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Blockade of Cannabinoid-Induced Antinociception by Naloxone Benzoylhydrazone (NalBZH)

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WELCH, S. P. *Blockade of cannabinoid-induced antinociception by naloxone benzoylhydrazone (NalBZH).* PHARMACOL BIOCHEM BEHAV **49**(4) 929-934, 1994.—We have recently shown that the antinociceptive effects, but not other behavioral effects of the intrathecally administered, but not intracerebroventricularly administered, cannabinoids, are blocked by the kappa antagonist, nor-binaltorphimine. We employed naloxone benzoylhydrazone, a $kappa_3$ agonist, and $kappa_1$, mu, and delta antagonist, to better characterize the interaction of cannabinoids with kappa receptors. Naloxone benzoylhydrazone blocked the antinociceptive effects of both intrathecally and intracerebroventricularly administered cannabinoids. Because the cannabinoids are not blocked by mu and delta antagonists, the effects of naloxone benzoylhydrazone are presumed to occur through interaction with kappa receptors. Because the data indicate that naloxone benzoylhydrazone does not block $kappa_3$ receptors, the data indicate that the cannabinoids may interact with $kappa_1$ receptors in the production of antinociception. However, differences in the profile of activity of naloxone benzoylhydrazone and the cannabinoids at kappa receptors exist. Thus, the exact nature of the interaction of the cannabinoids and the kappa receptors remains to be elucidated.

Cannabinoids Naloxone benzoylhydrazone U50,488H Antinociception Intrathecal administration Nor-binaltorphimine Kappa receptor subtypes

CANNABINOIDS produce potent antinociceptive effects in mice and rats through both spinal and supraspinal mechanisms (10,19,27,28). The mechanism by which cannabinoids produce antinociception has not been fully determined. Cannabinoid-induced antinociception in the rat has been proposed to involve the alpha-2 adrenergic receptor (11), as well as interaction with G_io proteins, calcium, c-AMP, and potassium channels (28). We have reported that a significant parallel leftward shift in the dose--effect curve of intrathecally administered (IT) morphine occurs following pretreatment with subthreshold doses of Δ^9-THC, Δ^8-THC, levonantradol, and 11-OH-Δ^9-THC, but not CP 55,940, CP 56,667, or dextronantradol (all IT) (27). Conversely, CP 55,940 and CP 56,667 produce greater than additive effects with morphine when all drugs are administered ICV (28). These data may indicate an interaction of morphine and the cannabinoids at a common site or via a common second messenger system. In addition, the lack of potentiation of morphine by CP 55,940 and CP 56,667 at spinal sites may indicate that different cannabinoid receptor subtypes exist in the spinal cord vs. in the brain. We have recently shown that the antinociceptive effects of cannabinoids administered IT, but not intracerebroventricularly (ICV), are blocked by the kappa antagonist, nor-binaltorphimine (nor-BNI, IT, but not ICV) (26,28). In addition, we have shown that nor-BNI (IT) blocks only the antinociceptive effects of the cannabinoids (IT), but not other behavioral effects of the drugs (21). Bidirectional crosstolerance between Δ^9-tetrahydrocannabinol (Δ^9-THC) and the kappa agonists, CI-977 (8) and U50,488H (25) has been shown (21). These data indicate a possible interaction of cannabinoids with kappa receptors in the production of antinociception.

Naloxone benzoylhydrazone (NalBZH) has been shown to bind to kappa, mu, and delta receptors (1). The antinociceptive effects of NalBZH have been evaluated (5,14). NalBZH acts as an agonist at $kappa_3$ receptors resulting in antinocicep-

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tion. In addition, NalBZH is an antagonist at mu, delta, and $kappa_1$ receptors. The use of NalBZH to characterize the $kappa_3$ binding site has been an important step in the classification of the subtypes of kappa receptors (1,15,18). Because the intrathecal administration (IT) of the cannabinoids results in antinociception that is blocked by nor-BNI at doses that are much higher than those required to block kappa agonists (20,28), there appear to be differences in the mechanism by which the cannabinoids and the kappa agonists produce antinociception. Nor-BNI has been shown in in vitro binding studies to interact with $kappa_1$ receptors (22) for which it has nearly a 30-fold greater affinity than for $kappa_3$ receptors (1), although it clearly binds both kappa receptor subtypes. However, the lack of nor-BNI-induced blockade of NalBZH antinociception indicates that nor-BNI may not interact in vivo with the $kappa_3$ receptor (14). We used NalBZH to better characterize the interaction of the cannabinoids with the kappa receptor. We reasoned that blockade of cannabinoid-induced antinociception by NalBZH would substantiate our previous findings of a kappa receptor/cannabinoid interaction because the binding of NalBZH is not displaced by a variety of other neuropeptides and neurotransmitters (1). In addition, since cannabinoid (IT and ICV)-induced antinociception has been shown to be insensitive to very high doses of either mu or delta antagonists (IT or ICV) (26,28), by the process of elimination, blockade of cannabinoid-induced antinociception by NalBZH, which is an antagonist at mu, delta, and kappa receptors, would appear to represent a blockade via interaction with the kappa receptor. In addition, because the data suggest that NalBZH is not an antagonist at the $kappa_3$ receptor, we might be able to attribute the block of cannabinoid-induced antinociception to interaction of the drugs at the $kappa_1$ receptor.

METHOD

Intrathecal injections were performed following the protocol of Hylden and Wilcox (9). Unanesthetized mice were injected between the L5 or L6 area of the spinal cord with a 30 gauge, 1/2 inch needle. Injection volumes of 5 μl were administered. Intraventricular injections were performed using the method of Pedigo et al. (16). Mice were injected ICV under light ether anesthesia at a point 2 mm lateral and 2 mm caudal to the bregma at a depth of 2 mm using a 26 gauge needle with a plastic sleeve attached to regulate the depth of the injection. Injection volumes were 5 μl/mouse. The cannabinoids were prepared in 100% DMSO. The cannabinoids or DMSO vehicle were administered 15 min prior to determination of the response latency of the mice in the tail-flick test. This time point represents the peak effect of the drugs as determined in previous studies in our laboratory (27). NalBZH was prepared in distilled water and administered subcutaneously (SC) at either 10 min prior to the administration of the cannabinoids (IT or ICV) or 10 min after the administration of the cannabinoids (IT). Vehicle controls received distilled water SC at either 10 min prior to or 10 min following the cannabinoids (ICV or IT) or DMSO vehicle (100% DMSO, 5 μl/mouse, IT or ICV). The distilled water SC/DMSO IT combination resulted in nonsignificant antinociceptive effects of less than 15% MPE. The distilled water SC/DMSO ICV combination resulted in nonsignificant antinociceptive effects of less than 5% MPE. Upon initial administration, DMSO produced some scratching that lasted 2 min following IT administration. No overt toxic effects of the DMSO were observed at any time point evaluated.

The tail-flick procedure used was that of D'Amour and Smith (2). Control reaction times of 2–4 s and a cut-off time of 10 s were employed. Antinociception was quantified as the %MPE as developed by Harris and Pierson (6) using the following formula:

$$\%MPE = 100 \times [(test - control)/(10 - control)].$$

Percent MPE was calculated for each mouse using at least 12 mice per dose. The AD_{50} for NalBZH blockade of the cannabinoids was determined by calculation of the percent antagonism of the cannabinoid-induced antinociception (using a dose of the cannabinoid that resulted in at least 80% MPE) according to the following formula:

$$\%antagonism = 100 \times 1 - [(\%MPE \text{ of antagonist} + cannabinoid)/(\%MPE \text{ of vehicle plus cannabinoid})].$$

Using at least three doses of NalBZH, AD_{50} values were determined by log-probit analysis (a modification of the Litchfield-Wilcoxon method omitting doses producing 100% or 0% MPE) and 95% CLs were determined using the method of Litchfield and Wilcoxon (12). At least 12 mice per dose were used for all determinations. Significant differences between treatment and control groups were determined using analysis of variance and the Dunnett's *t*-test (4).

The cannabinoids were obtained from the National Institute on Drug Abuse with the exception of CP-55,940 which was obtained from Dr. Lawrence Melvin, Pfizer Central Research. NalBZH was kindly donated by Dr. Gavril Pasternak, Sloan-Kettering Cancer Center, New York City, NY.

RESULTS

In order to determine the time of the peak blockade of the cannabinoids by NalBZH, several time points for administration of NalBZH prior to and following the administration of the cannabinoids were tested. Those time points showing significant blockade are shown in the figures. If NalBZH was administered at 15, 30, or 60 min prior to the cannabinoids, no significant blockade was observed, although with a 15 min pretreatment some attenuation in antinociception was observed. Administration of NalBZH at 5 min following the administration of the cannabinoids resulted in no greater blockade than that observed at 10 min following the administration of the cannabinoids. Thus, NalBZH appeared to produce a blockade for at least 25 min following administration SC and have an onset of 10 min. NalBZH administered 10 min prior to Δ^9-THC produced an initial enhancement of the antinociception following administration of 1 and 3 mg/kg, but a nearly complete blockade following administration of 5 mg/kg (Fig. 1). The enhancement of the effects of Δ^9-THC by NalBZH did not occur with the other cannabinoids tested. The enhancement observed was not statistically significant. Use of 10 mg/kg NalBZH prior to Δ^9-THC did not attenuate the antinociceptive effects of Δ^9-THC to any greater degree than that observed with 5 mg/kg. Doses of NalBZH higher than 15 mg/kg were not employed due to antinociceptive activity (% MPE greater than 25%), which confounded the study of antagonism of the cannabinoids. We were, thus, not able to generate an AD_{50} for NalBZH blockade of Δ^9-THC when the drug was administered prior to Δ^9-THC. However, the AD_{50} ($\pm$95% confidence limits, CLs) for blockade of Δ^9-THC-induced antinociception was 0.9 mg/kg (0.4–1.9) when NalBZH was administered 10 min after the IT administration of Δ^9-THC.

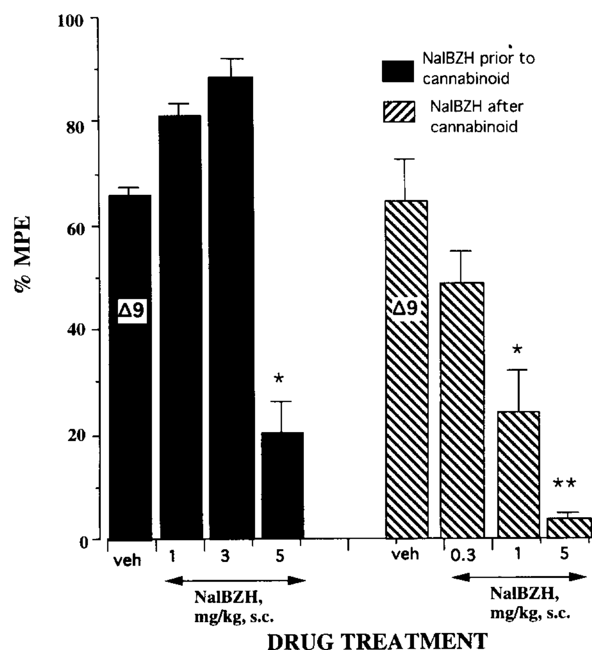

FIG. 1. Naloxone benzoylhydrazone antagonism of IT-administered Δ^9-THC. Dark bars: mice were pretreated SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min prior to Δ^9-THC (50 μg/mouse, IT). Injection of distilled water (SC) prior to DMSO (IT) resulted in a % MPE of 8 ± 3%. Striped bars: mice were injected SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min after the administration of Δ^9-THC (50 μg/mouse, IT). Injection of distilled water (SC) 10 min after DMSO (IT) resulted in a % MPE of 12 ± 4%. In both cases, the mice were tested using the tail-flick test at 15 min following IT injection of the cannabinoid. The data are presented as % MPE plus the standard error with at least 12 mice per dose. *$p < 0.05$ from distilled water/cannabinoid; **$p < 0.01$ from distilled water/cannabinoid.

In combination with Δ^8-THC, NalBZH blocked the antinociception produced by Δ^8-THC when NalBZH was administered either prior to or 10 min after Δ^8-THC. The AD_{50}s (±CLs) for NalBZH were 5 (2.6–10) and 3.5 (2–6), respectively, and did not differ significantly (Fig. 2). The responses of the mice were variable in response to the combination of the drugs, as evidenced by the large standard errors for each treatment group. In combination with CP 55,940, NalBZH blocked the antinociceptive effects of the cannabinoid when administered either prior to or 10 min after the IT administration of CP 55,940. The AD_{50}s (± CLs) for NalBZH were 1.8 (1.3–2.7) and 0.7 (0.3–2), respectively (Fig. 3) and did not differ significantly.

We performed a limited number of studies to evaluate the blockade of the ICV-administered cannabinoids by NalBZH SC at 10 min prior to the cannabinoid administration (Fig. 4). Administration of 5 mg/kg NalBZH prior to Δ^9-THC (50 μg/mouse), Δ^8-THC (100 μg/mouse), or CP 55,940 (8 μg/mouse) significantly blocked the antinociceptive effects of all three cannabinoids.

In a separate group of studies, spinal cord and whole brain synaptosomes were prepared from naive mice and the binding of the cannabinoid ^{3}H-CP,55940 was assessed. Scatchard analysis indicated a K_d of 1.24 nM and B_{max} of 133 pM for ^{3}H-CP,55940 in the spinal cord and a K_d of 0.24 nM and a B_{max} of 406 pM in the brain. NalBZH and nor-BNI (1 μM each) failed to displace ^{3}H-CP,55940 binding in either the brain or in the spinal cord.

DISCUSSION

Many investigators have reported the existence of subtypes of the kappa receptor in various tissues (1,3,13,23). The use of NalBZH has aided in the elucidation of the $kappa_3$ receptor subtype. Even though NalBZH is a mixed agonist/antagonist that is not selective in binding to the kappa receptor, it has been reported to produce antinociception by its interaction with the $kappa_3$ receptor, but does not appear to be a $kappa_3$ antagonist (14). Thus, the ability of NalBZH to block the antinociceptive effects of the cannabinoids, taken together with the blockade of cannabinoid-induced antinociception by nor-BNI, would appear to indicate an action at predominantly the $kappa_1$ receptor. It cannot be ruled out, however, that the interaction of the cannabinoids with nor-BNI may be nonopioid in nature. This would imply that both nor-BNI and NalBZH have actions at sites (unknown) other than the kappa receptor, although no reports indicate such an interaction for

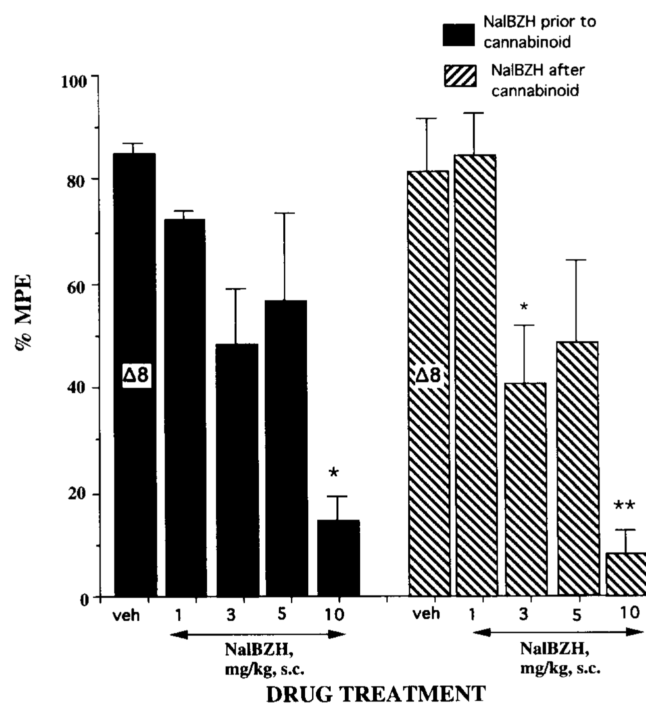

FIG. 2. Naloxone benzoylhydrazone antagonism of IT administered Δ^8-THC. Dark bars: mice were pretreated SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min prior to Δ^8-THC (100 μg/mouse, IT). Injection of distilled water (SC) prior to DMSO (IT) resulted in a % MPE of 10 ± 3%. Striped bars: mice were injected SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min after the administration of Δ^8-THC (100 μg/mouse, IT). Injection of distilled water (SC) 10 min after DMSO (IT) resulted in a % MPE of 4 ± 4 %. In both cases, the mice were tested using the tail-flick test at 15 min following IT injection of the cannabinoid. The data are presented as % MPE plus the standard error with at least 12 mice per dose. *$p < 0.05$ from distilled water/cannabinoid; **$p < 0.01$ from distilled water/cannabinoid.

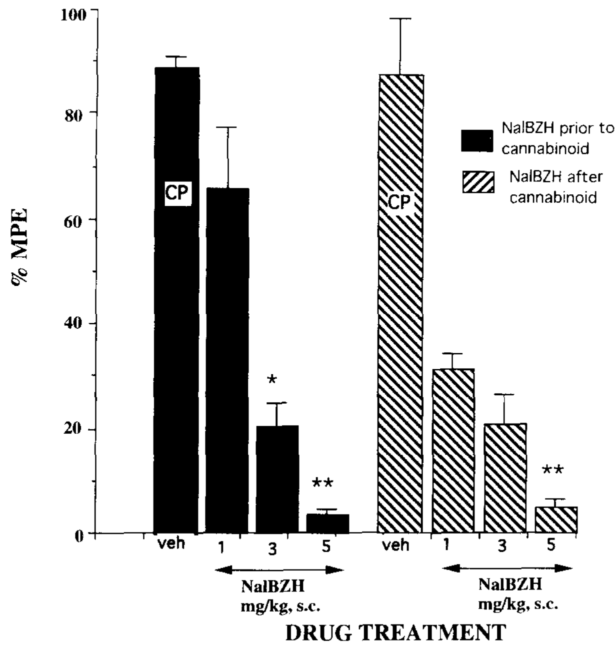

FIG. 3. Naloxone benzoylhydrazone antagonism of IT-administered CP 55,940. Dark bars: mice were pretreated SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min prior to CP 55,940 (8 μg/mouse, IT). Injection of distilled water (SC) prior to DMSO (IT) resulted in a % MPE of 13 $\pm$ 5 %. Striped bars: mice were injected SC with either distilled water vehicle or naloxone benzoylhydrazone (doses shown in mg/kg) 10 min after the administration of CP 55,940 (8 μg/mouse, IT). Injection of distilled water (SC) 10 min after DMSO (IT) resulted in a % MPE of 10 $\pm$ 4 %. In both cases, the mice were tested using the tail-flick test at 15 min following IT injection of the cannabinoid. The data are presented as % MPE plus the standard error with at least 12 mice per dose. *$p < 0.05$ from distilled water/cannabinoid; **$p < 0.01$ from distilled water/cannabinoid.

nor-BNI, and binding data have ruled out may neurotransmitters and neuromodulators as binding to the NalBZH binding site (1). Nor-BNI has high kappa/mu selectivity in a variety of in vivo and in vitro systems (17,22) and has been recently described as having an extremely long duration of action at the kappa receptor in vivo and in vitro (7).

However, some evidence indicates that the blockade of the cannabinoids by nor-BNI differs from the blockade of traditional kappa agonists like U50,488H. The doses of nor-BNI required to block the cannabinoids are higher than those required to increase the ED_{50} of the kappa agonist, U50,488H by 28-fold (22,20,26). Thus, nor-BNI is far more potent in blocking the antinociceptive effects of U50,488H than those of the cannabinoids. The spinal cord plays a predominant role in the antinociceptive effects of U50,488H, and nor-BNI only slowly diffuses within the central nervous system (22). Because the effects of the cannabinoids are both spinal and supraspinally mediated (20), nor-BNI (IT) could be less effective in blocking the effects of the cannabinoids due to a block of only the spinal component of the cannabinoid-induced antinociception, leaving an intact supraspinal component. In support of such a hypothesis is data indicating that nor-BNI (ICV) fails to block the effects of the cannabinoids (ICV) (26,28). Alternatively, we hypothesized that the cannabinoids might interact with a subtype of kappa receptor for which nor-BNI had less affinity. The logical possibilities were the $kappa_2$ (29) or the $kappa_3$ receptors for which nor-BNI has far less affinity. Due to the lack of an appropriate ligand for examination of the $kappa_2$ receptor in vivo, we evaluated the interaction of the cannabinoids with the $kappa_3$ receptor. Our data tend to negate the hypothesis of a $kappa_3$ interaction because NalBZH, which blocks the cannabinoids (IT and ICV), does not appear to block $kappa_3$- receptor-mediated antinociception (14). Thus, our data indicate a possible interaction of the cannabinoids with the $kappa_1$ receptor in the production of antinociception. In addition, NalBZH appears to differ from nor-BNI in that the ICV administration of NalBZH blocks cannabinoid-induced antinociception, while nor-BNI (ICV) fails to block cannabinoids (ICV or IT). Such data might indicate that the administration of nor-BNI (ICV) fails to distribute to the site of cannabinoid binding (although it is clear that ICV nor-BNI will block kappa agonists administered ICV). NalBZH (SC), which blocks the cannabinoids, would certainly distribute to the brain and the spinal cord and may, thus, distribute more readily to cannabinoid binding sites. Alternatively, nor-BNI and NalBZH may interact with subtypes of the $kappa_1$ receptor, although no evidence for such a hypothesis has been generated. Binding studies in mouse spinal cord and the brain indicate that NalBZH and nor-BNI do not directly interact with the cannabinoid receptor. Thus, a direct interaction of

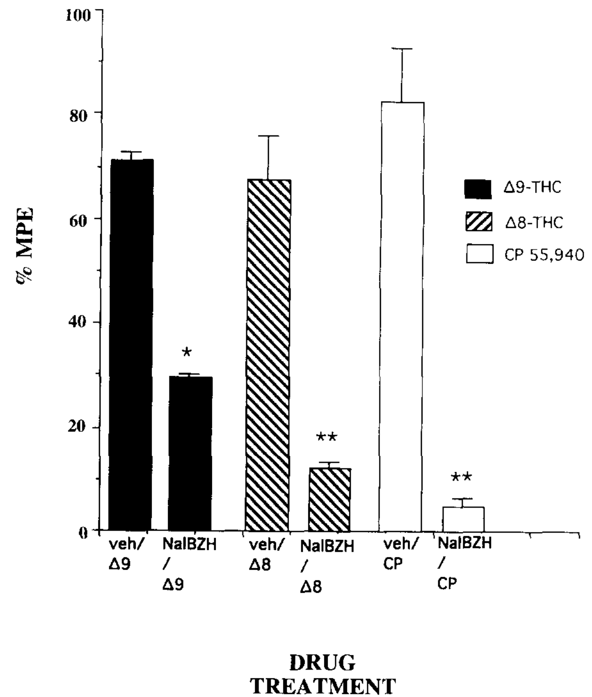

FIG. 4. Naloxone benzoylhydrazone antagonism of ICV-administered cannabinoids. Mice were pretreated SC with either distilled water vehicle or naloxone benzoylhydrazone (NALBZH, 5 mg/kg) 10 min prior to Δ^9-THC (dark bars, 50 μg/mouse, ICV), Δ^8-THC (striped bars, 100 μg/mouse), or CP 55,940 (clear bars, 8 μg/mouse). Injection of distilled water (SC) prior to DMSO (ICV) resulted in a % MPE of 17 $\pm$ 3 %. The mice were tested using the tail-flick test at 15 min following ICV injection of the cannabinoid. The data are presented as % MPE plus the standard error with at least 12 mice per dose. *$p < 0.05$ from distilled water/cannabinoid **$p < 0.01$ from distilled water/cannabinoid.

these kappa ligands with the cannabinoid receptor, which binds ^{3}H-CP,55940, does not appear to occur. Other previous binding studies have shown that kappa opioid binding is unaffected by Δ^9-THC (24) in rat brain and that conversely, cannabinoid binding is unaffected by U50,488H or nor-BNI in rat brain (26).

However, several lines of evidence indicate that NalBZH and the cannabinoids may not interact at a kappa$_1$ receptor. NalBZH has been reported to produce antinociception that is resistant to nor-BNI-induced blockade (22), while the cannabinoids are effectively blocked by nor-BNI. Such a difference, however, may be attributed to the location of the kappa receptor subtypes. The kappa$_3$-mediated antinociceptive effects of NalBZH appear to be supraspinal in location, rather than spinal. The cannabinoids administered IT appear to produce antinociception predominantly at the spinal cord level (19). NalBZH induces tolerance upon chronic administration, but is not crosstolerant to U50,488H (5). The cannabinoids are crosstolerant to both U50,488H and the kappa agonist, CI-977 (21), but not mu and delta receptor-selective agonists. The major dissimilarity between kappa opioid-induced antinociception and cannabinoid-induced antinociception is that kappa opioids produce naloxone-blockable antinociception while cannabinoids are not blocked by very high doses of naloxone (27). NalBZH-induced antinociception is blocked by beta-funaltrexamine (14), while mu and delta antagonists fail to block cannabinoid-induced antinociception (26).

In summary, the antinociceptive effects of several cannabinoids were blocked by NalBZH, as well as nor-BNI, but not the delta antagonist, ICI 174,864, or high doses of naloxone. These data, taken together with the bidirectional cross tolerance of Δ^9-THC to kappa agonists, strongly indicates that the cannabinoids interact with kappa opioid systems in the production of antinociception. Although a possible site of interaction appears to be the kappa$_1$ receptor, the exact nature of such an interaction remains to be evaluated by binding studies to rule out interactions with other kappa receptor subtypes. Further evaluation of the interaction of cannabinoids with kappa opioids may provide leads as to the pathways involved in nociceptive processes.

ACKNOWLEDGEMENT

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