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Involvement of Dynorphin B in the Antinociceptive Effects of the Cannabinoid CP55,940 in the Spinal Cord[1]

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ABSTRACT

Intrathecal administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) but not the cannabinoid agonist CP55,940 enhances the antinociception produced by morphine. In addition, CP55,940- and Δ^9-THC-induced antinociception is blocked by the *kappa* opioid antagonist norbinaltorphimine, and both cannabinoids are cross-tolerant to *kappa* agonists but do not act directly at the *kappa* receptor. Previous work in our laboratory has implicated dynorphins in the antinociceptive effects of Δ^9-THC and its enhancement of morphine-induced antinociception. The goal of the present study was to evaluate the role of dynorphins in the antinociceptive effects of CP55,940 at the spinal level. Pretreatment of mice with antisera to dynorphin A(1–17), dynorphin A(1–8) or α-neoendorphin, all of which have been shown to retain specificity for blockade of their respective peptide *in vivo*, blocked the antinociceptive effects of Δ^9-THC but not CP55,940. Dynorphin B produced antinociceptive effects on intrathecal administration to mice. Like CP55,940, dynorphin B failed to enhance the antinociceptive effects of morphine, whereas dynorphin A(1–17) and α-neoendorphin enhanced the antinociceptive effects of morphine. Using spinal catheterization of the rat, CP55,940 administration was shown to produce a significant release of dynorphin B concurrent with the production of antinociception. Our data suggest that CP55,940 induces a release of spinal dynorphin B that contributes at least in part to its antinociceptive effects in the spinal cord.

Cannabinoids are active as antinociceptive drugs when injected i.t. (Gilbert, 1981; Lichtman and Martin, 1991a, 1991b; Welch *et al.*, 1995; Welch and Stevens, 1992; Yaksh, 1981). Intrathecally administered cannabinoids appear to act at predominantly spinal sites in the production of antinociception (Smith and Martin, 1992). The mechanisms by which the cannabinoids produce antinociception are as yet unclear. The identification of cannabinoid receptors has been the topic of intense investigation leading to the cloning of two distinct cannabinoid receptors; one is predominantly located in the central nervous system (Matsuda *et al.*, 1990), and the other is found on immune cells and on peripheral tissues (Munro *et al.*, 1993). In addition, a splice variant of the CB1 receptor termed the CB1A receptor has been identified (Shire *et al.*, 1995). When the sequence for the cannabinoid receptor was published, Gérard *et al.* (1990) reported they had isolated the human homolog of this receptor. The discovery of the cannabinoid antagonist SR141716A (Rinaldi-Carmona *et al.*, 1994) and the discovery of an endogenous cannabinoid-like ligand, anandamide, (Devane *et al.*, 1992) have greatly facilitated work with the cannabinoids and complements the discovery and cloning of the cannabinoid receptors.

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Our previous data indicate that the cannabinoids produce antinociception by indirect interaction with *kappa* opioids in the spinal cord after i.t. administration. The *kappa* antagonist nor-BNI and dynorphin antisera block Δ^9-THC-induced i.t. antinociception but not THC-induced catalepsy, hypothermia or hypoactivity (Smith *et al.*, 1994; Pugh *et al.*, 1996). Such data represent the first time that the behavioral effects of the cannabinoids had been separated. In addition, the discovery of the bidirectional cross-tolerance of THC and CP55,940 to *kappa* agonists using the tail-flick test (Smith *et al.*, 1994) indicates that cannabinoids interact with *kappa* opioids. The attenuation of the antinociceptive effects of THC by antisense to the *kappa*-1 receptor further implicates the release of endogenous *kappa* opioids in the mechanism of action of the cannabinoids (Pugh *et al.*, 1995). Because nor-BNI-induced blockade of dynorphin-induced antinociception has been documented and the principle site of action of nor-BNI is at the *kappa* receptor (Clark *et al.*, 1989), nor-BNI-induced blockade of cannabinoid antinociception is likely the result of a block of the effects of dynorphin at the *kappa* receptor. The blockade of cannabinoid-induced antinociception by the *kappa*-1 antagonist naloxone benzoylhydrazone also implicates the *kappa*-1 receptor in the effects of the cannabinoids (Welch, 1994). In addition, dynorphin antibodies block cannabinoid-induced antinociception, and pre-

ABBREVIATIONS: i.t., intrathecally; THC, Δ^9-tetrahydrocannabinol, nor-BNI, norbinaltorphimine; MPE, maximum possible effect; CL, confidence limit; DMSO, dimethylsulfoxide; CSF, cerebrospinal fluid.

vention of the metabolism of dynorphin A(1–17) to dynorphin(1–8) or to leucine enkephalin prevents the enhancement of morphine-induced antinociception by THC (Pugh *et al.*, 1996).

High levels of dynorphins exist in the dorsal horn of the spinal cord as well as in the brain, where they produce diverse effects on nociception (Fujimoto *et al.*, 1990; Fujimoto and Arts, 1990; Fujimoto and Holmes, 1990; Piercey *et al.*, 1982; Song and Takemori, 1991; Stevens and Yaksh, 1986; Tulanay *et al.*, 1981). The dynorphins have high affinity for the *kappa* receptor (for a review, see Hollt, 1986). Cleavage of the large precursor prodynorphin results in the release of various dynorphins, including dynorphin A(1–17), which has been proposed to be the endogenous ligand for the *kappa* receptor (Chavkin *et al.*, 1982). The breakdown of dynorphin A(1–17) into dynorphin A(1–8) and subsequently into leucine enkephalin has been shown (Dixon and Traynor, 1990; Hollt, 1986). Both dynorphin A(1–8) and analogs of leucine enkephalin produce antinociception when administered i.t., as tested in the tail-flick test. The antinociceptive effects of both dynorphin A(1–8) and dynorphin A(1–13) at spinal sites have been shown to result from interaction with *kappa* receptors (although other opioid receptor subtypes have been shown to bind these dynorphins), and both ligands have been shown to enhance the antinociceptive effects of morphine at spinal sites after i.t. administration (Jen *et al.*, 1986; Jhamandas *et al.*, 1986; Pugh *et al.*, 1996). Dynorphin B and α-neoendorphin are other products of the prodynorphin precursor. It has been shown that dynorphin B produces antinociception when administered i.t., as measured by the tail-flick test (Nakazawa *et al.*, 1991; Spampinato *et al.*, 1988).

Despite all the data indicating involvement of *kappa* (and/or *delta*) opioids in cannabinoid-induced antinociception, *mu* and *delta*, but not *kappa*, receptor-selective opioids have been shown to be displaced by the cannabinoids in brain, albeit at relatively high cannabinoid concentrations (Vaysse *et al.*, 1987). *Delta* opioids are not displaced by cannabinoids in neuroblastoma cells (Devane *et al.*, 1986). In addition, binding of the cannabinoid CP55,940 in the spinal cord is not displaced by *kappa* agonists or the *kappa* antagonist nor-BNI (Welch *et al.*, 1995). Thus, we have accumulated considerable evidence suggesting a link of the cannabinoids to the dynorphins that requires further investigation.

The potent, synthetic cannabinoid CP55,940 was instrumental in demonstrating that cannabinoid binding sites are present in the substantia gelatinosa, an area involved with the transmission of pain signals (Herkenham *et al.*, 1990). In addition, CP55,940 produces many of the behavioral and physiological effects characteristic of THC. Despite these similarities, we found that THC and CP55,940 differ in their interaction with morphine in the spinal cord (Welch and Stevens, 1992). Pretreatment of mice with CP55,940 i.t. does not enhance the antinociceptive effects of morphine i.t., whereas pretreatment with THC produces a 10-fold decrease in the morphine ED_{50}. Our data indicate that THC enhances the antinociception of morphine through the release of endogenous dynorphin (Pugh *et al.*, 1996) Unfortunately, the role of endogenous opioids, particularly the dynorphins, in the antinociceptive effects of CP55,940 is unclear. In the present investigation, we examined the role of dynorphins in CP55,940-induced antinociception.

Methods

Animals. Male ICR mice (Harlan Laboratories, Indianapolis, IN) with a weight range of 23 to 27 g were housed 6 or 8 to a cage in animal care quarters maintained at 22 ± 2°C on a 12-hr light/dark cycle. Food and water were available *ad libitum*.

Intrathecal injections. Intrathecal injections were performed according to the protocol of Hylden and Wilcox (1983). Unanesthetized mice were injected between the L5 or L6 area of the spinal cord with a 30-gauge, 0.5-inch needle. Injection volumes of 5 μl were administered. THC and CP55,940 were prepared in 100% DMSO. Dynorphins and α-neoendorphin were prepared in distilled water plus Triton X-100 (0.01%). Dynorphin antisera, morphine sulfate and nor-BNI were prepared in distilled water. All drugs were kept in plastic tubes on ice and were prepared fresh daily. In studies evaluating the effects of various dynorphin antisera on the antinociceptive effects of THC and CP55,940 alone, a 45- to 60-min pretreatment time of the antisera was used before testing the animals in the tail-flick test. This time course was consistent with our previous studies (Pugh *et al.*, 1996) and those of others that indicate that peak blockade of antinociception occurs when the antibodies are injected 60 min before testing (Fujimoto *et al.*, 1990). α-Neoendorphin (75 μg/mouse i.t.) was administered at 10 min before testing after a 45-min vehicle (distilled water i.t.) or antisera (10 μg/mouse i.t.) or IgG (10 μg/mouse i.t.) pretreatment and tested for antinociception using the tail-flick test. Dynorphin B (85 μg/mouse i.t.) was tested similarly in combination with vehicle, IgG or dynorphin B antisera. Other doses of antisera ($\leq$100 μg/mouse) and time points of pretreatment of $\leq$2 hr were evaluated. For studies of the combination of morphine with dynorphins, the highest inactive dose of the respective dynorphins was administered 10 min before morphine. Inactive doses of the dynorphins (μg/mouse) were as follows: dynorphin A(1–8), 10; dynorphin A(1–17), 1; dynorphin B, 10; and α-neoendorphin, 10. At 10 min after the morphine administration, the mice were tested using the tail-flick test.

The dynorphins produce splaying of the hindlimbs at doses that produce >80% MPE. Clearly, the motor effects at such doses could contribute to the antinociceptive effects of the drugs. However, we can block the antinociceptive effects of the dynorphins at high doses with nor-BNI (*kappa* antagonist), which implies that the antinociception produced at such high doses may not be related to nonspecific toxic effects. At the low, inactive doses used in combination with morphine, there are no toxic effects of the dynorphins observed. The dynorphin antisera were devoid of any observable side effect.

Tail-flick test. The tail-flick procedure was performed according to D'Amour and Smith (1941). Control reaction times of 2 to 4 sec and a cutoff time of 10 sec were used. Antinociception was quantified as the % MPE as developed by Harris and Pierson (1964) using the following formula: % MPE = $100 \times [(\text{test} - \text{control})/(10 - \text{control})]$.

Percentage of MPE was calculated for each mouse using at least 6 mice per dose. By using the % MPE for each mouse, the mean effect and S.E.M. were calculated for each dose. Dose-response curves were generated using at least three doses of test drug. ED_{50} values were determined by log-probit analysis, and 95% CLs were determined using the method of Litchfield and Wilcoxon (1949).

Tolerance to THC. Mice were rendered tolerant to the effects of Δ^9-THC by repetitive administration of 15 mg/kg s.c. Δ^9-THC over a 7-day period according to the method of Tsou *et al.* (1995). The animals received two subcutaneously administered injections per day at 8:00 a.m. and 6:00 p.m. for the first 6 days and a single injection at 8:00 a.m. on day 7. Testing was performed at 8:00 a.m. on day 8. Control groups receiving appropriate vehicle administration were also tested. Dynorphin B was administered i.t. to THC-tolerant and nontolerant mice, and the antinociceptive effects were evaluated 10 min later.

Spinal cord perfusion and quantification of dynorphin B release. Spinal dynorphin release in rat has been documented in superfused isolated spinal cords (Song and Takemori, 1992) and

spinal cord slices (Przewlocka *et al.*, 1990), and it has been directly released from rat spinal cord in response to clonidine (Xie *et al.*, 1986) at levels consistent with sensitivity of our radioimmunoassays. Using the methods of Tseng (1989), rats were injected with sodium barbital (300 mg/kg i.p.) and methylatropine bromide (2.0 mg/kg i.p.) and placed on a 37°C heating pad. Administration of CP55,940 (100 μg/rat i.t.) or DMSO vehicle to the rat was performed according to the method of Yaksh (1981) by the insertion of an indwelling intrathecal cannula *via* incision on the basal occipital membrane and insertion of PE-10 polyethylene tubing caudally into the subarachnoid space. (The dose of CP55,940 was the ED_{80} dose in the rat as previously determined by Lichtman and Martin, 1991a). The catheter was designed to be 8.5 cm in length and extend into the lumbar enlargement and was prefilled with artificial CSF. A peristaltic pump perfused artificial CSF or drugs at a rate of 30 μl/min. Drug and DMSO vehicle were administered in a 30-μl volume. The artificial CSF was composed of 125 mM Na^+, 2.6 mM K^+, 0.9 mM Mg^{++}, 1.3 mM Ca^{++}, 122.7 mM Cl^-, 21.0 mM $NaHCO_3$, 2.4 mM sodium phosphate buffer, 120 μg/ml bovine serum albumin, 30 μg/ml bacitracin and 0.01% Triton X-100 to prevent sticking of the dynorphin to the tubing, and bubbled with 95% O_2/5% CO_2 immediately before use. Outflow for CSF occurred by making an midline skull incision to expose the bregma and cisterna. The cisternal membrane was opened and PE-50 tubing was placed in the open cisternal space. The outflow cannula rapidly collected perfusate (one 1.5-ml aliquot in 1 min) into polypropylene tubes on ice. The antinociceptive effects of CP55,940 are significant for ≥30 min after spinal perfusion. Thus, collection of the CSF was performed at a time point when CP55,940 produced antinociception. The fractions were boiled at 99°C for 5 min to destroy any enzymatic activity and centrifuged in a microfuge, and the supernatant was frozen at −70°C for later lyophilization and analysis of dynorphin B *via* radioimmunoassay.

Radioimmunoassay measurement of dynorphin B was performed using kits and protocols obtained from Peninsula Laboratories, Inc. (Belmont, CA). The values were expressed as spinal peptide content (pg/ml) per CSF fraction across the linear portion of the standard curve (0.1–32 pg/ml). Dynorphin B antisera has 12% cross-reactivity to "big dynorphin" and dynorphin B(1–29) but no cross-reactivity to the enkephalins or other dynorphins, the cannabinoids or DMSO vehicle.

Drugs. Δ^9-THC and morphine sulfate were from National Institute on Drug Abuse (Rockville, MD), dynorphins and dynorphin antibodies were from Peninsula Laboratories (Belmont, CA) and CP55,940 was from Pfizer Central Research (New York, NY).

Results

If cannabinoids produce antinociceptive effects *via* interaction with dynorphins, we would expect the dynorphins to induce antinociception. Our previous work indicates that dynorphins A(1–8), A(1–13) and A(1–17) produce antinociception (Pugh *et al.*, 1996). We evaluated the antinociceptive effects of two additional dynorphins, α-neoendorphin and dynorphin B (Figure 1) and blockade of those effects by the respective antisera to the dynorphins (Figure 2). Our results indicated that α-neoendorphin (75 μg/mouse) produced a 78% MPE in the tail-flick test at 10 min before testing (time point of maximal antinociception) and after a 45-min vehicle (distilled water i.t.) pretreatment. The ED_{50} value for α-neoendorphin was 38 μg/mouse (95% CLs, 20–71; fig. 1). Pretreatment of mice with the *kappa* antagonist nor-BNI (3 μg/mouse i.t.) or distilled water vehicle at 5 min before α-neoendorphin (75 μg/mouse i.t.) significantly attenuated the antinociception produced by this endogenous opioid peptide (MPE = 12 ± 3%; fig. 2). The effects of a 5-min distilled water vehicle pretreatment (not shown in fig. 2) did not differ from a 45-min vehicle pretreatment (data shown in fig. 2). Pretreatment of mice with α-neoendorphin antisera (10 μg/mouse i.t.) 45 min before α-neoendorphin injection significantly decreased the antinociception from 78 ± 7% MPE to 23 ± 8% MPE in the tail-flick test (fig. 2). Dynorphin B i.t. produced

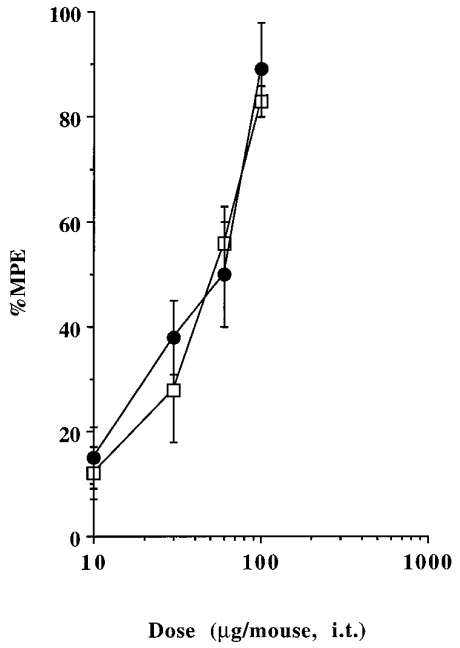

Fig. 1. Dose-effect curves for α-neoendorphin and dynorphin B after i.t. administration to mice. The peptides were administered i.t., and antinociception was quantified 10 min later using the tail-flick test. □, Dynorphin B. ●, α-Neoendorphin. Eight mice were used per dose. The ED_{50} values were calculated as described in the text.

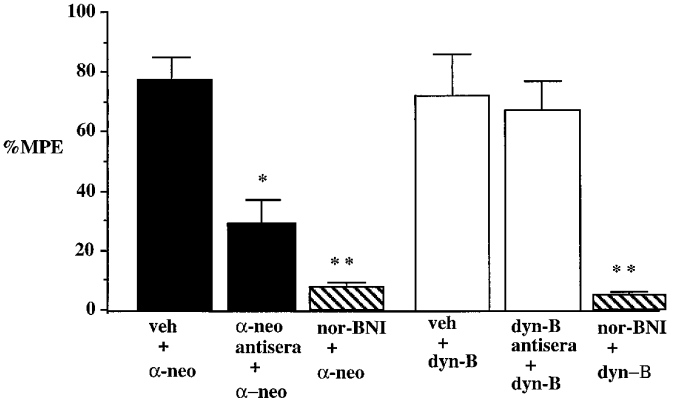

Fig. 2. Blockade of the antinociceptive effects of α-neoendorphin (α-neo), but not dynorphin B (dyn-B), by the respective antisera for the dynorphins administered i.t. to the mouse. α-Neoendorphin (75 μg/mouse i.t.) was administered at 10 min before testing after a 45-min vehicle (veh) (distilled water i.t.) or antisera (10 μg/mouse i.t.) pretreatment and tested for antinociception using the tail-flick test. Dynorphin B (85 μg/mouse i.t.) was tested similarly in combination with vehicle or dynorphin B antisera. Other doses of antisera (≤100 μg/mouse) and time points of pretreatment ≤2 hr were evaluated. Pretreatment of the mice with the *kappa* antagonist nor-BNI (3 μg/mouse i.t.) or distilled water vehicle i.t. 5 min before α-neoendorphin or dynorphin B was also evaluated. The effects of a 5-min vehicle pretreatment (data not shown) did not differ from a 45-min vehicle pretreatment (shown). The % MPE ± S.E.M. values were determined and compared as described in the text using 8 mice per dose. *$P < 0.05$ from vehicle pretreatment; **$P < 0.01$ from vehicle pretreatment.

TABLE 1

Characterization of the selectivity of dynorphin antisera administered i.t. to mice

Mice were injected i.t. with dynorphin antisera or IgG at either 60 min [dynorphin A(1–8) or A(1–17) antisera or IgG] or 45 min (α-neoendorphin or dynorphin B antisera) before various dynorphins. At 10 min later, the mice were tested using the tail-flick test. All the dynorphins tested represented the ED_{80} dose range for the peptides. Because antisera to dynorphin B failed to attenuate the antinociceptive effects of dynorphin B, no further testing was performed with antisera to dynorphin B.

	Dynorphin A(1–8) (125 μg/mouse)	Dynorphin A(1–17) (50 μg/mouse)	α-Neoendorphin (75 μg/mouse)	Dynorphin B (85 μg/mouse)
Vehicle (distilled H_2O) pretreatment	97 ± 8%	88 ± 6%	78 ± 7%	75 ± 10%
Antisera to:				
Dynorphin A(1–8) (30 μg/mouse)	15 ± 5%[a,b]	90 ± 3%	83 ± 8%	80 ± 9%
Dynorphin A(1–17) (30 μg/mouse)	99 ± 2%	30 ± 5%[a,b]	81 ± 9%	71 ± 5%
α-Neoendorphin (10 μg/mouse)	95 ± 5%	82 ± 7%	23 ± 8%[b]	76 ± 3%
Dynorphin B (10 μg/mouse)				69 ± 9%
IgG (10 μg/mouse)	94 ± 4%[a]	89 ± 4%[a]	78 ± 2%	79 ± 9%

[a] Data from Pugh *et al.* (1996).
[b] Significant ($P < 0.05$) decrease from vehicle or IgG control pretreatments.

antinociception (ED_{50} = 44 μg/mouse i.t., 95% CLs, 26–73). The ED_{80} dose was determined to be 85 μg/mouse. Pretreatment of mice with the *kappa* antagonist nor-BNI (3 μg/mouse i.t.) or distilled water vehicle 5 min before dynorphin B (85 μg/mouse i.t.) significantly attenuated the antinociception produced by this endogenous opioid peptide (%MPE = 8 ± 1%; fig. 2). The effects of a 5-min vehicle pretreatment (data not shown) did not differ from those of a 45-min vehicle pretreatment (data shown). Administration i.t. of dynorphin B (85 μg/mouse) 10 min after distilled water vehicle injection i.t. produced a 75 ± 10% MPE in the tail-flick test. Pretreatment of mice with the dynorphin B antisera (10 μg/mouse) 45 min before injection of dynorphin B (85 μg/mouse) failed to attenuate the antinociceptive effects of dynorphin B. Other doses of antisera and time points of administration were evaluated, but the dynorphin B antisera failed to alter the antinociceptive effects of dynorphin B. Thus, dynorphin B antisera could not be used to characterize the interaction with CP55,940 because the antisera does not block its respective peptide in the tail-flick test (fig. 2).

In addition, we characterized the specificity of antisera to all of the dynorphins *in vivo*. It was critical to our understanding of the effects of the dynorphin antisera in combination with the cannabinoids to first determine that the antisera were active as blockers of dynorphins and selective when administered i.t. to the whole animal. As a control, IgG was administered. No dynorphin antisera produced intrinsic antinociceptive effects. Table 1 includes data on antisera to dynorphins A(1–8) and A(1–17) and IgG from Pugh *et al.* (1996). These data are included along with new data such that a complete picture of the selectivity of the antisera for dynorphin peptides *in vivo* is available. ED_{80} doses of all dynorphins were administered i.t. 10 min before testing in the tail-flick test. Pretreatment i.t. with antisera to the dynorphins occurred at the time observed for peak blockade of dynorphin-induced antinociception (45 min to 1 hr before dynorphins depending on the respective dynorphin antibody). IgG was administered at 1 hr before the dynorphins. All of the antisera tested retained selectivity for their respective dynorphin fragment. However, because dynorphin B antisera was inactive *vs.* dynorphin B, no further evaluation of antisera to dynorphin B was performed.

Table 2 shows the effects of dynorphin antisera i.t. or IgG (control, 10 μg/mouse) on the antinociceptive effects of THC and CP55,940 (both i.t.). CP55,940 (2.5 μg/mouse i.t.) administered 10 min before testing resulted in 78 ± 8% MPE in the tail-flick test. Antisera to dynorphins (30 μg/mouse i.t.) were evaluated alone; all were found to be devoid of antinociceptive properties. Previous work has demonstrated that antisera to dynorphins A(1–8) and A(1–17) attenuate the antinociceptive effects of THC (Pugh *et al.*, 1996). We now show that the antinociceptive effects of an ED_{80} dose of THC (50 μg/mouse) are blocked totally (% MPE reduced to 14 ± 4%) by antisera to α-neoendorphin. The antinociceptive effects of an ED_{80} dose of CP55,940 (2.5 μg/mouse) are not altered by pretreatment with any of the dynorphin antisera (% MPE remained >73 ± 10% after all pretreatments with antisera). Thus, CP55,940, unlike THC, does not appear to interact with such dynorphins in the production of antinociception.

We have previously shown that dynorphins A(1–8) and A(1–13) enhance the antinociceptive effects of morphine (Pugh *et al.*, 1996). Experiments were designed to extend such work to evaluate the effects of low, inactive doses of α-neoendorphin, dynorphin A(1–17) and dynorphin B on morphine-induced antinociception. The ED_{50} values observed for morphine i.t. are listed in table 3. Data on dynorphin A(1–8) are from Pugh *et al.* (1996) for the purpose of comparison with the other dynorphins. Each i.t. pretreatment of mice with the highest inactive dose of dynorphins

TABLE 2

Characterization of the blockade of THC- and CP55-induced antinociception by dynorphin antisera administered i.t. to mice

Mice were injected i.t. with dynorphin antisera or IgG at either 60 min [dynorphin A(1–8)or A(1–17) antisera or IgG] or 45 min (α-neoendorphin antisera) before the cannabinoids. At 10 min later, the mice were tested using the tail-flick test. The cannabinoids were tested using the ED_{80} dose range for each (50 μg/mouse for THC and 2.5 μg/mouse for CP55). Because antisera to dynorphin B failed to attenuate the antinociceptive effects of dynorphin B, no further testing was performed with antisera to dynorphin B.

	Cannabinoid	
	THC (50 μg/mouse)	CP55,940 (2.5 μg/mouse)
Vehicle (distilled H_2O) pretreatment	97 ± 8%	88 ± 6%
Antisera to:		
Dynorphin A(1–8) (30 μg/mouse)	19 ± 4%[a,b]	73 ± 10%
Dynorphin A(1–17) (30 μg/mouse)	23 ± 8%[a,b]	75 ± 8%
α-Neoendorphin (10 μg/mouse)	14 ± 4%[b]	80 ± 4%
IgG (10 μg/mouse)	79 ± 5%	86 ± 6%

[a] Data from Pugh *et al.* (1996).
[b] Significant ($P < .05$) decrease from vehicle or IgG control pretreatments.

TABLE 3

Enhancement of morphine-induced antinociception by dynorphin A(1–8), dynorphin A(1–17) and α-neoendorphin but not by dynorphin B after i.t. administration to mice

The highest inactive dose of the respective dynorphins was administered 10 min before morphine. Doses of the dynorphins (μg/mouse) were as follows: dynorphin A(1–8), 10; dynorphin A(1–17), 1; dynorphin B, 10; and α-neoendorphin, 10. All injections were i.t. At 10 min after the morphine administration, the mice were tested using the tail-flick test. ED_{50} values (μg/mouse) and 95% CLs for morphine were calculated as described in the text.

Pretreatment (i.t.)	Morphine (i.t.) ED_{50} values (μg/mouse)
Distilled water	0.62 (0.13–2.82)
Dynorphin A(1–8)[a]	0.04 (0.02–0.09)
Dynorphin A(1–17)	0.02 (0.01–0.10)
α-Neoendorphin	0.11 (0.09–1.17)
Dynorphin B	0.50 (0.12–1.76)

[a] Data from Pugh *et al.* (1996).

A(1–8), A(1–13) A(1–17) and α-neoendorphin enhanced the antinociceptive potency of morphine as observed by a decrease in the ED_{50} of morphine (table 3). The effects observed with dynorphins A(1–8), (1–13), and (1–17) were significant. The effect with α-neoendorphin approached significance (nearly a 5-fold shift in the morphine ED_{50} value) but due to variability and wider 95% CLs was not a significant effect. However, using the highest inactive dose of dynorphin B (10 μg/mouse), no enhancement of the antinociceptive potency of morphine was observed.

We also performed a limited number of experiments to evaluate whether the enhancement of CP55,940- or dynorphin A(1–17)- or dynorphin B-induced antinociception could be enhanced by a low dose of morphine i.t. We have previously shown that an inactive dose of morphine i.t. shifts the dose-effect curve of THC to the left but that the shift produces a flattening of the slope of the THC curve and results in a nonsignificant (wide intervals) shift in the ED_{50} value for THC (Welch and Stevens, 1992). Using a 10-min pretreatment with an inactive dose of morphine (0.1 μg/mouse) before dynorphin A(1–17), the ED_{50} value was shifted from 20 (11–36) to 5 (2–10) μg/mouse. Unlike our previous results with THC, the effect was significant. Thus, dynorphin A appeared to somewhat mimic the effects of THC in terms of enhancement by morphine. The ED_{50} value for dynorphin B was not shifted significantly [41 (26–54) μg/mouse] by morphine pretreatment. The ED_{50} value for CP55,940 was also not altered by morphine pretreatment [1.5 (0.5–2.8) in the presence of morphine *vs.* 1.3 (0.2–2) in the presence of vehicle]. Thus, the enhancement of morphine-induced antinociception by dynorphin A(1–17) was bidirectional, whereas CP55,940 and dynorphin B were unaffected by pretreatment with morphine.

To further characterize the lack of interaction of dynorphin B with THC, we evaluated the cross-tolerance of dynorphin B to THC (fig. 3). Animals were rendered tolerant to THC as described in "Methods." The ED_{50} value (μg/mouse i.t.) for THC was significantly shifted by 6.7-fold in THC-tolerant mice [ED_{50} = 11.5 (5.8–22.9) *vs.* 77.7 (45.6–132.5)]. Dynorphin B showed no cross-tolerance to THC. The ED_{50} values (μg/mouse i.t.) for dynorphin B in the nontolerant *vs.* the THC-tolerant mice were 40 (21.7–75.8) and 49 (28.1–86), respectively. We have previously demonstrated that dynorphin A(1–17) is cross-tolerant to THC (Welch, in press).

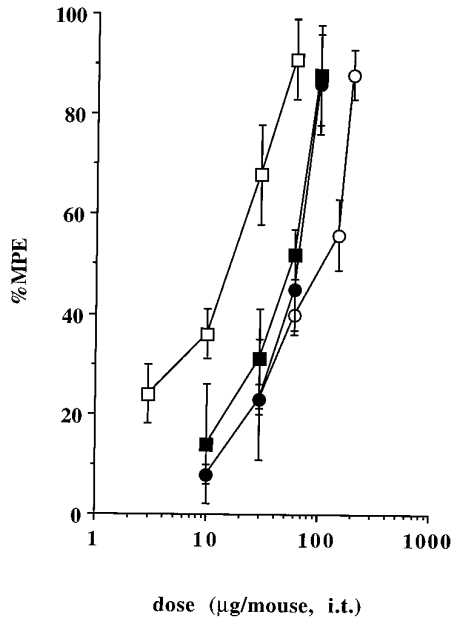

Fig. 3. Lack of cross-tolerance of dynorphin B to THC. Mice were rendered tolerant to THC using the protocol as described in the text. Mice were also treated chronically with vehicle (non-THC-tolerant mice). On the test day, dynorphin B and THC were administered i.t. at 10 min before testing using the tail-flick test in both the mice chronically treated with THC and those treated chronically with vehicle. Eight mice per dose were used. ED_{50} values were calculated as previously described. □, Nontolerant mice challenged with THC on test day. ○, Tolerant mice challenged with THC on test day. ■, Nontolerant mice challenged with dynorphin B on test day. ●, Tolerant mice challenged with dynorphin B on test day.

Cross-tolerance to CP55,940 was not evaluated due to the lack of adequate drug supplies of CP55,940 for such studies.

Because dynorphin B, like CP55,940, failed to enhance morphine-induced antinociception, we hypothesized that CP55,940 might release dynorphin B (because the dynorphin A antisera studies appeared to rule out dynorphin A release by CP55,940). For these studies, the rat was used to obtain adequate dynorphin B for testing. Spinal cord perfusion with a 100 μg/rat dose of CP55,940 (ED_{80} = CP55,940 in the rat; Lichtman and Martin, 1991a) resulted in an antinociceptive effect of 79 ± 2% MPE (n = 5 rats) *vs.* 16 ± 8% MPE (n = 7 rats) for the 100% DMSO vehicle. Dynorphin B levels were increased significantly from 5.4 ± 0.4 pg/ml in the DMSO-treated rats to 14.0 ± 2.8 pg/ml in the CP55,940-pretreated rats (fig. 4).

Discussion

Several attempts have been made to understand how the cannabinoids produce their pharmacological effects, particularly antinociception. Intrathecal administration of the cannabinoids in spinally transected rats has shown that both spinal and supraspinal mechanisms are involved in cannabinoid-induced antinociception (Lichtman and Martin, 1991a). In addition, it has been shown that cannabinoid and opiate receptors are co-localized in areas involved with the transmission of pain signals (Herkenham *et al.*, 1990). Based on these studies, it is not unlikely that an interaction would occur between the cannabinoids and opiates in the production of antinociception. Additional evidence that indicates the

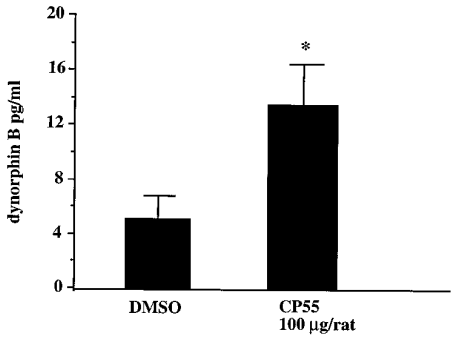

Fig. 4. Release of dynorphin B by CP55,940 (CP55) in rat spinal cord. Rats were used to obtain adequate dynorphin B for testing. The rat spinal cord was perfused with a 100 μg/rat dose of CP55,940 or DMSO vehicle in 30-μl volumes. CSF was perfused and collected (1.5 ml/min) for analysis of dynorphin B at 10 min later. Concurrent with the drawing of the CSF, the rats were evaluated in the tail-flick test for antinociceptive effects. Spinal cord perfusion with a 100 μg/rat dose of CP55,940 resulted in an antinociceptive effect of 79 $\pm$ 2% MPE (n = 5 rats) *vs.* 16 $\pm$ 8% MPE (n = 7 rats) for the 100% DMSO vehicle. Dynorphin B levels in pg/ml were determined as described in the text using 5 rats for CP55,940 and 7 rats for DMSO administration. *$P < 0.05$ from DMSO pretreatment.

existence of a cannabinoid/opiate functional interaction is the observation that THC ameliorates naloxone-precipitated withdrawal (Bhargava, 1976). Vaysee *et al.* (1987) have shown that high concentrations of THC inhibit agonist binding at *mu* and *delta* receptors but not *kappa* receptors. The *kappa* antagonist nor-BNI does not displace cannabinoid binding in brain or spinal cord (Welch, 1993); however, the *kappa* receptor seems to be important in mediating cannabinoid-induced antinociception. It was observed that the *kappa* receptor antagonist nor-BNI specifically blocked the antinociceptive effects of THC without altering its hypothermic, hypoactive or cataleptic effects (Smith *et al.*, 1994). Subsequent studies designed to determine the nature of the THC/*kappa* receptor interaction indicate that THC interacts indirectly with the *kappa* receptor through endogenous opioid release (Pugh *et al.*, 1996).

The endogenous opioid peptides are derived from three different gene families; each has a distinct anatomical distribution (Akil *et al.*, 1984). Prodynorphin produces three main [Leu^5]enkephalin-containing peptides: α/β neoendorphin, dynorphin A and dynorphin B. High levels of dynorphins are found in the brain as well as the dorsal horn of the spinal cord (Lewis *et al.*, 1982; Slater and Patel, 1983; Weber *et al.*, 1982), show a high affinity for the *kappa* receptor and have been suggested as the endogenous ligands for the *kappa* receptor (Chavkin *et al.*, 1982; Chavkin and Goldstein, 1981). In addition, the dynorphin A fragments, as well as dynorphin B, and α/β-neoendorphins have been shown to produce antinociception when administered i.t. (Han and Xie, 1982; Piercey *et al.*, 1982). The release of *kappa* opioids by THC, in combination with the activation of *mu* receptors by morphine, has been attributed to the greater-than-additive antinociceptive effect produced by the THC/morphine combination.

The synthetic cannabinoid CP55,940 is more potent than THC in both *in vivo* and *in vitro* assays and has been useful in determining the site and mechanism of action of the cannabinoids (Welch, 1993; Welch *et al.*, 1995; Welch and Stevens, 1992). The block of CP55,940-induced antinociception with nor-BNI and the lack of a greater-than-additive effect between CP55,940 and morphine in antinociceptive tests was hypothesized to be due to the release of a pool of endogenous *kappa* opioids that do not enhance morphine-induced antinociception. We concluded that dynorphin A fragments are not involved in mediating the antinociceptive effects of CP55,940 on the basis of antisera studies. Furthermore, on the basis of data from previous experiments, we would not have expected CP55,940-induced antinociception to be mediated by such dynorphins because all of these dynorphin peptides administered i.t. enhance the antinociceptive potency of morphine in the spinal cord. In subsequent experiments, we examined the role of a different prodynorphin product, α-neoendorphin, on CP55,940-induced antinociception. We were able to demonstrate that α-neoendorphin does enhance the antinociceptive effects of morphine in the spinal cord and that antisera to this peptide fail to alter CP55,940-induced antinociception. Thus, we concluded that CP55,940 does not modulate the activity of α-neoendorphin in the spinal cord.

Xie *et al.* (1986) have shown that dynorphin B produces antinociception in the spinal cord. Our studies replicate those of Xie *et al.* We observed that dynorphin B, unlike any of the other dynorphins we tested in combination with morphine, does not increase the antinociceptive potency of morphine. Similarly, morphine fails to enhance the antinociceptive effects of dynorphin B, an effect also observed with CP55,940. Thus, the effects of dynorphin B are similar to those of CP55,940 with respect to modulation by morphine. Dynorphin B is not cross-tolerant to THC, even though CP55,940 is cross-tolerant to THC and THC displaces CP55,940 binding (Smith *et al.*, 1994). Dynorphin A is cross-tolerant to THC (Welch, 1996). Thus, THC appears linked in some unknown way to the modulation of dynorphin A, whereas CP55,940 appears to be linked to modulation of dynorphin B. Clearly, an interesting but technically difficult study would be to evaluate the cross-tolerances of dynorphins A and B to each other. If such a cross-tolerance were to be observed, it might enhance our understanding of the cross-tolerance of CP55,940 and THC.

Fujimoto *et al.* (1990) have also shown that dynorphin B does not enhance the antinociception of morphine in the spinal cord. We hypothesized that CP55,940-induced release of dynorphin B could account for the observed nor-BNI blockade of CP55,940, as well as for the lack of enhancement produced by the combination of CP55,940 and morphine in combination. Direct measurement of dynorphin B release by CP55,940 in animals that also showed antinociceptive effects of the peptide appears to confirm a role for dynorphin B in the action of CP55,940.

The existence of multiple cannabinoid receptor subtypes may underlie the differences seen between THC and CP55,940 in the spinal cord. We hypothesize that with the existence of multiple cannabinoid receptor subtypes, different pools of endogenous opioids may be altered by THC and CP55,940. Thus, the antinociceptive potency of morphine could be modulated differently depending on whether CP55,940 or THC pretreatment is evaluated.

We envision release of dynorphins as an indirect process due to the disinhibition of yet unknown neuronal processes. The localization of the cannabinoid receptors involved in

dynorphin release is not known. We hypothesize that in the spinal cord, cannabinoids produce antinociceptive effects *via* the direct interaction of cannabinoids with $G_{i/o}$ proteins, resulting in a decreased cAMP production (Welch *et al.*, 1995), as well as hyperpolarization *via* interaction with specific potassium channels (Deadwyler *et al.*, 1993). Thus, the cannabinoids produce disinhibition by decreasing the release of an inhibitory neurotransmitter in dynorphinergic pathways. The net result of such an effect is an increase in dynorphin release. The events that follow the release of dynorphin also remain unclear. The dynorphin most likely is a modulator of other "downstream" systems (possible substance P release or interaction with N-methyl-D-aspartate-mediated events) that culminate in antinociception on administration of cannabinoids.

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