

Peripheral Opioid Receptors Mediating Antinociception in Inflammation. Evidence for Activation by Enkephalin-Like Opioid Peptides After Cold Water Swim Stress¹

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ABSTRACT

This study utilized inhibitors of the enzymatic degradation of enkephalins to investigate the possibility that this class of opioid peptides contributes to the stress-induced antinociception seen in inflamed peripheral tissues of rats with Freund's complete adjuvant-initiated unilateral hind paw inflammation. Following a 1-min cold water swim stress, rats previously injected in both hind paws with vehicle showed a transient elevation of paw pressure threshold, which was much greater in inflamed than in noninflamed paws and returned to control levels within 15 min. This preferential antinociception was significantly pronounced and prolonged in rats previously injected bilaterally with a cocktail

of the enkephalinase inhibitors thiorphan (0.2 mg intraplantar) and bestatin (0.2 mg intraplantar). The enhancement of stress-induced antinociception by thiorphan/bestatin was dose-dependently antagonized by tertiary naloxone (0.125–2 mg kg⁻¹ s.c.). Evidence for a peripheral site of action of enkephalin-like peptides in this model was provided by the antagonism of the actions of thiorphan/bestatin by quaternary naltrexone (10–20 mg kg⁻¹ s.c.). Systemic administration of the orally active enkephalinase inhibitor SCH 34826 (5–40 mg kg⁻¹ i.p.) was also able to dose-dependently potentiate the preferential stress-induced antinociception in a naloxone (1 mg kg⁻¹ s.c.) reversible manner.

In recent studies on a model of unilateral inflammatory 'pain', we have shown that the stress of a forced cold water swim induces a greater elevation of paw pressure threshold in inflamed than in noninflamed paws (Parsons *et al.*, 1990; Stein *et al.*, 1990). This effect seems to be mediated, at least in part, by direct activation of opioid receptors in inflamed tissues (Ferreira and Nakamura, 1979; Joris *et al.*, 1987; Russell *et al.*, 1987; Stein *et al.*, 1988, 1989). Furthermore, several lines of evidence indicate that the endogenous opioid peptide mediating this effect could be hypophysial β -endorphin (Rossier *et al.*, 1977; Millan *et al.*, 1981; Przewlocki *et al.*, 1982; Höllt *et al.*, 1986). However, when tested *i.v.* over a wide dose range in the same model, β -endorphin was not able to induce a magnitude or time course of antinociception in the inflamed paw similar to that seen after cold water swim. Moreover, the effects of intravenous β -endorphin in this model were not naloxone-reversible (Parsons *et al.*, 1990). It therefore seems likely that a pool of endogenous opioid peptides other than hypophysial β -endorphin is important in mediating this effect. More recent evidence from our laboratory indicates that β -endorphin may

be released directly within the inflamed paw and bind with μ -receptors therein to mediate this peripheral stress-induced antinociception (Stein *et al.*, 1990).

Enkephalinergic systems are also activated by stressful stimuli, and are widely reported to mediate stress-induced antinociception via interactions with opioid receptors within the central nervous system (CNS) (see Millan, 1981, 1986; Terman *et al.*, 1984; Porro and Carli, 1988). Enkephalins are rapidly inactivated *in vivo* by a variety of enzyme systems (Schwartz *et al.*, 1981; see Schwartz, 1983; Turner *et al.*, 1985) and, as such, the antinociceptive effects of both exogenously administered and endogenously released enkephalins are generally of short duration (*e.g.*, Roques *et al.*, 1980; Chipkin *et al.*, 1982; Yaksh and Harty, 1982; Lecomte *et al.*, 1986). However, the half-life of enkephalins *in vivo* can be prolonged by the use of a variety of inhibitors of their enzymatic degradation (Barclay and Philipps, 1980; Roques *et al.*, 1980; Schwartz *et al.*, 1981; Fournié-Zaluski *et al.*, 1984; Chipkin *et al.*, 1988; see Schwartz, 1983, Schwartz *et al.*, 1985). In fact, it is due largely to the use of these inhibitors that a functional role of endogenous enkephalins in stress- and stimulation-produced analgesia has been ascribed (Chipkin *et al.*, 1982; Greenberg and O'Kiefe, 1982; O'Connor and Chipkin, 1984). The rapid inactivation of enkephalins fits well with the abrupt time course of the antin-

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ABBREVIATIONS: CNS, central nervous system; EC. 3.4.24.11, endopeptidase EC.3.4.24.11 "enkephalinase"; i.pl., intraplantar; ANOVA, analysis of variance.

ociception seen in our previous studies and therefore points towards a functional role of enkephalins in mediating this effect.

Although a plethora of enzymes seem to be capable of degrading enkephalins *in vitro*, only two seem to be of any real physiological significance for the *in vivo* degradation of these opioid peptides, namely, aminopeptidase MII and endopeptidase EC. 3.4.24.11 ("enkephalinase"; Malfroy *et al.*, 1978; Schwartz *et al.*, 1981; Hersh, 1984, 1985; see Schwartz, 1983; Hersh, 1986a and b). The relative importance of these two enzymes in this regard varies from tissue to tissue; therefore, a common approach in the study of physiological roles for endogenous enkephalins has been to block both enzymes concomitantly (*e.g.*, Zhang *et al.*, 1982; Chaillet *et al.*, 1983; De La Baume *et al.*, 1983). This can be accomplished either 1) with a cocktail of an inhibitor of aminopeptidase MII, *e.g.*, bestatin (Barclay and Philipps, 1980) and an inhibitor of EC. 3.4.24.11, *e.g.*, thiorphan (Roques *et al.*, 1980), or 2) with more recently developed compounds, such as kelatorphan, which are capable of blocking both enzymes (Bouboutou *et al.*, 1984; Fournié-Zaluski *et al.*, 1984; Waksman *et al.*, 1985). Unfortunately, these newer compounds are not commercially available.

We therefore tested for the involvement of endogenous enkephalins in mediating stress-induced antinociception in inflamed tissues of rats by direct injection of a thiorphan/bestatin cocktail into both hind paws of rats previously unilaterally inoculated with Freund's complete adjuvant. However, the effects of the above-mentioned inhibitors are not limited to the degradation of enkephalins (Hersh, 1984, 1985, see Schwartz, 1983; Schwartz *et al.*, 1985; Turner 1985). Thus, it was important to test the opioid specificity of this effect by the systemic administration of naloxone and quaternary naltrexone.

Recently, more selective inhibitors of EC. 3.4.24.11 have been developed. Two compounds of particular interest are SCH 34826 and SCH 32615, inasmuch as they also show a much increased potency over both thiorphan and kelatorphan following systemic administration (Chipkin *et al.*, 1988). In addition, SCH 34826 has the therapeutic advantage of being orally active. Therefore, we further characterized the role of enkephalinergic peptides in mediating stress-induced antinociception in our model of unilateral inflammatory pain with *i.p.* administered SCH 34826. The opioid specificity of this effect was again tested with *s.c.* naloxone.

Methods

Full details of the methods used have been given previously (Parsons *et al.*, 1990). Briefly, male Wistar rats (150–200 g) were housed individually within an environmentally controlled laboratory with free access to water and chow. Unilateral inflammation was induced by intraplantar (*i.p.l.*) injection of 0.15 ml Freund's complete adjuvant (Calbiochem, La Jolla, CA) under brief ether anesthesia. In order to characterize further if the anatomical changes observed following this treatment really model an inflammatory process, changes in paw volume and paw temperature were compared over a 4-day postinoculation period; paw volume was measured with a plethysmometer (Ugo Basile, Comerio, Italy) and paw temperature with an infrared meter (Ultraküst, Rühmannsfelden, FRG). All behavioral testing was performed in the light phase of a 12-hr light/dark cycle, 4 days after the induction of inflammation. Ethical guidelines as recommended by Zimmermann (1983) were adhered to and each rat was stressed only once.

The algometric test chosen for this study was paw pressure threshold measured with an automated gauge (Ugo Basile, Comerio, Italy). Mean paw pressure thresholds were evaluated from three tests

separated by 10-sec intervals. Cutoff was 250 g and data were normalized to a percentage maximal possible effect. In successive rats the sequence of inflamed/noninflamed paw testing was alternated to preclude order effects. All experiments were performed blind to the experimenter and under similar laboratory conditions with each animal serving as its own control.

In studies on thiorphan/bestatin, control paw pressure thresholds for each rat were evaluated immediately before injection in the plantar surface of both hind paws (0.1 ml/paw) with either this enkephalinase inhibitor cocktail or vehicle under brief ether anesthesia. In initial studies, the effects of these procedures alone on paw pressure threshold were monitored every 5 min for the next 40 min. Modulation of differential stress-induced antinociception by these manipulations was assessed by subjecting the animals to a 1-min cold water swim, 20 min after injection; the antinociception induced thereby was then assessed 1, 5, 15 and 30 min post-cold water swim. These studies were further extended by testing the actions of naloxone, quaternary naltrexone or saline injected *s.c.* in a volume of 0.2 ml 5 min prior to the cold water swim.

To evaluate the effects of SCH 34826, rats were injected *i.p.* with this relatively potent, systemically active inhibitor or vehicle (2 ml) immediately after assessment of control paw pressure thresholds. In initial studies, any actions of SCH 34826 on paw pressure threshold in the absence of stress were assessed for 60 min after injection. The effects of these manipulations on stress-induced antinociception were then determined by subjecting rats to a 1-min cold water swim 60 min after injection; paw pressure thresholds were then recorded 1, 5, 15 and 30 min after cold water swim. The opioid nature of any effects of SCH 34826 were tested with naloxone/NaCl given *s.c.* 5 min. prior to the cold water swim.

Two nonparametric tests were used to assess the significance of changes in paw pressure threshold, paw volume and paw temperature at set time points. Parametric ANOVA was used to test for differences in the time course of antinociception. Between inflamed and noninflamed paws within experimental groups, the paired Wilcoxon test and two factor interactions ANOVA were utilized. Normal ANOVA and the Mann-Whitney *U* test were used for similar paws between experimental groups.

Drug doses refer, where appropriate, to the salts of the compounds used. Sources were as follows: Naloxone HCl (DuPont, Geneva, Switzerland); naltrexone methobromide (Boehringer, Ingelheim, FRG); thiorphan and bestatin (Sigma, Deisenhofen, FRG), SCH 34826 (Schering, Bloomfield, MI). Thiorphan was solubilized to 16 mg ml⁻¹ in 0.9% NaCl first brought to a pH of 9.5 with 2 M NaOH. In contrast, bestatin was only readily soluble at 16 mg ml⁻¹ in 0.9% NaCl after decreasing the pH to 2 by the addition of 2 M HCl. The solutions were then buffered with 50% 1 M Tris before combining to obtain a stock solution of bestatin 8 mg ml⁻¹ and thiorphan 8 mg ml⁻¹, pH 7.4. SCH 34826 was homogenized in 0.4% aqueous methylcellulose to final concentrations between 0.5 and 4.0 mg ml⁻¹. Vehicles were prepared as above without the addition of drugs and were then used for further dilutions of the stock solutions and control studies.

Results

Development of Inflammation

In 24 rats, paw size and paw temperature were recorded immediately prior to inoculation with Freund's complete adjuvant and 1, 2, 4, 18, 24, 50 and 90 hr thereafter (see fig. 1). The development of unilateral inflammation was already pronounced 2 hr after inoculation and continued linearly over the course of the next 18 hr so that there was a good correlation between the difference in paw volume and the difference in paw temperature (slope 3.876°C ml⁻¹, *r* = 0.78, *P* < .001). At this time point, the difference in paw temperature was 3.93 ± 0.18°C and the difference in paw volume was 1.25 ± 0.03 ml. Thereafter, the difference in paw volume remained constant,

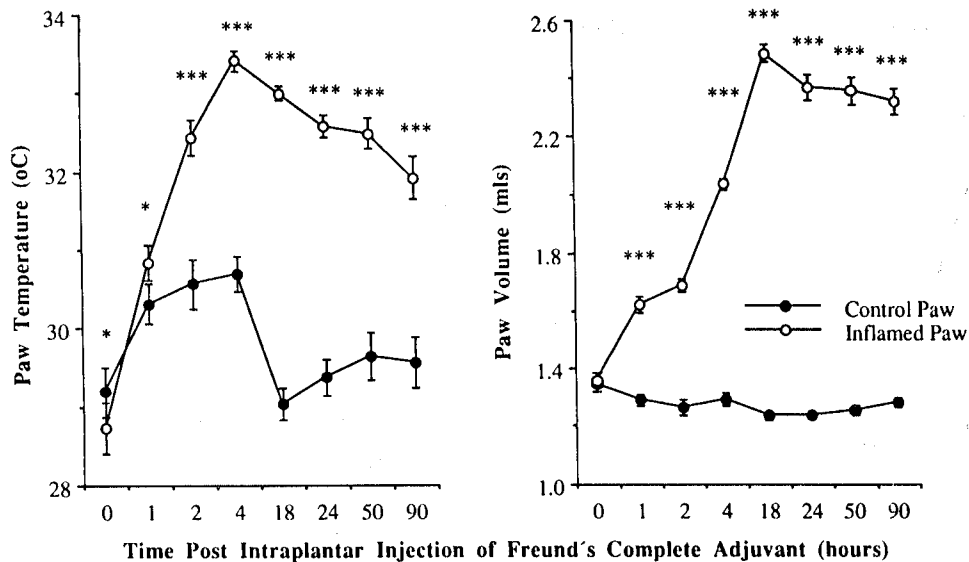


Fig. 1. Development of inflammation in rats unilaterally injected with 0.15 ml of Freund's complete adjuvant ($n = 24$). The left panel illustrates the temperature ($^{\circ}\text{C}$), and the right panel the volume of injected (open circles) and noninjected paws (filled circles) plotted against the time post injection (hr). Error bars represent S.E.M. The asterisks above each data pair represent the two-tailed probabilities of a difference between inflamed and noninflamed paws as determined by the Wilcoxon test (* $P < .05$, *** $P < .001$).

whereas the difference in paw temperature gradually decreased so that on the day of behavioral testing, these values were 1.06 ± 0.05 ml and $2.38 \pm 0.28^{\circ}\text{C}$ respectively.

Thiorphan/bestatin Cocktail

Effects of enkephalinase inhibitors on paw pressure threshold in nonstressed rats. Control studies revealed that the stress of transient ether anesthesia was sufficient to cause a nonselective and very brief elevation of paw pressure threshold which was nonselectively potentiated to a small degree by thiorphan (0.2 mg i.p.)/bestatin (0.2 mg i.p.). However, paw pressure thresholds had returned to control values 20 min postinjection (inflamed $-0.7 \pm 2.3\%$ maximal possible effect, noninflamed $6.5 \pm 3.7\%$ maximal possible effect). Therefore, in all further tests on the potentiation of stress-induced antinociception by thiorphan/bestatin, rats were subjected to a 1-min cold water swim stress 20 min after i.p. injection of this mixture.

Effects of enkephalinase inhibitors on paw pressure threshold in cold water swim-stressed rats. The results presented in figure 2 illustrate that a cocktail of thiorphan/bestatin was able to prolong the time course of the elevation of inflamed paw pressure threshold induced by a 1-min cold water swim when injected concomitantly 20 min beforehand at a dosage of 0.2 mg of each (approximately 1 mg kg^{-1}). Thus, at 1, 5, 15 and 30 minutes after cold water swim, the elevation of paw pressure threshold in the inflamed paws of thiorphan/bestatin-pretreated rats was significantly greater than that in the inflamed paws of vehicle-treated rats [Mann-Whitney U test, $P < .04$ at all time points; ANOVA (1,94), $F = 18.299$, $P < .0001$]. In contrast, there was no difference in magnitude or time course of effects seen in the noninflamed paws [Mann-Whitney U test, $P > .1$ at all time points; ANOVA (1,94), $F = 0.248$, $p = .625$]. This relatively low dose of the enkephalinase inhibitor cocktail was already maximally effective, inasmuch as thiorphan/bestatin produced an almost identical pattern of potentiation when the dose was doubled [ANOVA 0.2 mg vs. 0.4 mg (1,94), inflamed, $F = 0.2$, $P = .650$; noninflamed, $F = 1.1$, $p = .063$; data not represented].

In contrast, bestatin administered alone at a dose of 0.8 mg ($\approx 4 \text{ mg kg}^{-1}$) had no effect on the pattern of stress-induced

antinociception seen after cold water swim. Thus, the time course and magnitude of elevations of paw pressure threshold in like paws were similar in vehicle- and bestatin-treated rats [ANOVA (1,70), inflamed, $F = 0.10$, $P = .801$; noninflamed paws, $F = 1.02$, $P = .324$].

Antagonism of the effects of thiorphan/bestatin by tertiary naloxone. The potentiation of differential cold water swim stress-induced antinociception by thiorphan/bestatin was readily reversed by the tertiary opioid antagonist naloxone 1 mg kg^{-1} s.c. (fig. 3, left and middle panels). These actions of naloxone were manifested exclusively in inflamed paws [ANOVA saline vs. naloxone(1,46), inflamed, $F = 11.1$, $P = .002$; noninflamed, $F = 1.3$, $P = 0.117$].

The antagonism of the effects of thiorphan/bestatin by naloxone was dose-dependent. This is illustrated in figure 4. The left panel shows that although the elevation of inflamed paw pressure threshold seen immediately after cold water swim was dose-dependently antagonized by naloxone, this effect reached a plateau around 1 mg kg^{-1} and 50% MPE [ANOVA (5,30), $F = 3.0$, $P = .027$]. Thus, a component of the thiorphan/bestatin-potentiated stress-induced antinociception seen in the inflamed paw immediately after cold water swim seems not to be opioid in nature. In contrast, the stress-induced elevation of inflamed paw pressure threshold seen 5 and 15 min after cold water swim was completely and dose-dependently antagonized by naloxone and, as such, appears to be purely opioid in nature [ANOVA (5,30), 5 min after cold water swim, $F = 7.0$, $P \leq .001$; 15 min after cold water swim, $F = 2.6$, $P = .047$]. Any stress-induced alterations of noninflamed paw pressure threshold were unaffected by naloxone and, therefore, probably not manifested by activation of endogenous opioid systems [ANOVA (5,30), 1 min post cold water swim, $F = 0.2$, $P = .957$; 5 min post cold water swim, $F = 0.6$, $P = .686$; 15 min post cold water swim, $F = 1.9$, $P = .160$].

Antagonism of the effects of thiorphan/bestatin by quaternary naltrexone. The effects of thiorphan/bestatin in potentiating cold water swim stress-induced antinociception in the inflamed paws of Freund's complete adjuvant-inoculated rats was also reversed by systemic administration of quaternary naltrexone at doses that were previously shown to have no effect on the antinociceptive actions of morphine mediated by

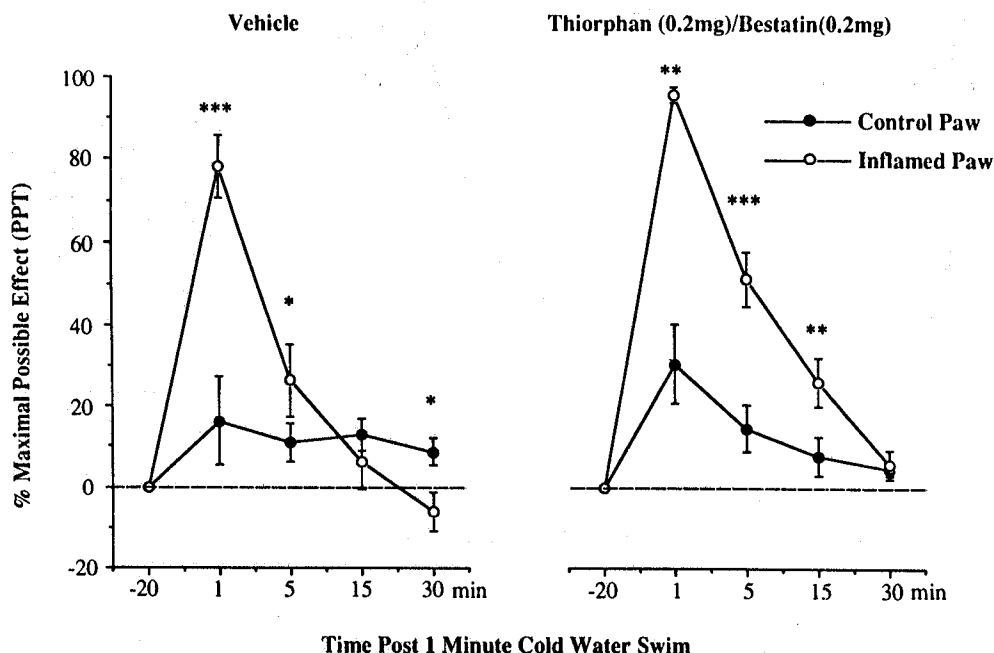


Fig. 2. Effects of thiorphan/bestatin on the elevation of paw pressure threshold induced by a 1-min cold water swim in rats with unilateral inflammation. The mean elevation of paw pressure threshold was measured 1, 5, 15 and 30 min after a 1-min cold water swim and normalized to a percent maximal possible effect with respect to control values. Error bars indicate S.E.M. The significance of differences between inflamed (open circles) and noninflamed paws (closed circles) were tested using the two-tailed Wilcoxon test and are indicated for each time point (* $P < .05$, ** $P < .01$, *** $P < .001$). The left panel illustrates the time course of the preferential elevation of paw pressure threshold seen in inflamed paws of vehicle-treated rats ($n = 12$, ANOVA (3,44) inflamed $F = 26.727$, $P < .001$; noninflamed $F = 0.246$, $P = .863$). The right panel illustrates potentiation and prolongation of this effect in rats injected i.p. with thiorphan(0.2 mg)/bestatin(0.2mg) 20 min prior to cold water swim ($n = 12$, ANOVA (3,44), inflamed $F = 36.648$, $P < .001$; noninflamed $F = 3.507$, $P = .023$). Two factor interactions ANOVA of inflamed vs. noninflamed (3,88) vehicle $F = 12.54$, $P < .001$; thiorphan/bestatin $F = 6.857$, $P < .001$. A very similar potentiation and prolongation of this effect in rats treated with two times this dose of thiorphan/bestatin (data not shown, $n = 12$, ANOVA (3,44), inflamed $F = 16.857$, $P < .001$, noninflamed $F = 3.695$, $P = .019$). Control paw pressure thresholds (in grams \pm S.E.M.) were as follows: Vehicle—inflamed paw: 50.3 ± 6.5 , noninflamed paw: 58.5 ± 3.2 ; Thiorphan(0.2mg)/Bestatin(0.2mg)—inflamed paw: 47.7 ± 5.7 , noninflamed paw: 64.6 ± 4.9 ; Thiorphan(0.4mg)/Bestatin(0.4mg)—inflamed paw: 48.3 ± 5.4 , noninflamed paw: 62.5 ± 1.7 .

opioid receptors within the CNS of rats without unilateral inflammation (data not shown). Thus, the complete block of thiorphan/bestatin-potentiated differential cold water swim stress-induced antinociception by quaternary naltrexone (20 mg kg^{-1}) illustrated in the right panel of figure 3 provides strong evidence that the effects of the enkephalinase inhibitor cocktail were mediated within the injected inflamed paw. This effect of quaternary naltrexone was also dose-dependent. Thus, when tested at half of the dose illustrated, quaternary naltrexone was somewhat less effective (data not represented). The antagonistic actions of both doses of quaternary naltrexone tested were mediated exclusively in inflamed paws [ANOVA saline vs. quaternary naltrexone (1,46), inflamed, 10 mg kg^{-1} , $F = 6.4$, $P = 0.014$, 20 mg kg^{-1} , $F = 15.073$, $P < .001$; non-inflamed, 10 mg kg^{-1} , $F = 2.1$, $P = .115$, 20 mg kg^{-1} , $F = 1.984$, $P = .199$]

Potentiation of Differential Cold Water Swim Stress-induced Antinociception by SCH 34826

Effects of SCH 34826 on paw pressure threshold in cold water swim-stressed rats. As can be seen from the results presented in figure 5, when administered systemically 60 min prior to cold water swim, SCH 34826 ($5\text{--}40 \text{ mg kg}^{-1}$ i.p.) dose-dependently potentiated the stress-induced antinociception apparent 1 min (left panel), 5 min (middle panel) and 15 min (right panel) after cold water swim. This effect was evident to some degree in both paws immediately after the cold water

swim stress [ANOVA (4,55), inflamed, $F = 3.168$, $P = .021$; noninflamed, $F = 2.197$, $P = .081$], whereas 5 min after cold water swim, the potentiation of stress-induced antinociception by SCH 34826 was far greater in the inflamed than in the noninflamed paw [ANOVA (4,55), inflamed, $F = 3.907$, $P = .007$; noninflamed, $F = 0.664$, $P = .620$]. These dose-dependent, selective effects of SCH 34826 in potentiating the elevation of inflamed paw pressure threshold by induced by cold water swim were still evident 15 min after the stress [ANOVA (4,55), inflamed, $F = 3.454$, $P = .012$; noninflamed, $F = 1.473$, $P = .233$].

Antagonism of the effects of SCH 34826 by tertiary naloxone. The opioidergic nature of the potentiating effects systemic SCH 34826 on cold water swim stress-induced antinociception was tested with either NaCl or tertiary naloxone 1 mg kg^{-1} given s.c. 5 min prior to cold water swim (fig. 6). In rats pretreated s.c. with NaCl, SCH 34826 20 mg kg^{-1} i.p. selectively potentiated the magnitude and prolonged the time course of cold water swim stress-induced antinociception evident in inflamed paws [ANOVA SCH 34826 vs. vehicle (1,94), inflamed, $F = 8.658$, $P = .004$; noninflamed, $F = 0.811$, $P = .057$]. The potentiated and prolonged stress-induced antinociception produced by this dose of SCH 34826 (left vs. middle panel) was completely abolished by s.c. naloxone 1 mg kg^{-1} (right panel, two factor interactions ANOVA inflamed vs. noninflamed (3,88); $F = 1.512$, $P = .219$). Moreover, the antagonistic actions of naloxone were manifested exclusively by decreasing the stress-induced antinociception evident in the in-

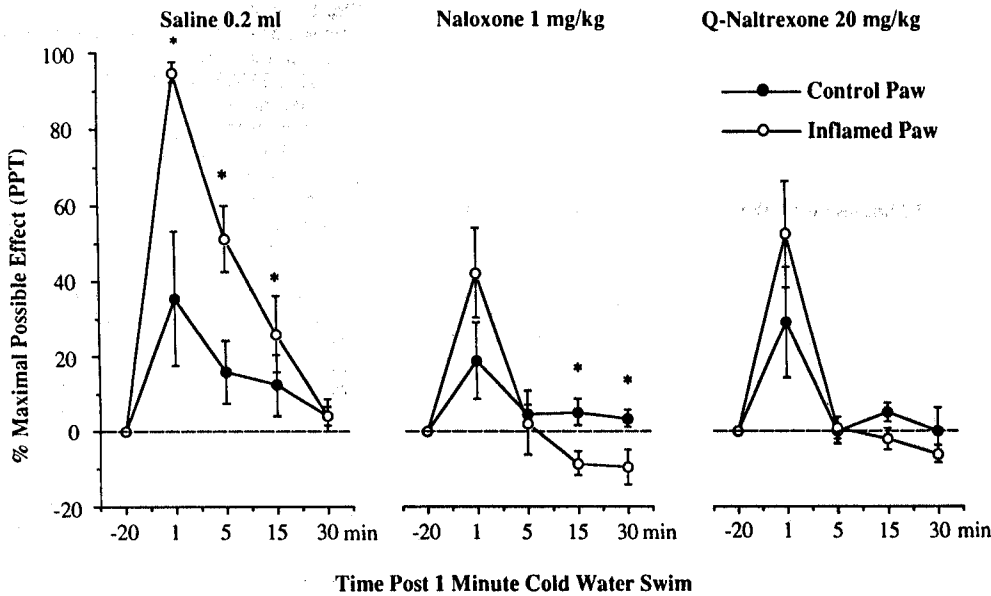


Fig. 3. Reversal of the effects of thiorphan/bestatin by tertiary naloxone and quaternary naltrexone. The left panel illustrates the lack of effect of NaCl (0.2 ml s.c., $n = 6$) on the potentiation and prolongation of stress-induced antinociception seen in the inflamed paws following treatment with thiorphan (0.2mg i.pl.)/bestatin (0.2mg i.pl.). The middle and right panels show that the effects of the enkephalinase inhibitor cocktail were antagonized by naloxone (1 mg kg⁻¹ s.c., $n = 6$) and quaternary naltrexone (20 mg kg⁻¹ s.c., $n = 6$) given 5 min prior to the cold water swim. Two factor interactions ANOVA inflamed vs. non-inflamed (3,40) NaCl $F = 4.0$, $P = .014$; naloxone $F = 1.612$, $P = 203$; quaternary naltrexone $F = 3.243$, $P = .032$. See legend to figure 2 for more details.

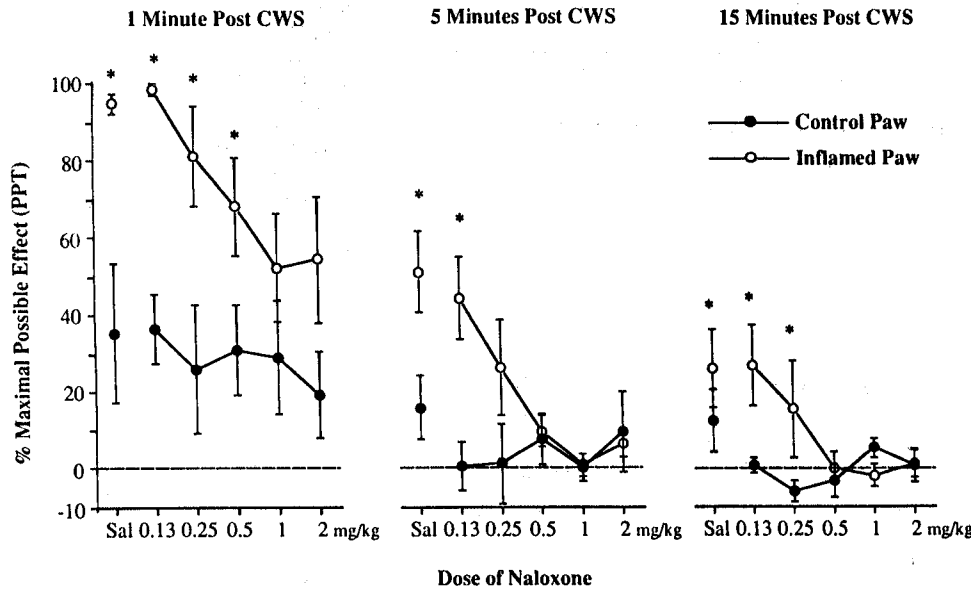


Fig. 4. Dose-dependent antagonism of the effects of thiorphan/bestatin by tertiary naloxone. Freund's complete adjuvant-inoculated rats were injected with thiorphan (0.2 mg i.pl.)/bestatin (0.2 mg i.pl.) 20 min prior to stress and either NaCl or naloxone (0.125–2 mg kg⁻¹ s.c.; $n = 6$ for each dose) 5 min prior to stress. The mean stress-induced elevation of paw pressure threshold assessed 1 (left panel), 5 (middle panel) and 15 (right panel) min after a 1-min cold water swim was normalized to a percent maximal possible effect and plotted against the dose of naloxone. See legend to figure 2 for more details.

inflamed paw (ANOVA naloxone vs. vehicle on SCH 34826 (1,94); inflamed, $F = 33.966$, $P < .001$; noninflamed, $F = 1.302$, $P = .100$).

Discussion

The results of this study provide further evidence that the stress of a forced cold water swim induces a release of opioid peptides which act directly at opioid receptors within inflamed peripheral tissues to mediate antinociception (see also Parsons *et al.*, 1990; Stein *et al.*, 1990). Furthermore, evidence has been provided that the opioid peptides mediating this effect could be at least partly enkephalinergic in nature.

Thus, the magnitude and time course of the preferential stress-induced antinociception evident in inflamed paws of Freund's complete adjuvant-inoculated rats after cold water swim were, respectively, somewhat potentiated and substantially prolonged by i.pl. injection of a cocktail of the enkephalinase inhibitors thiorphan and bestatin (see fig. 2). These two compounds were most likely exerting their major effects by

inhibiting the *in vivo* degradation of enkephalin-like opioid peptides by EC. 3.4.24.11 and aminopeptidase MII respectively (Malfroy *et al.*, 1978; Hersh, 1984, 1985; see Schwartz *et al.*, 1985). However, two points should be noted. First, both compounds show some inhibitory effects on other enzyme systems. Second, both of the above-mentioned enzymes are capable of metabolizing other biologically active peptides such as substance-P, somatostatin and angiotensin I (Schwartz *et al.*, 1981; Schwartz, 1983; Hersh, 1984, 1985; for review, see Schwartz *et al.*, 1985; Turner *et al.*, 1985).

Therefore, it was particularly important to test the effects of thiorphan/bestatin with opioid antagonists. From these studies it is evident that the stress-induced antinociception observed immediately after cold water swim was not purely opioidergic in nature. Thus, the slight elevation of non-inflamed paw pressure threshold was unaffected by naloxone, and dose-dependent reduction of the elevation of inflamed paw pressure threshold reached a plateau around 1 mg kg⁻¹ and 50% maximal possible effect (see fig. 4). It should be noted that, even in

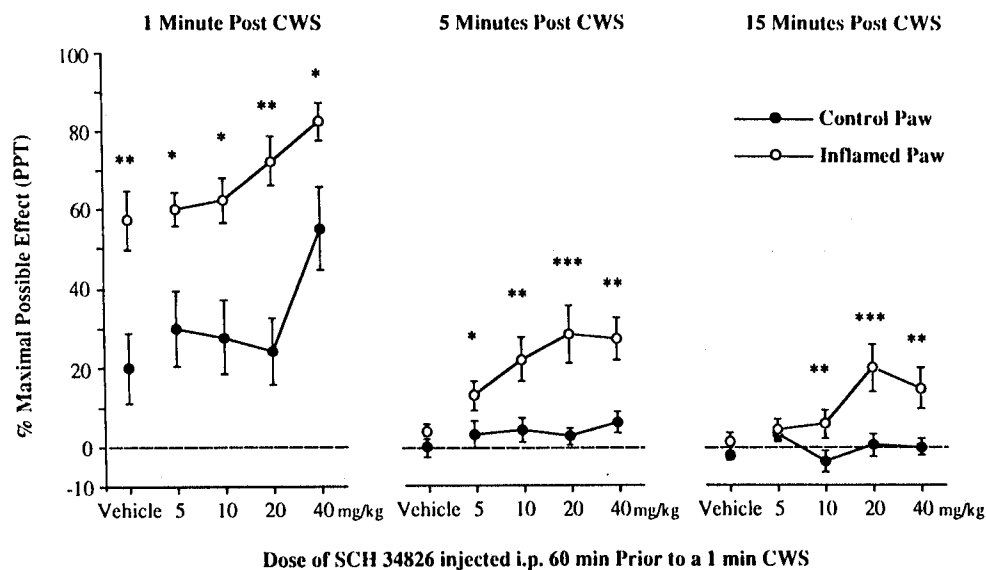


Fig. 5. Dose-dependent potentiation of cold water swim stress-induced antinociception by SCH 34826. In initial studies, SCH 34826 40 mg kg⁻¹ was found to have no effect on paw pressure threshold in the absence of cold water swim when investigated every 10 min for 60 min after i.p. injection. In subsequent studies, Freund's complete adjuvant-inoculated rats were injected i.p. with either SCH 34826 (5–40 mg kg⁻¹) or 0.4% methyl cellulose vehicle 60 min prior to a 1-min cold water swim. The mean stress-induced elevation of paw pressure threshold assessed 1 (left panel), 5 (middle panel) and 15 (right panel) min after a 1-min cold water swim were normalized to a percent maximal possible effect and plotted against the dose of SCH 34826. Error bars indicate S.E.M. The significance of differences between inflamed (open circles) and noninflamed paws (closed circles) were tested using the two-tailed Wilcoxon test and are indicated for each dose (* P < .05, ** P < .01, *** P < .001). Control paw pressure thresholds (in grams ± S.E.M.) were as follows: Vehicle (n = 12): inflamed paw (50.8 ± 3.9), noninflamed paw (70.6 ± 3.9); SCH 34826 5 mg kg⁻¹ (n = 12) inflamed paw (58.8 ± 9.4), noninflamed paw (68.9 ± 6.1); SCH 34826 10 mg kg⁻¹ (n = 12) inflamed paw (59.2 ± 9.8), noninflamed paw (68.9 ± 6.0); SCH 34826 20 mg kg⁻¹ (n = 12) inflamed paw (49.6 ± 3.2), noninflamed paw (70.8 ± 4.6); SCH 34826 40 mg kg⁻¹ (n = 12) inflamed paw (49.4 ± 5.9), noninflamed paw (75.3 ± 3.4).

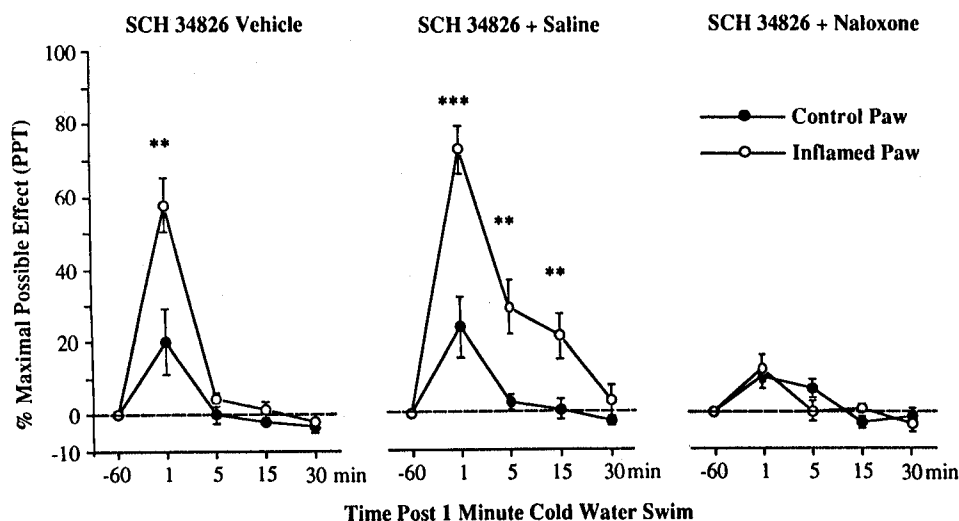


Fig. 6. Reversal of the effects of SCH 34826 20 mg kg⁻¹ by tertiary naloxone 1 mg kg⁻¹. The left panel illustrates the nonpotentiated stress-induced antinociception seen in rats treated with vehicle alone (n = 12). The middle panel illustrates potentiation and prolongation of the stress-induced antinociception seen in the inflamed paws of rats injected with SCH 34826 20 mg kg⁻¹ i.p. 60 min prior to cold water swim and NaCl (0.2 ml s.c.) 5 min prior to cold water swim (n = 12). The right panel shows that this effect of the enkephalinase inhibitor was completely reversed by naloxone (1 mg kg⁻¹ s.c.) given 5 min prior to the cold water swim (n = 12). See legend to figure 2 for more details.

vehicle treated rats, the stress-induced antinociception was somewhat greater in magnitude than that observed previously in our laboratory (see also left panel of fig. 6). While the nonselective effects of ether anesthesia alone had returned to control after 20 min, it seems likely that this "conditioning" stress resulted in the priming of nonopioid antinociceptive mechanisms which were then coactivated with the enkephalinergic mechanisms by cold water swim stress (see also Terman *et al.*, 1986). However, as would be expected of inhibitors of the enzymatic degradation of biologically active substances, the

specific effects of thiorphan/bestatin were more clearly manifested by selectively prolonging the time course of the elevation of inflamed paw pressure threshold. Importantly, this effect was dose-dependently and completely antagonized by naloxone (see fig. 4). Further evidence for a functional role of peripheral enkephalinergic peptides in mediating stress-induced antinociception in our model was provided by the antagonism of the effects of local i.p. thiorphan/bestatin by s.c. quaternary naloxone at doses shown previously to act exclusively outside the CNS.

Bestatin given alone was without effect in our model. It therefore seems likely that EC. 3.4.24.11. plays a more important role than aminopeptidase MII in the degradation of the enkephalinergic peptides responsible for the peripheral stress-induced antinociception observed (Schwartz *et al.*, 1981; Hersh 1984, 1985; for review see Schwartz, 1983; Schwartz *et al.*, 1985). Because SCH 34826 is both more potent and more selective than thiorphan in inhibiting the actions of EC. 3.4.24.11 (Roques *et al.*, 1980 *cf.* Chipkin *et al.*, 1988), we decided to extend our studies with this compound administered systemically. SCH 34826 40 mg kg⁻¹ i.p. had no effect on paw pressure threshold in its own right. Thus, the systemic administration of SCH 34826 proved somewhat better as a means of assessing whether the opioid peptides responsible for mediating cold water swim stress-induced antinociception in inflamed tissues are enkephalinergic in nature. SCH 34826 (5 to 40 mg kg⁻¹ i.p.) dose-dependently potentiated and prolonged the time course of the cold water swim stress-induced elevation of inflamed paw pressure threshold, this effect being most evident 5 minutes after cold water swim (see fig. 5). In contrast to thiorphan/bestatin, the effects of SCH 34826 20 mg kg⁻¹ were completely reversed by naloxone 1 mg kg⁻¹ at all time points (see fig. 6). Again, this is a reflection of the more purely enkephalinergic nature of the cold water swim stress-induced antinociception seen in the inflamed paw in the studies with SCH 34826.

The effects of both thiorphan (plus bestatin) and SCH 34826 were most likely mediated by inhibiting the degradation of endogenous opioid peptides by EC. 3.4.24.11 (Roques *et al.*, 1980; Chipkin *et al.*, 1988; Schwartz *et al.*, 1985). EC. 3.4.24.11 degrades not only the classical enkephalins but also longer opioid peptides such as Met⁵ Arg⁶ Phe⁷ enkephalin, γ -endorphin and β -lipotrophin, whereas levels of β -endorphin and dynorphin 1⁻¹³ are unaffected by this enzyme (Schwartz *et al.*, 1981; Hersh 1984; for review see Schwartz, 1983; Turner, 1985). Therefore, it is difficult to ascertain the precise nature of the opioid peptides responsible for mediating the stress-induced antinociception although it seems most likely that enkephalin-like peptides are involved.

These results provide strong evidence that stress of cold water swim causes an activation of enkephalinergic systems which, in turn, produces a selective opioid receptor-mediated antinociception within the inflamed paws of rats unilaterally inoculated with Freund's complete adjuvant. Previously we have shown that this cold water swim stress-activated, peripheral, opioidergic antinociception is dependent on the activity of corticotrophic cells in the anterior lobe of the hypophysis (Parsons *et al.*, 1990). Which of the many factors released by the hypophysis following stressful stimuli is responsible for mediating this effect is not yet known, although it seems unlikely that any of the opioid peptides released from this tissue following stress directly activate peripheral opioid receptors in peripheral inflamed tissues (see Millan 1981, 1986). It seems more likely that prolactin (Rossier *et al.*, 1980; Höllt *et al.*, 1986), or some similar hypophysial factor, is released into the circulation and in turn, signals the release of opioid peptides in the periphery, possibly directly within the inflamed tissues. The possibility of such a peripheral release of the opioid peptides involved is supported by recent studies from our laboratory which indicate a role for β -endorphin released directly within inflamed tissues to mediate cold water swim stress-induced antinociception (Stein *et al.*, 1990). Further studies shall address the nature of this releasing factor and the source/

nature of the peripheral enkephalin- and β -endorphin-like peptides involved.

In conclusion, cold water swim stress-induced antinociception seen in the inflamed paws of rats unilaterally injected with Freund's complete adjuvant is prolonged by the administration of peptidase inhibitors. The effects are opioidergic, and possibly enkephalinergic, in nature and are mediated in the periphery.

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