beam of light was positioned under either hindpaw, and the time for the rat to remove the paw from the thermal stimulus was recorded as the paw withdrawal latency (PWL). The intensity of the stimulus was set to produce a PWL between 8 and 12 sec in a naive rat. If the rat did not withdraw its paw from the stimulus by 20 sec, the test was terminated, and the rat was assigned this cutoff value. After determination of the PWL, 150 μl of either CFA (100 μg *Mycobacterium butyricum*, 85% Marcol 52 and 15% Aracel A mannide monoemulsifier; Calbiochem, La Jolla, CA) or saline (0.9%) was then injected into the plantar surface of one hindpaw of each rat under brief halothane anesthesia. The rats were then divided into three groups and returned to their home cages for a period of either 4 hr, 4 d, or 2 weeks. Longer periods of inflammation were not examined to avoid possible systemic spread of the CFA and induction of a polyarthritic state.

On return to the test environment, body weight was recorded and measurements of paw diameter and PWL were made. Paw diameter was measured at the point of maximal inflammation along the dorsal–ventral axis of the hindpaw using calipers (FST Instruments, Foster City, CA). After determination of PWL, saline, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) [3 pg-40 ng; lot 121H58153; molecular weight (MW) = 513.6], or [D-Ala^2,Glu^4]deltorphin (DELT) (16 ng–1.25 μg; lot nos. 44H08641 and 88H13351; MW = 782.5) was microinjected into the RVM. These doses of DAMGO and DELT were determined to selectively activate μ and δ_2 opioid receptors, respectively, after administration into the RVM in a concurrent study (Hurley and Hammond, 1998). These drugs were purchased from Sigma (St. Louis, MO), dissolved in saline, pH 7.4, and delivered in a volume of 0.25 μl via a 33 gauge stainless steel injector that extended 3 mm beyond the tip of the guide cannula. The injector was left in place for another 60 sec to allow the drug to diffuse locally and to limit its diffusion up the injection track. Paw withdrawal latency was then redetermined 15, 30, and 60 min later. At the conclusion of testing, the rats were euthanized by CO_2 inhalation, and the brains were removed for histological localization of the microinjection sites as previously described (Hurley et al., 1999). The location of each microinjection site was verified by a person unaware of the treatment.

Agonist-induced alterations in body temperature can confound measurements of thermal nociceptive thresholds (Berge et al., 1988). Therefore, an ancillary study was conducted to determine whether microinjection of opioid receptor agonists in the RVM decreased cutaneous skin temperature and inflammation of the hindpaw because these effects could "masquerade" as antinociception or antihyperalgesia. Paw temperature measurements were performed with an infrared thermometer (model OS-604; Omega Engineering, Stamford, CT) held 0.5 cm from the ventral surface of the hindpaw. After baseline measurements of paw diameter and skin temperature were taken, the rats received an intraplantar injection of CFA into one hindpaw. Four hours later, paw

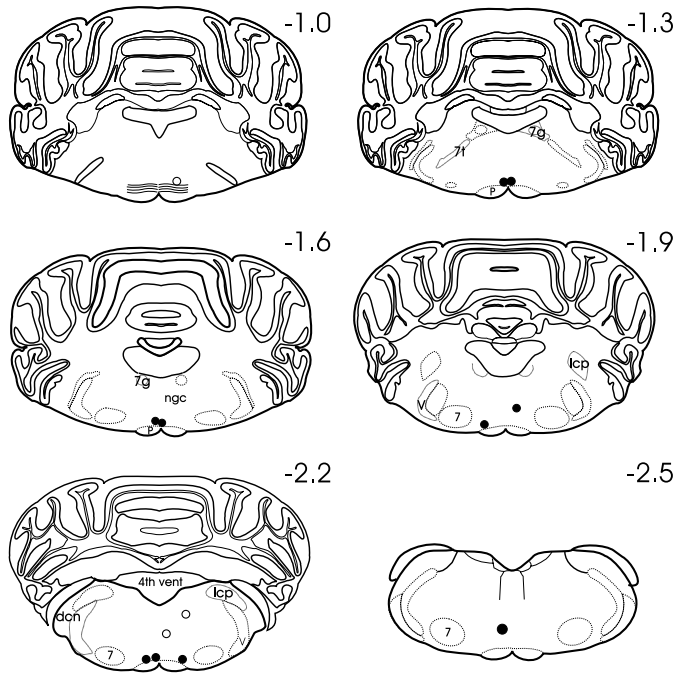

Figure 1. Rostrocaudal distribution of the sites in the medulla at which 0.63 μg DELT was microinjected 2 weeks after an intraplantar injection of saline. *Solid* and *open circles* represent injection sites within and outside of the RVM, respectively. *Numbers* to the *right* of each section refer to the distance caudal to the interaural line. *4th vent*; Fourth ventricle; *7*, facial motor nucleus; *7g*, genu of the seventh cranial nerve; *7t*, tract of the seventh cranial nerve; *dcn*, dorsal cochlear nucleus; *icp*, inferior cerebellar peduncle; *ngc*, nucleus reticularis gigantocellularis; *P*, pyramid; *V*, spinal trigeminal nuclei.

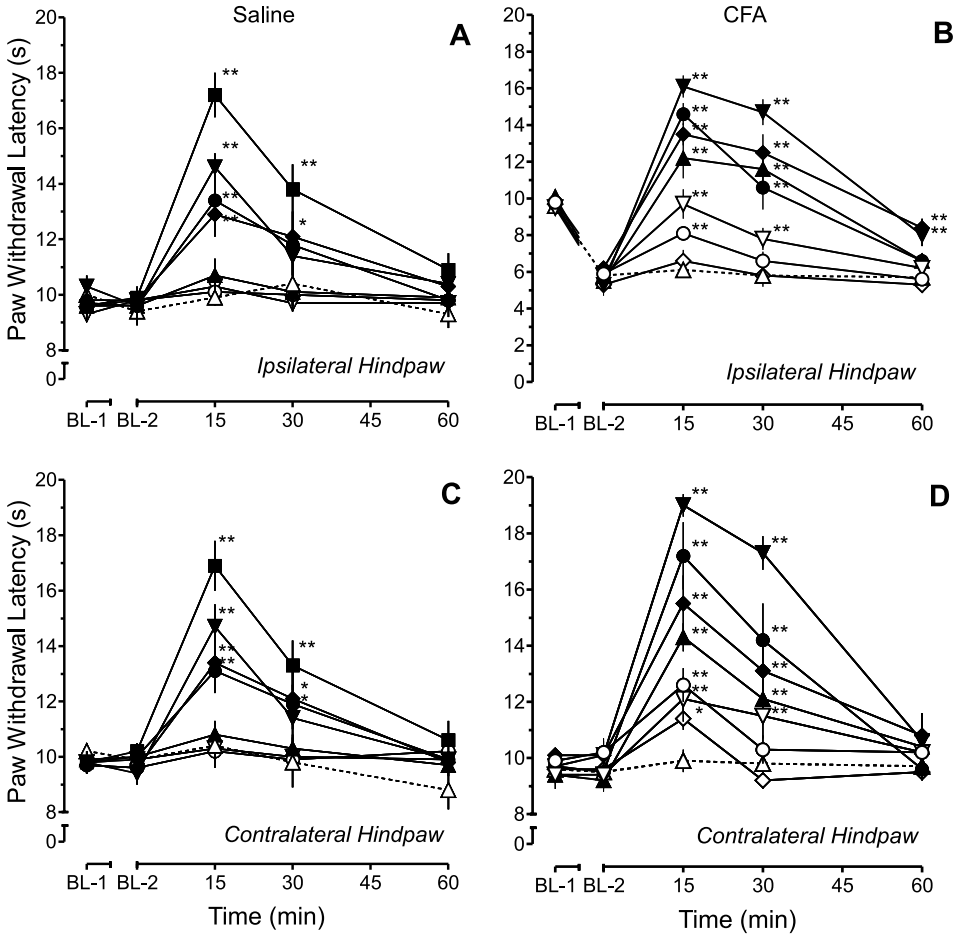

Figure 2. Time course of the increase in PWL produced by microinjection of either 0.003 ng (◇), 0.03 ng (○), 0.1 ng (□), 0.3 ng (▽), 1 ng (▲), 3 ng (◆), 10 ng (●), 20 ng (▼), or 40 ng (■) of DAMGO into the RVM of rats that received an intraplantar injection of saline (*A, C*) or CFA (*B, D*) in the left hindpaw 2 weeks earlier. The lack of effect of saline (△, *dashed line*) is depicted for comparison. *A* and *B* depict the PWL of the ipsilateral hindpaw of saline- and CFA-treated rats, respectively. *C* and *D* depict the PWL of the contralateral hindpaw of saline- and CFA-treated rats, respectively. *BL-1* represents the baseline PWL determined before intraplantar injection of saline or CFA. *BL-2* represents the baseline PWL after the intraplantar injection of saline or CFA, and before the microinjection of DAMGO. Each symbol represents the mean ± SEM of determinations in 6–11 rats. *Asterisks* indicate values that are significantly different from values at the corresponding time point in rats in which saline was microinjected in the RVM: $^{*}p < 0.05$, $^{**}p < 0.01$.

temperature and diameter were redetermined for both hindpaws; 10 ng of DAMGO, 1.25 μg of DELT, or saline was then microinjected in the RVM. Paw temperature and diameter were redetermined 15, 30, 45, and 60 min later. This study was restricted to 4 hr after injection of CFA to minimize the number of animals used.

Statistical analyses. Two-way ANOVA was used to compare the effects of CFA injection with that of saline on body weight and hindpaw diameter. Two-way ANOVAs for repeated measures were used to compare the effects of DAMGO or DELT on response latency, paw temperature, or paw diameter with that of saline. The Newman–Keuls test was used for *post hoc* comparisons among the individual group means. Dose–response relationships for the agonists were determined by linear regression analysis using the individual PWLs measured at the time of peak effect. These times corresponded to 15 and 30 min in the case of DAMGO and DELT, respectively. The ED_{50} value was defined as the dose of agonist that produced 50% of the maximum possible increase in PWL. In the case of 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 case of the inflamed hindpaw, the average baseline PWL was 3.7, 4.9, and 5.7 sec at 4 hr, 4 d, or 2 weeks after injection of CFA. Because we were interested in the antihyperalgesic (as opposed to the antinociceptive) 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, 4 d, or 2 weeks after injection of CFA was therefore set to 6.6, 7.3, and 7.9 sec, respectively. Fieller's theorem as applied by Finney (1964) was used to determine the 95% confidence limits. Calculation of the ED_{50} values for the antihyperalgesic and antinociceptive effects of DAMGO and DELT were based on the entire dose-effect relationship.

RESULTS

Characteristics of the inflammatory injury

Intraplantar injection of CFA induced significant and persistent inflammation, erythema, and hyperalgesia that were restricted to the injured hindpaw (Table 1). The increase in paw diameter was maximal at 4 hr, persisted through 4 d, and began to diminish by 2 weeks. The decrease in PWL was also maximal at 4 hr, persisted through 4 d, and was modestly diminished at 2 weeks. The persistence of significant hyperalgesia 2 weeks after the injection of CFA differs from a previous report (Iadarola et al., 1988a). However, this difference is likely attributable to the use of a larger dose of CFA in the present study that was administered in a different vehicle undiluted by saline. The small differences in PWL between the 4 hr and 4 d treatment groups (1.2 sec) and between the 4 d and 2 week treatment groups (0.8 sec) were statistically significant. This finding is most likely a consequence of the large sample size ($n > 60$). There was also a significant difference between PWL of the 4 hr and 2 week treatment groups.

Neither the diameter nor the PWL of the contralateral hindpaw of CFA-treated rats differed from baseline values or values for the corresponding hindpaw of saline-treated rats at any treatment time. This finding and the fact that rats that received an intraplantar injection of CFA gained an amount of weight similar to that of their saline-treated counterparts (Table 1) indicate that CFA did not exert a significant systemic effect as late as 2 weeks after inoculation. No significant changes in diameter or PWL of either

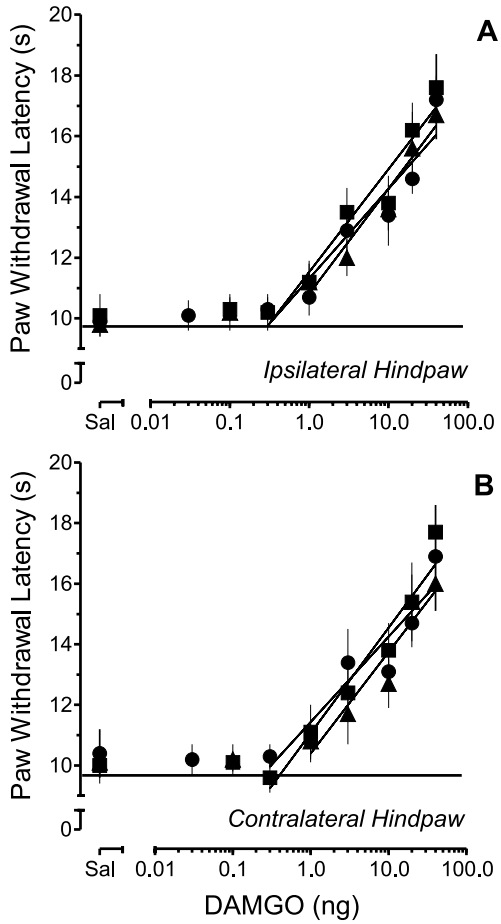

Figure 3. Dose–response relationships of DAMGO microinjected in the RVM of rats that received an intraplantar injection of saline in one hindpaw 4 hr (■), 4 d (▲), or 2 weeks (●) earlier. *Sal* represents mean PWL determined 15 min after microinjection of saline in the RVM. Doses of DAMGO are plotted on a logarithmic scale. The potency and maximal effect of DAMGO are similar for the (*A*) ipsilateral and (*B*) contralateral hindpaw at each time point and are independent of time after intraplantar injection of saline. Doses <0.3 ng, which did not lie on the linear part of the dose–response relationship, were not included in the linear regression. *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 six to nine rats. The *horizontal line* in each panel represents the average baseline PWL of rats determined after the intraplantar injection of saline and before the microinjection of DAMGO.

hindpaw occurred at any time after intraplantar injection of saline (Table 1).

Distribution of microinjection sites

The sites at which DAMGO or DELT were microinjected were predominantly distributed throughout the rostrocaudal extent of the nucleus raphe magnus, with a smaller percentage of sites within the adjacent nucleus reticularis gigantocellularis pars α. The very large number of rats, doses, and treatment conditions precluded illustration of all the microinjection sites in this study. Therefore, because there were no systematic differences in the distribution of sites at which saline, DAMGO, or DELT was microinjected among the different treatment conditions, only the distribution of microinjection sites for the 0.63 μg dose of DELT is presented (Fig. 1). Microinjection of DAMGO or DELT at sites outside these two nuclei, such as the pyramids, trapezoid body, medial longitudinal fasciculus, or dorsal or lateral aspects of the nucleus reticularis gigantocellularis, did not significantly increase PWL in saline-treated rats. These sites were excluded from further analysis.

Effect of DAMGO in saline-treated rats

Microinjection of 0.3–40 ng of DAMGO in the RVM of rats 4 hr, 4 d, or 2 weeks after the unilateral intraplantar injection of saline produced a dose-dependent increase in PWL of the ipsilateral and contralateral hindpaw. Because the time course of this increase was similar at all three time points examined, only the data for the 2 week treatment group are shown (Fig. 2*A*,*C*). Peak effect consistently occurred within 15 min and was considerably diminished by 30 min and essentially absent by 60 min. Figure 3 illustrates the dose–response relationship of DAMGO determined for the ipsilateral and contralateral hindpaws of rats either 4 hr, 4 d, or 2 weeks after the injection of saline in one hindpaw. There was no difference in the potency or efficacy of DAMGO as a function of time after the intraplantar injection of saline ($p > 0.2$ for each hindpaw) (Fig. 3*A*,*B*; Table 2). Similarly, the effects of DAMGO did not differ between the ipsilateral and contralateral hindpaws at any time ($p > 0.2$ each time point) (Figs. 2, 3; Table 2). These data attest to the precision and reliability of the microinjection technique and validate comparison of the dose–response relationships of DAMGO determined at various times after the injection of CFA.

Effect of DAMGO in CFA-treated rats: antihyperalgesia and the ipsilateral hindpaw

Microinjection of very low doses of DAMGO produced a dose-dependent reversal of the hyperalgesia induced 4 hr, 4 d, or 2

Table 2. ED_{50} values (and 95% confidence limits) for DAMGO microinjected in the RVM of rats that received an intraplantar injection of saline or CFA

Intraplantar treatment interval	Intraplantar saline		Intraplantar CFA	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
4 hr	10.7	13.6	0.65	5.5**
	(6.7 –20.1)	(8.2 –27.8)	(0.4 –1.0)	(3.3 –8.5)
4 d	16.2	23.7	0.21[a]	9.2*
	(10.1 –32.0)	(12.7 –72.6)	(0.09 –0.4)	(5.1 –18.4)
2 weeks	17.6	18.5	0.02[a]	1.9**[a]
	(10.3 –37.6)	(10.1 –46.4)	(0.009 –0.04)	(1.1 –3.1)

ED_{50} values are expressed as nanograms. Asterisks indicate ED_{50} values for the contralateral, uninflamed hindpaw of CFA-treated rats that differ from that of the contralateral, noninflamed hindpaw of saline-treated rats: $^{*}p < 0.05$, $^{**}p < 0.01$.

[a]Indicates ED_{50} values that significantly differ from the previous time point, $p < 0.01$.

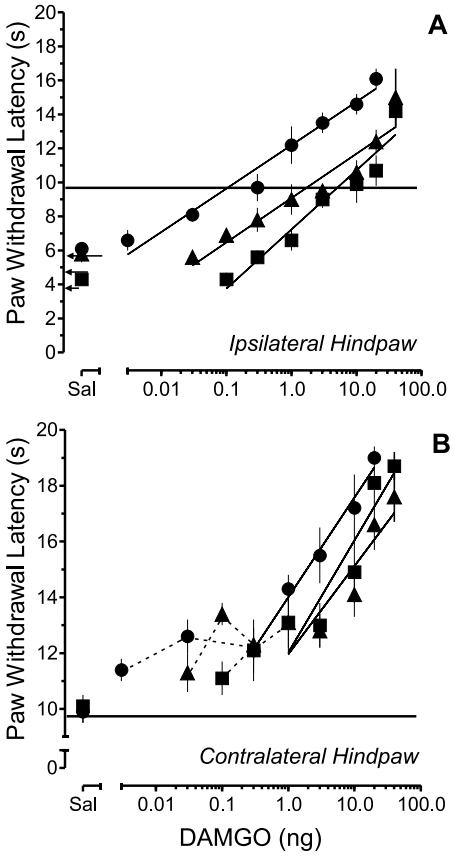

Figure 4. Dose–response relationships of DAMGO microinjected in the RVM of rats that received an intraplantar injection of CFA in one hindpaw 4 hr (■), 4 d (▲), or 2 weeks (●) earlier. Doses of DAMGO are plotted on a logarithmic scale. *A* depicts the progressive leftward shift in the dose–response relationship for the antihyperalgesic effect of DAMGO on the ipsilateral hindpaw as a function of time after injection of CFA. The *horizontal arrows* indicate the mean baseline PWL for the ipsilateral hindpaw determined 4 hr, 4 d, or 2 weeks (*shortest* to *longest arrows*; also see Table 1) after the injection of CFA and before the microinjection of DAMGO. The *horizontal line* represents the average baseline PWL for the ipsilateral hindpaw determined before the injection of CFA. *B* depicts the progressive leftward shift in the dose–response relationship for the antinociceptive effect of DAMGO on the contralateral hindpaw of the same animals as a function of time. The *horizontal line* represents the average baseline PWL for the contralateral hindpaw determined after injection of CFA and before the microinjection of DAMGO. The *dashed lines* that connect the lowest doses of DAMGO represent the non-dose-dependent portion of the dose–response relationship of DAMGO. In both panels, the dose-dependence was established by least squares linear regression of the individual data at 15 min, the time of peak effect. *Sal* represents the mean PWL determined 15 min after microinjection of saline in the RVM. Each symbol represents the mean ± SEM of determinations for 6–11 rats.

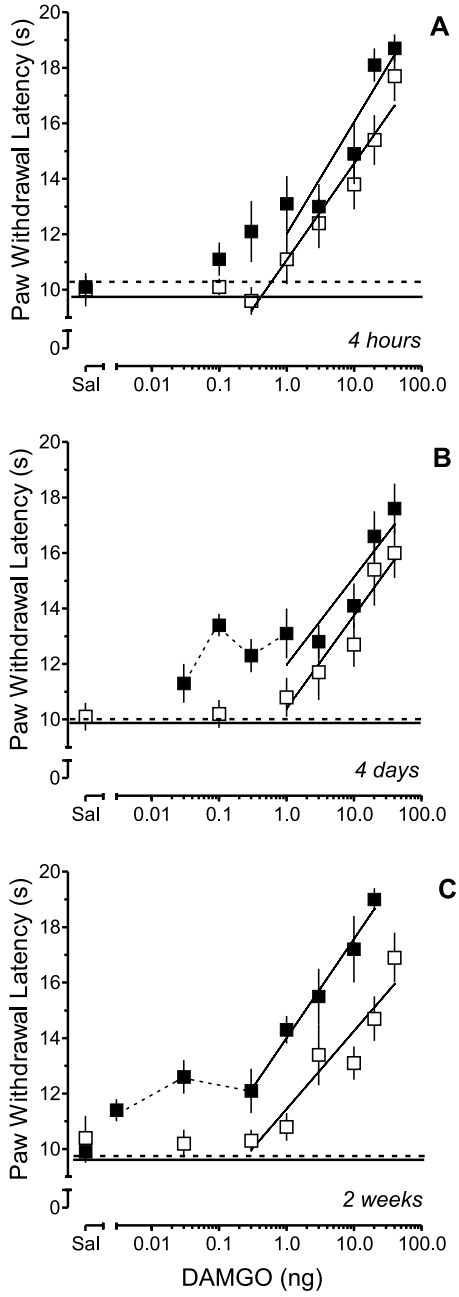

Figure 5. Dose–response relationships of DAMGO for the contralateral hindpaw 4 hr (*A*), 4 d (*B*), or 2 weeks (*C*) after intraplantar injection of saline (□) or CFA (■). Doses of DAMGO are plotted on a logarithmic scale. Note that the dose–response relationships for the antinociceptive effects of DAMGO in the contralateral hindpaw of CFA-treated rats are shifted to the *left* of that in the saline-treated rats. *Dashed lines* connect the very low doses of DAMGO and depict the non-dose-dependent portion of the dose–response relationship of DAMGO. Note that the effects of very low doses of DAMGO are greatly enhanced in the CFA-treated rats. *Sal* represents mean PWL determined 15 min after microinjection of saline in the RVM. The *horizontal solid* and *dashed lines* represent the average baseline PWL of rats after the intraplantar injection of saline or CFA, respectively, and before the injection of DAMGO. Each symbol is the mean ± SEM for determinations in 6–11 rats.

weeks after the intraplantar injection of CFA. Higher doses of DAMGO further increased PWL beyond normal baseline values. The peak effect occurred within 15 min of microinjection, persisted through 30 min, and was substantially diminished by 60 min. Figure 1*B* illustrates these effects of DAMGO in the 2 week treatment group. The potency of DAMGO was highly dependent on the time after injection of CFA (Fig. 4*A*; Table 2). Direct comparisons among all three treatment groups were confounded by the 2 sec difference between the baseline PWL of rats injected 4 hr and those injected 2 weeks earlier with CFA (Table 1). However, comparisons could be made between consecutive time points where the baseline PWLs differed by only ~1 sec, a difference that is unlikely to be biologically relevant. These comparisons revealed that the potency of DAMGO progressively increased as a function of time (Fig. 4*A*; Table 2). For example,

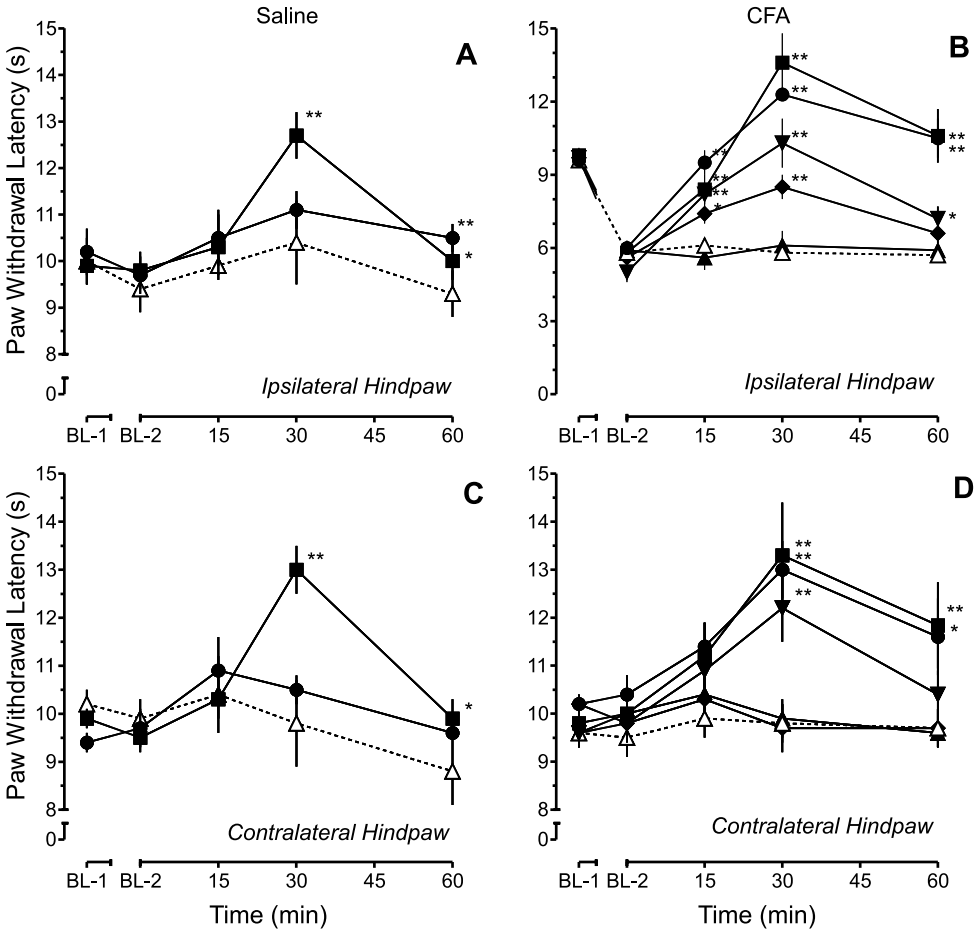

Figure 6. Time course of the increase in PWL produced by microinjection of 0.016 μg (▲), 0.063 μg (♦), 0.16 μg (▼), 0.63 μg (●), or 1.25 μg (■) of DELT into the RVM of rats that received an intraplantar injection of saline (*A, C*) or CFA (*B, D*) in one hindpaw 2 weeks earlier. The lack of effect of saline (△, *dashed line*) is depicted for comparison. *A* and *B* depict the mean PWLs of the ipsilateral hindpaw of saline- and CFA-treated rats, respectively. *C* and *D* depict the mean PWLs of the contralateral hindpaw of saline- and CFA-treated rats, respectively. *BL-1* represents the mean baseline PWL determined before intraplantar injection of saline or CFA. *BL-2* represents the mean baseline PWL after the intraplantar injection of saline or CFA, and before the microinjection of DELT. Each symbol represents the mean ± SEM of determinations in 6–11 rats. *Asterisks* indicate values that are significantly different from values at the corresponding time point in rats in which saline was microinjected in the RVM; $^{*}p < 0.05$, $^{**}p < 0.01$. Note that the ordinates have been truncated at 15 sec.

the dose–response relationship of DAMGO determined 4 d after induction of inflammation was shifted threefold to the left of that determined 4 hr after the induction of inflammation ($p < 0.01$). The dose–response relationship of DAMGO determined 2 weeks after inflammatory injury was shifted 10-fold to the left of that determined 4 d after inflammatory injury ($p < 0.01$).

Effect of DAMGO in CFA-treated rats: antinociception and the contralateral hindpaw

The antinociceptive potency of DAMGO, determined for the hindpaw contralateral to the inflammatory injury, was also increased as a function of time after injection of CFA (Figs. 2*D*, 4*B*). Comparisons of the ED_{50} of DAMGO could be made among all three treatment groups because the baseline PWL of these animals did not differ (Table 1). The ED_{50} of DAMGO did not differ at 4 hr and 4 d after the injection of CFA ($p > 0.2$). However, at 2 weeks the ED_{50} of DAMGO for the contralateral hindpaw was shifted to the left of that at 4 hr and 4 d by three- to fivefold ($p < 0.01$) (Fig. 4*B*; Table 2). Additional support for an enhancement of DAMGO-induced antinociception was evident in the effect of extremely low doses of DAMGO in CFA-treated rats. Doses of 0.3 ng or less significantly increased the PWL of the contralateral hindpaw of rats that received CFA either 4 hr, 4 d, or 2 weeks earlier (Figs. 1*D*, 4*B*, *dashed lines*) ($p < 0.05$). By comparison, doses in this range were completely ineffective in rats that received an injection of saline in one hindpaw (Fig. 1, compare *D*, *C*; Fig. 5*A-C*). However, the magnitude of the increase produced by these very low doses was not dose-dependent.

The enhanced antinociceptive effects of DAMGO in CFA-treated rats were also evident when the dose–response relationship of DAMGO in the contralateral hindpaw of CFA-treated rats was compared with that of DAMGO in the contralateral hindpaw of saline-treated rats (Fig. 5*A-C*; Table 2). At 4 hr, there was a 2.5-fold leftward shift in the dose–response relationship of DAMGO in the rats that received CFA ($p < 0.01$). Similarly, at 4 d, the dose–response relationship of DAMGO was shifted 2.5-fold to the left of that in saline-treated rats ($p < 0.025$) (Table 2). Also, very low doses of DAMGO (e.g., 0.1 ng) that were without effect in saline-treated rats significantly increased PWL in the contralateral hindpaw of the CFA-treated rats ($p < 0.05$) (Fig. 5*B*). Finally, by 2 weeks, the dose–response relationship of DAMGO was shifted nearly 10-fold to the left in CFA-treated rats compared with that in saline-treated rats ($p < 0.01$) (Fig. 5*C*). Again, very low doses of DAMGO on the order of 0.03 ng of DAMGO, which were without effect in saline-treated rats, significantly increased PWL in the contralateral hindpaw of CFA-treated rats ($p < 0.05$) (Fig. 5*C*).

Effects of DELT in saline-treated rats

Microinjection of DELT in the RVM of rats that received an intraplantar injection of saline 4 hr, 4 d, or 2 weeks earlier produced a dose-dependent increase in PWL of both hindpaws in rats. Paw withdrawal latency was maximally increased within 30 min and returned to baseline by 60 min (Fig. 6*A,C*). Because the antinociception produced by DELT did not differ among the three time points, its time course is illustrated only for the 2 week time point. The effects of DELT were similar in the ipsilateral and contralateral hindpaws (Figs. 6*A,C*, 7*A,B*). Although DELT ap-

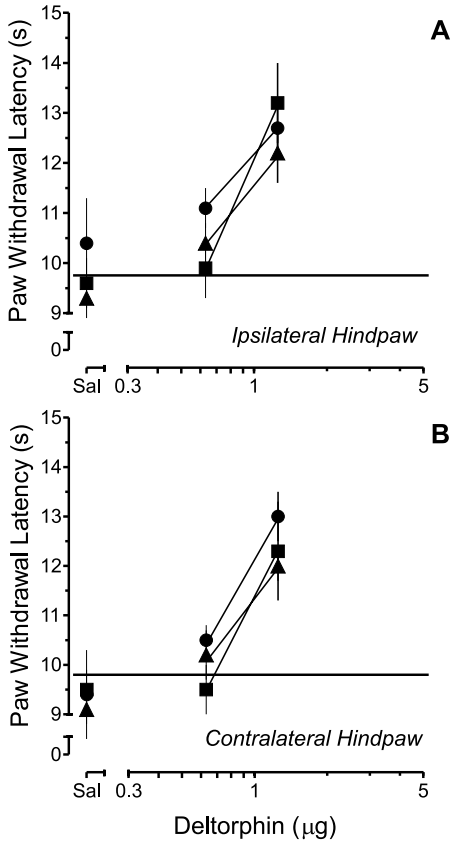

Figure 7. Dose–response relationships of DELT microinjected in the RVM of rats that received an intraplantar injection of saline in one hindpaw 4 hr (■), 4 d (▲), or 2 weeks (●) earlier. The potency and maximal effect of DELT are similar for the (*A*) ipsilateral and (*B*) contralateral hindpaws at each time point and are independent of time after injection of saline. *Sal* represents mean PWL determined 30 min after microinjection of saline in the RVM. Each symbol represents the mean ± SEM of determinations in 6–10 rats. The *horizontal line* represents the average baseline PWL for that hindpaw of rats determined after the intraplantar injection of saline and before the microinjection of DELT. Note that the ordinates have been truncated at 15 sec.

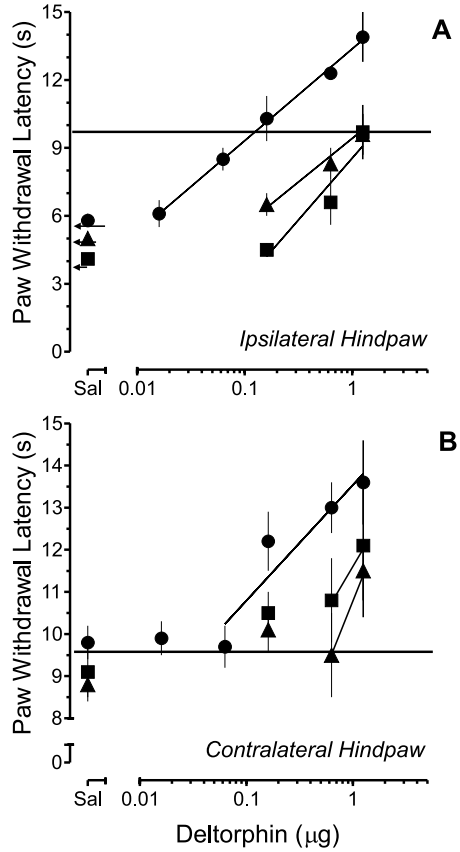

Figure 8. Dose–response relationships of DELT microinjected in the RVM of rats that received an intraplantar injection of CFA in one hindpaw 4 hr (■), 4 d (▲), or 2 weeks (●) earlier. *A* depicts the leftward shift in the dose–response relationship for the antihyperalgesic effect of DELT on the ipsilateral hindpaw 2 weeks after the injection of CFA. The *horizontal arrows* indicate the mean baseline PWL for the ipsilateral hindpaw determined 4 hr, 4 d, or 2 weeks (*shortest* to *longest arrows*; also see Table 1) after the injection of CFA and before the microinjection of DAMGO. The *horizontal line* represents the average baseline PWL for the ipsilateral hindpaw determined before the injection of CFA. *B* depicts the leftward shift in the dose–response relationship for the antinociceptive effect of DELT on the contralateral hindpaw 2 weeks after the injection of CFA. The *horizontal line* represents the average baseline PWL for the contralateral hindpaw determined after injection of CFA and before the microinjection of DAMGO. In both panels, dose-dependence was established by least squares linear regression of the individual data at 30 min, the time of peak effect. *Sal* represents the mean PWL determined 30 min after the microinjection of saline in the RVM. Each symbol represents the mean ± SEM of determinations for 6–11 rats. Note that the ordinates have been truncated at 15 sec.

peared to be less efficacious than DAMGO, doses higher than 1.25 μg could not be microinjected because of solubility limitations. It was not possible to calculate ED_{50} values for DELT because PWL at the maximum dose of 1.25 μg did not exceed the criterion value of 15 sec.

Effects of DELT in CFA-treated rats: antihyperalgesia and the ipsilateral hindpaw

Microinjection of DELT into the RVM of CFA-treated rats produced a dose-dependent reversal of the hyperalgesia on the ipsilateral hindpaw induced by injection of CFA 4 hr, 4 d, or 2 weeks earlier. Figure 6*B* illustrates the time course of DELT in the 2 week treatment group. As in saline-treated rats, the peak effect occurred at 30 min. However, the duration of effect was more prolonged and persisted through 60 min. Figure 8*A* illustrates the leftward shift in the dose–response relationship for the antihyperalgesic effects of DELT as a function of time after injection of CFA. The ED_{50} (and 95% confidence limits) of DELT at 4 hr and 4 d were 0.6 (0.34–1.0) μg and 0.38 (0.13–0.62) μg, respectively. The difference between these values was of marginal significance ($p = 0.05$). At 2 weeks, the dose–response relationship of DELT in the ipsilateral hindpaw was shifted further to the left as compared with that at 4 d ($p < 0.01$) (Fig. 8*A*). The ED_{50} value was decreased by nearly sixfold to 0.06 (0.03–0.09) μg. In addition, the efficacy of DELT appeared to be increased because doses of 0.63 and 1.25 μg actually prolonged PWL beyond normal baseline latencies.

Effects of DELT in CFA-treated rats: antinociception and the contralateral hindpaw

The antinociceptive potency of DELT, determined for the hindpaw contralateral to the inflammatory injury, was also increased as a function of time after injection of CFA. This enhancement was only apparent 2 weeks after the injection of CFA (Figs. 8*B*, 9*A-C*). For example, microinjection of a dose as low as 0.16 μg in the RVM of rats treated 2 weeks earlier with CFA significantly increased PWL, whereas this dose was without effect in rats

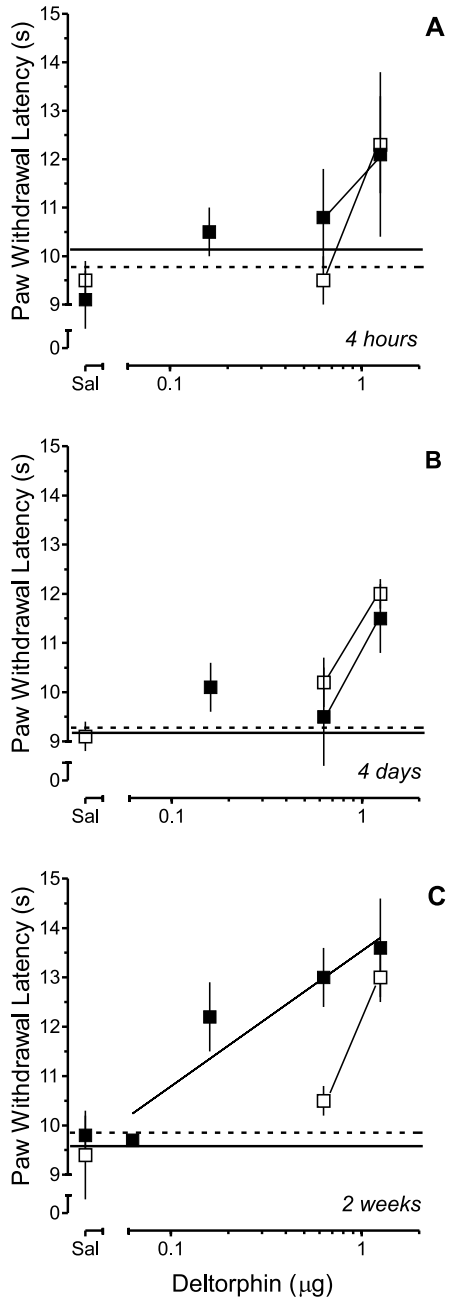

Figure 9. Dose–response relationships of DELT for the contralateral hindpaw 4 hr (*A*), 4 d (*B*), or 2 weeks (*C*) after intraplantar injection of saline (□) or CFA (■). Note that the dose–response relationship for the antinociceptive effects of DELT in the contralateral hindpaw of CFA-treated rats is shifted to the *left* of that in the saline-treated rats at 2 weeks. The *horizontal solid* and *dashed lines* represent the average baseline PWL of rats after the intraplantar injection of saline or CFA, respectively, and before the injection of DELT. Note that the ordinates have been truncated at 15 sec.

treated 4 hr or 4 d earlier with CFA (Fig. 8*B*). The enhanced antinociceptive effect of DELT in the contralateral hindpaw of CFA-treated rats 2 weeks after the induction of inflammation was also evident when the dose–response relationship of DELT in the contralateral hindpaw of CFA-treated rats was compared with that of DELT in the contralateral hindpaw of saline-treated rats (Fig. 9*C*). Although the potency of DELT was increased, there was no change in the apparent efficacy of DELT at this time. Thus, the highest dose of DELT, 1.25 μg, did not increase PWL beyond latencies observed in saline-treated rats (Fig. 9*C*).

Lack of effect of DAMGO or DELT on paw diameter and skin temperature

Microinjection of 10 ng of DAMGO in the RVM did not attenuate the increase in paw diameter or increase in cutaneous skin temperature produced by injection of CFA in the left hindpaw ($p > 0.3$ and $p > 0.2$, respectively). For example, the diameter and temperature of the left hindpaw determined 15 min after the microinjection of DAMGO in CFA-treated rats was 9.6 ± 0.2 mm and 34 ± 0.4°C. Corresponding values in CFA-treated rats 15 min after microinjection of saline in the RVM were 9.5 ± 0.2 mm and 34.6 ± 0.2°C. Microinjection of 1.25 μg of DELT in the RVM also did not attenuate the increase in paw diameter or the increase in skin temperature produced by injection of CFA in the left hindpaw ($p > 0.6$ and $p > 0.5$, respectively). Thirty minutes after the microinjection of DELT in CFA-treated rats, the diameter and temperature of the left hindpaw were 9.6 ± 0.1 mm and 34.5 ± 0.4°C. Corresponding values in CFA-treated rats 30 min after microinjection of saline in the RVM were 9.5 ± 0.2 mm and 34.0 ± 0.2°C. Neither the diameter nor the skin temperature of the contralateral hindpaw was altered after intraplantar injection of CFA as compared with baseline values. The diameter and skin temperature of the contralateral hindpaw remained unchanged after microinjection of DAMGO, DELT or saline in the RVM ($p > 0.2$ all conditions; data not shown).

DISCUSSION

Recent studies of persistent nociception suggest that the activity of RVM neurons that give rise to the pain inhibitory (Ren and Dubner, 1996; Wei et al., 1999) as well as pain facilitatory (Urban et al., 1996; Mansikka and Pertovaara, 1997; Wiertelak et al., 1997; Pertovaara, 1998; Wei et al., 1999) pathways is increased during inflammatory nociception. These studies inferred alterations in the activity of supraspinal neurons on the basis of lesion-induced changes in response latency or the responses of the dorsal horn neurons to which they project. Although valid, this approach is nonetheless indirect, and the findings are restricted to those neurons with spinal projections. Furthermore, such studies provide no insight into the functional ramifications of persistent inflammatory nociception for the complex pharmacology of excitatory and inhibitory inputs that modulate the activity of the RVM neurons that give rise to these pathways (Fields and Basbaum, 1994).

Among the inputs that modulate the activity of RVM neurons are endogenous opioid peptides acting at μ or δ opioid receptors (Kiefel et al., 1993; Kalyuzhny et al., 1996; Roychowdhury and Fields, 1996; Kalyuzhny and Wessendorf, 1998; Hirakawa et al., 1999). This study used a direct pharmacological approach to assess changes in the potency and efficacy of μ and δ opioid receptor agonists that were administered into the RVM of rats with inflammatory nociception of 4 hr, 4 d, or 2 weeks duration. The results indicate that persistent inflammatory nociception leads to alterations in the pharmacology and physiology of medullary neurons that enhance the production of antihyperalgesia and antinociception by supraspinally administered μ as well as δ opioid receptor agonists. These changes and the magnitude of the enhancement are highly dependent on the chronicity of the injury. Furthermore, these changes have ramifications not only for the alleviation of hyperalgesia at the site of injury, but also for the

production of opioid-mediated antinociception at sites remote to the injury as revealed by increases in the potency of opioid agonists to suppress nociceptive responses of the contralateral, uninjured hindpaw. Finally, these data indicate that supraspinal mechanisms are also likely to contribute to the enhanced antinociceptive effects of systemically administered opioids during inflammatory nociception.

Persistent inflammatory nociception markedly increased the antihyperalgesic potencies of DAMGO and DELT as assessed by the ipsilateral hindpaw. A parallel increase in the antinociceptive potency of both agonists also occurred for the contralateral, uninjured hindpaw. For DAMGO, this increase occurred as early as 4 hr, was maximal at 2 weeks, and was evident as a parallel leftward shift in its dose–effect relationship. It was also evident in the ability of low doses of DAMGO, which were ineffective in saline-treated rats, to increase PWL in the contralateral hindpaw of CFA-treated rats. For DELT, the increase was evident only at 2 weeks. The enhancement of the antinociceptive effects of both DAMGO and DELT on the contralateral hindpaw is noteworthy. First, it serves as an important control for the confound introduced by the progressive increase in the baseline PWL of the ipsilateral hindpaw of CFA-treated rats, which is consistent with an attenuation of thermal hyperalgesia. It could be argued that DAMGO's potency increased simply because the effective intensity of the noxious stimulus decreased over time (Saeki and Yaksh, 1993). However, the occurrence of a parallel increase in the potency of DAMGO on the contralateral hindpaw of CFA-treated rats, whose baseline PWL did not differ from those of other CFA- or saline-treated rats, negates this argument. Second, and more importantly, the enhancement of DAMGO's antinociceptive effects on the contralateral hindpaw indicates that inflammatory injury facilitates the ability of DAMGO to activate bulbospinal pain inhibitory neurons. The majority of microinjections in the RVM were made into the nucleus raphe magnus, which projects bilaterally through the dorsolateral funiculus to the spinal cord dorsal horn (Basbaum et al., 1978; Jones and Light, 1990; Hama et al., 1997). The consequence of increases or decreases in the activity of these neurons should therefore be evident ipsilaterally as well as contralaterally. Indeed, lesions of serotoninergic neurons in the RVM produce small increases in the number of Fos-immunoreactive neurons in the contralateral spinal cord and decreases in the PWL of the contralateral hindpaw of CFA-treated rats (Wei et al., 1999). These data lead to the prediction that the antinociceptive effects of systemically administered morphine, which distributes to supraspinal sites, should also be enhanced on the contralateral hindpaw of rats with unilateral injection of carrageenan or CFA. Such data are lacking. Of the three known studies, all were conducted 2 hr to 6 d after inflammation, when activation of the bulbospinal pain inhibitory pathways by DAMGO is not yet maximal, and none included a comparison of dose–effect curves with those in uninflamed rats (Stein et al., 1988; Barthó et al., 1990; Joris et al., 1990). Thus, an enhancement would not be readily apparent.

The decrease in the ED_{50} values of DAMGO with time suggests that different mechanisms contribute to the increase in DAMGO's potency at different times. A similar conclusion was reached for differential induction of diffuse noxious inhibitory controls in rats with acute versus chronic monoarthritis (Danziger et al., 1999). At its most obvious, the increased potency of DAMGO could result from an increase in the affinity or numbers of μ opioid receptors in the RVM. However, this mechanism is more likely to contribute to the enhancement observed at 2 weeks rather than at 4 hr and 4 d, because increases in opioid receptor binding in the PAG do not occur until 3 weeks after the induction of polyarthritis (Millan et al., 1987). More immediate changes occur in the levels of endogenous opioid peptides and their mRNA. In the ventrolateral PAG, the basal release of [Met^5]enkephalin is increased 24 hr after the induction of inflammation by CFA (Williams et al., 1995). Also, neurons of the microcellular tegmentum, which project to the RVM (Williams and Klobuchar, 1998), exhibit increases in mRNA for preproenkephalin (Bellavance et al., 1996). Endogenously released enkephalins act preferentially at δ opioid receptors in both the brain and spinal cord (Takemori and Portoghese, 1993; Tseng et al., 1995). Furthermore, coincident activation of μ and δ opioid receptors at either supraspinal (Miaskowski et al., 1991; Adams et al., 1993; Rossi et al., 1994) or spinal (Horan et al., 1992; Malmberg and Yaksh, 1992) sites can produce antinociception in a synergistic manner. Thus, the increased potency of DAMGO at early time points may result from the synergistic interaction of the exogenously administered μ opioid receptor agonist with endogenous enkephalins, whose release in the RVM is increased as a consequence of inflammation, resulting in an additional activation of δ opioid receptors. Indeed, coadministration of the δ opioid receptor antagonist naltriben with DAMGO in the RVM abolishes the enhancement of DAMGO's effects in CFA-treated rats (Hurley and Hammond, 1999). A similar proposal has been put forth to explain the ability of naltrindole, a δ opioid receptor antagonist, to antagonize the enhanced antinociceptive effects of systemically administered morphine in rats with carrageenan-induced inflammation (Ossipov et al., 1995). The greater enhancement observed at 2 weeks likely reflects the recruitment of additional mechanisms such as increases in the number or affinity of opioid receptors in the RVM, or an increased efficiency of coupling to subcellular effectors. Opioids are hypothesized to activate spinally projecting RVM neurons and produce antinociception by inhibition of tonically active GABAergic inputs to these neurons (i.e., by disinhibition) (Fields and Basbaum, 1994). Lesion studies indicate that the activity of bulbospinal pain inhibitory pathways that arise in the RVM is increased shortly after the induction of inflammation. Such an increase in activity could come about as the result of an increased tonic release of enkephalins in the RVM. Finally, the possibility that DAMGO's enhanced potency results from an increased sensitivity of dorsal horn neurons to bulbospinal inputs cannot be excluded. However, because the enhancement occurred contralaterally as well as ipsilaterally, it is unlikely to be mediated by a solely spinal mechanism.

The antihyperalgesic and antinociceptive effects of DELT were enhanced only at 2 weeks. The fact that DAMGO and DELT did not exhibit parallel changes in potency suggests that different mechanisms are likely to mediate the enhancement of the effects of δ opioid receptor agonists. The lack of enhancement at early time points suggests that the exogenously administered δ_2 opioid receptor agonist may interact in an additive or possibly subadditive manner with endogenously released enkephalins. A subadditive interaction would result if the endogenously released enkephalins function as partial agonists at the same receptor at which DELT, a full agonist, binds in the RVM (Szekeres and Traynor, 1997). Subsequent increases in the number or affinity of δ opioid receptors in the RVM may be the predominant mechanism by which the antihyperalgesic and antinociceptive effects of DELT are enhanced at 2 weeks.

In summary, these results indicate that persistent inflammatory nociception alters the activity of RVM neurons in a manner that is consistent with an enhancement of the antinociceptive effects of both μ and δ opioid receptor agonists, presumably by facilitating their ability to activate bulbospinal pain inhibitory systems that arise in the RVM.

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