Pergamon

Pharmacology Biochemistry and Behavior, Vol. 49, No. 4, pp. 1093–1100, 1994

0091-3057/94 $6.00 + .00

0091-3057(94)00261-4

Modulation of Free Intracellular Calcium and cAMP by Morphine and Cannabinoids, Alone and in Combination in Mouse Brain and Spinal Cord Synaptosomes

GEORGE PUGH, JR.,[1] SANDRA P. WELCH AND PAM P. BASS

Department of Pharmacology and Toxicology, Medical College of Virginia/Virginia Commonwealth University, Richmond, VA

Received 5 November 1993

PUGH, G., JR., S. P. WELCH AND P. B. BASS. *Modulation of free intracellular calcium and cAMP by morphine and cannabinoids, alone and in combination in mouse brain and spinal cord synaptosomes.* PHARMACOL BIOCHEM BEHAV **49**(4) 1093–1100, 1994.—Changes in $[Ca^{++}]_i$ and cAMP were evaluated as possible mechanisms by which the cannabinoids enhance the antinociception of morphine. The addition of subactive concentrations of Δ9- (THC) and morphine in combination to brain synaptosomes did not result in an enhanced decrease in $[Ca^{++}]_i$; however, this drug combination enhanced decreases in $[Ca^{++}]_i$ in spinal cord synaptosomes. The combination of CP55,940 and morphine produced enhanced decreases in $[Ca^{++}]_i$ in both brain and spinal cord synaptosomes. In brain synaptosomes, the combination of Δ9-THC and morphine produced an additive decrease in cAMP accumulation, whereas no significant change was observed with this combination in the spinal cord. Thus, the difference in the modulation of $[Ca^{++}]_i$ but not cAMP in the brain in vitro may be a predictor of the greater-than-additve antinociceptive effects observe in vivo.

Calcium cAMP Synaptosomes Forskolin Intrathecal Intracerebroventricular Antinociception Cannabinoids

CANNABIS SATIVA (marijuana) is one of the oldest and most widely used drugs in the world (10,22). The major psychoactive ingredient found in cannabis is Δ9-tetrahydrocannabinol (THC). Δ9-THC and other cannabimimetic drugs produce a variety of pharmacologic effects on the central nervous system, among which is the production of antinociception. Although considerable effort has been expended to develop a therapeutically useful cannabinoid, the site of action and mechanism by which the cannabinoids produce many of their pharmacologic effects, including antinociception, remains unknown. Cannabinoid receptors have been cloned (20), and the endogenous ligand for the cannabinoid receptor has been identified (7) and has been shown to produce cannabinoid-like behavioral effects (8). The potent, synthetic cannabinoid, CP55,940, produces many of the behavioral and physiologic effects characteristic of the naturally occurring cannabinoids. In addition, CP55,940 has been instrumental in elucidating the site of action of the cannabinoids. It has been shown that cannabinoid-binding sites are present in the substantia gelatinosa, an area involved with the transmission of pain signals (12). CP55,940-induced antinociception is partially attenuated in spinalized mice (17). Furthermore, Δ9-THC produces the same degree of antinociception in mice that are spinalized as compared with mice that have the spinal cord intact (28). This indicates that the antinociceptive effects of intrathecally administered Δ9-THC are predominantly spinally mediated. These data indicate that the cannabinoids produce antinociception via both spinal and supraspinal mechanisms (17).

The pharmacologic profiles of the cannabinoids and morphine have been extensively studied, and the cannabinoids have been shown to produce some pharmacologic effects similar to the opiates. Some of these similarities include antino-

[1] To whom requests for reprints should be addressed at the Department of Pharmacology and Toxicology, Box 613 MCV Station, Richmond, VA 23298-0613.

ciception (2,3,4,6), hypothermia (35), and attenuation of the morphine abstinence syndrome (13). CP55,940 displays high-affinity binding in the substantia nigra and substantia gelatinosa, which is also the principle binding site of the opiates (9). In the brain Δ9-THC inhibits agonist binding at μ- and δ-opioid receptors (30). Studies have been done to determine the nature of the cannabinoid–opiate interaction in the brain and spinal cord. We have previously shown that pretreatment of mice with various cannabinoids enhances the antinociception of morphine (32). However, the enhancement of morphine-induced antinociception by cannabinoids differs in the brain vs. the spinal cord. Intrathecal administration of subantinociceptive doses of Δ9-THC and morphine in combination results in a greater-than-additive antinociceptive effect, whereas intracerebroventricular (ICV) administration of subantinociceptive doses of both Δ9-THC and morphine produces only an additive antinociceptive effect. These findings indicate a possible interaction between the cannabinoids and opiates in the production of antinociception. To explain the greater-than-additive antinociceptive effect seen after intrathecal administration of the combination of Δ9-THC and morphine, a point of interaction must be shared by both drugs. This common point of interaction may be intracellular second-messenger systems.

Free intracellular calcium ($[Ca^{++}]_i$) and cAMP are two second messengers that may serve as a possible point of interaction whereby the cannabinoid–opiate interaction in the spinal cord produces a greater-than-additive antinociceptive effect. $[Ca^{++}]_i$ has been shown to be modulated by morphine (5,10,36) and cannabinoids (11). Furthermore, the opioids and cannabinoids have both been shown to decrease cAMP accumulation by acting on inhibitory G-proteins (1). Because both drug classes individually decrease cAMP and $[Ca^{++}]_i$, we hypothesized that interactions of the drugs might occur at these two intracellular systems. Thus, our experiments were designed to evaluate the effects of both drugs, alone and in combination, on $[Ca^{++}]_i$ and cyclic AMP accumulation in vitro. We tried to correlate changes in cAMP or $[Ca^{++}]_i$ seen in vitro to the antinociceptive effect seen in vivo after administration of both drugs in combination.

METHODS

Preparation of Synaptosomes

Synaptosomes were prepared as described by McGovern et al. (21). Mouse whole brains were homogenized in 0.32 M sucrose/Kreb's buffer, pH 7.4. The Kreb's buffer was composed of (in mM): NaCl 120; KCl 5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 1; glucose 10; and $NaHCO_3$ 20. The homogenate was centrifuged at 3000 × *g* for 10 min. The resultant supernatant was centrifuged at 11,000 × *g* for 20 min. The resultant pellet was resuspended in 16% Ficoll in sucrose buffer. A 7.5% Ficoll suspension was layered over the 16% Ficoll. The gradient was centrifuged at 40,000 × *g* for 1 h. The synaptosomes banded at the interface of the layers and were removed and resuspended in Kreb's buffer, pH 7.4.

Spinal cord synaptosomes were prepared by the homogenization of 10 mouse spinal cords in 0.32 M sucrose/Kreb's buffer, pH 7.4. The homogenate was centrifuged at 3500 × *g* for 10 min. The resultant supernatant was then centrifuged at 11,000 × *g* for 20 min. The resultant pellet (P-2) was resuspended in Kreb's buffer, pH 7.4, and served as a crude spinal cord synaptosomal preparation. The crude P-2 preparation was used because of the low yield of pure synaptosomes from the spinal cord and the large number of mice that would have been required to generate adequate tissue from the spinal cord to prepare spinal synaptosomes.

Measurement of Cyclic AMP Accumulation

A cyclic AMP assay kit (Diagnostic Products, Los Angeles, CA) was used to determine cAMP accumulation under various conditions. Samples used in determining cAMP accumulation under each condition (basal, forskolin-stimulated, and drug treated + forskolin) were arranged in triplicate. Basal cAMP levels were determined through the addition of a 50 μl aliquot of synaptosomes to 450 μl of buffer containing Tris HCl (100 mM), EDTA (1 mM), $MgSO_4$ (7.5 mM), adenosine triphosphate (0.625 mM), guanosine triphosphate (0.0125 mM), creatinine phosphate (12.5 mM), creatinine phosphokinase (12.5 IU), and theophylline (12.5 mM). Forskolin served as a positive control. The percent increase in cAMP accumulation resulting from forskolin treatment was assessed by the addition of a 50-μl aliquot of synaptosomes to tubes containing 400 μl of buffer and 50 μl forskolin (10 μM). The percent change in cAMP accumulation due to Δ9-THC and morphine, both alone, was evaluated by adding a 50-μl aliquot of synaptosomes to assay tubes containing 50 μl of forskolin (10 μM), 350 μl of buffer, and 50 μl of either δ9-THC or morphine. The percent change in cAMP accumulation attributable to the combination of Δ9-THC and morphine was evaluated by adding a 50-μl aliquot of synaptosomes to assay tubes containing 50 μl forskolin (10 μM), 300 μl of buffer, 50 μl of Δ9-THC, and 50 μl of morphine. The total volume in all assay tubes was 500 μl. All assay tubes were incubated in a water bath for 12 min at 37°C. Tubes containing Δ9-THC and morphine were allowed to incubate for 2 min before the addition of synaptosomes. cAMP was allowed to accumulate during an incubation period of 5 min. The reaction was terminated by boiling the sample tubes. The samples were centrifuged for 10 min at 3000 × *g*. Aliquots of supernatant (100 μl) were taken from each tube and added to tubes that contained ^{3}H-cAMP (100 μl). Twelve standard assay tubes were arranged in duplicate containing cAMP at concentrations of 27, 9, 3, 1, 0.33 and 0.10 pmol/tube. Maximum binding was determined by adding to duplicate tubes 400 μl Tris-EDTA buffer, 100 μl ^{3}H-cAMP, and 100 μl cAMP-dependent protein kinase. Nonspecific binding was determined by adding 500 μl Tris-EDTA buffer and 100 μl ^{3}H-cAMP to duplicate assay tubes. Samples were counted using a Beckman (Palo Alto, CA) scintillation counter and were averaged and plotted to yield a standard curve. All standard curves generated had a correlation coefficient of at least 0.90. The protein concentration was determined via a Bio-Rad assay kit (Richmond, CA). cAMP accumulation was expressed as picomoles per milligram per minute for the 5-min incubation.

Measurement of $[Ca^{++}]_i$

Brain $[Ca^{++}]_i$ levels were determined by preparing synaptosomes on a Ficoll density gradient and spinal cord $[Ca^{++}]_i$ measurements were determined by using the P-2 pellet as described previously. The synaptosomes were in Kreb's buffer, which was composed of (in mM): NaCl 120; KCl 5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 1; glucose 10, and $NaHCO_3$ 20. A 1-ml aliquot of the synaptosomes was incubated with fura-2AM (5 μM, Molecular Probes, Eugene, OR) for 35 min at 37°C. The synaptosomes were then washed to facilitate the removal of extrasynaptosomal fura-2. A 500-μl aliquot of the samples was resuspended in buffer and allowed to equilibrate for 5 min at 37°C in a quartz cuvette before measurement of basal

$[Ca^{++}]_i$. Free intracellular calcium levels were determined using the SPEX Cation Measurement System (Spex Industries, Edison, NJ). Basal and KCl-stimulated (50 mM) levels of $[Ca^{++}]_i$ were determined for each preparation. Drugs were added in 20-μl aliquots to a total volume of 2 ml of synaptosomes (1 mg/ml protein) in each cuvette. The maximum binding of fura-2 (F_{max}) was determined by lysis of the synaptosomes with 0.1% sodium dodecyl sulfate and the addition of a saturating concentration of calcium. The minimum binding of fura-2 to calcium (F_{min}) was determined by adding an excess of EGTA prepared in 3 mM Tris buffer. Free intracellular calcium was determined using the following formula: $[Ca^{++}]_i = K_d \times (F - F_{min})/(F_{max} - F) \times Sf2/Sb2$, where K_d = 224 nM and Sf2 and Sb2 represent fluorescences at unsaturated dye conditions and saturated dye conditions of 380 nm, respectively.

Drugs

All of the drugs were obtained from the National Institute on Drug Abuse, with the exception of CP55,940, which was obtained from Dr. Lawrence Melvin, Pfizer Central Research (Groton, CT). All cannabinoids were prepared in 100% dimethyl sulfoxide (DMSO). Morphine sulfate was prepared in distilled water.

RESULTS

Effects of the Combination of Δ9-THC and Morphine on $[Ca^{++}]_i$ in Brain and Spinal Cord Synaptosomes

An inactive concentration of cannabinoids was used in combination with an inactive concentration of morphine to determine whether this interaction resulted in a greater-than-additive decrease in free intracellular calcium. We determined inactive drug concentrations of Δ9-THC and morphine from concentration-response data taken from brain and spinal cord synaptosomes. In the cannabinoid–opiate interaction studies, we used those drug concentrations that failed significantly to block KCl-induced rises in $[Ca^{++}]_i$. In brain synaptosomes, basal $[Ca^{++}]_i$ was approximately 200 nM. Depolarization of the synaptosomes with 20 μl of 50 mM KCl produced a 62% increase in $[Ca^{++}]_i$, resulting in a concentration increase from 200 nM to approximately 320 nM (Fig. 1, panels A and B). DMSO and dH20 did not attenuate KCl-induced rises in $[Ca^{++}]_i$. KCl-stimulated rises in $[Ca^{++}]_i$ were 58% (± 3.1) in the presence of vehicle controls. The results from our concentration response data using brain synaptosomes showed that Δ9-THC significantly, and in a concentration-related manner, decreased KCl-induced rises in calcium at concentrations ≥ 10^{-5} M (Fig. 1, panel A). Lower concentrations of Δ9-THC were inactive in blocking KCl-induced rises in calcium. The morphine concentration-response data in brain synaptosomes showed that morphine blocked KCl-induced rises in $[Ca^{++}]_i$ at concentrations ≥ 10^{-7} M (Fig. 1, panel B). Morphine failed to attenuate depolarized-induced rises in $[Ca^{++}]_i$ at lower drug concentrations. For the cannabinoid–opiate interaction studies, the addition of the highest subactive concentration of morphine (10^{-8} M) in combination with the highest subactive concentration of Δ9-THC (10^{-6} M) did not result in a greater-than additive decrease in $[Ca^{++}]_i$ in mouse brain synaptosomes. KCl was able to stimulate a 50% (± 5.8) increase in $[Ca^{++}]_i$ in the presence of the combination of Δ9-THC and morphine in the brain (Fig. 2, panel A). This increase was not statistically different from vehicle control-treated synaptosomes.

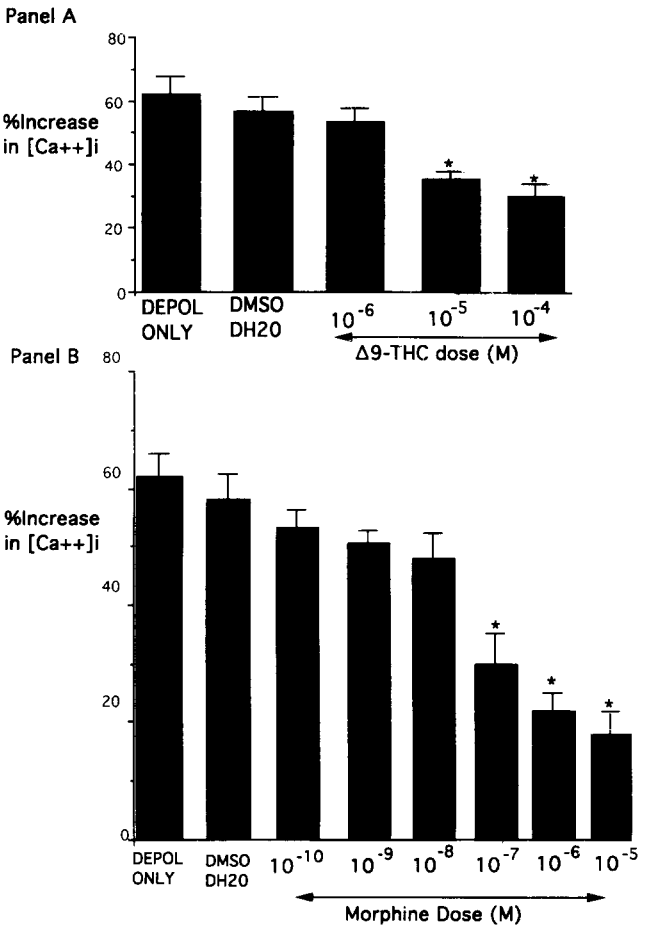

FIG. 1. Concentration-response data showing the effects of Δ9-THC alone and morphine alone on KCl-stimulated increases in $[Ca^{++}]_i$ in the brain. Panel A: Samples were treated with Δ9-THC and then depolarized with 50 mM KCl. Samples treated with Δ9-THC were compared with samples treated with vehicles dimethyl sulfoxide and DH20. $n = 7$. $^*p < 0.05$. Panel B: Synaptosomes were deplarized with 50 mM KCl. Samples treated with morphine were compared with samples treated with vehicles dimethyl sulfoxide and DH20. $n = 7$. $^*p < 0.05$.

When the cannabinoid–opiate interaction was evaluated in the spinal cord, concentration-response data showed that Δ9-THC blocked KCl-induced rises in $[Ca^{++}]_i$ at concentrations ≥ 10^{-5} M and morphine blocked KCl-induced rises in $[Ca^{++}]_i$ at concentrations ≥ 10^{-7} M (Fig. 2, panel B; Fig. 3, panel A). However, in contrast to our results obtained using brain tissue, the combination of subactive concentrations of both Δ9-THC and morphine produced a greater-than-additive effect in blocking depolarized-induced rises in $[Ca^{++}]_i$ in the spinal cord. KCl-stimulated rises in $[Ca^{++}]_i$ of only 28% (± 4.3) were observed in the presence of the combination of Δ9-THC and morphine in the spinal cord (Fig. 3, panel B). This percent change in $[Ca^{++}]_i$ was statistically significant from that observed using the DMSO/dH20 control group.

Effects of the Combination of CP55,940 and Morphine on $[Ca^{++}]_i$ in Brain and Spinal Cord Synaptosomes

Concentration-response curves for both CP55,940 and morphine were used in determining the inactive concentrations

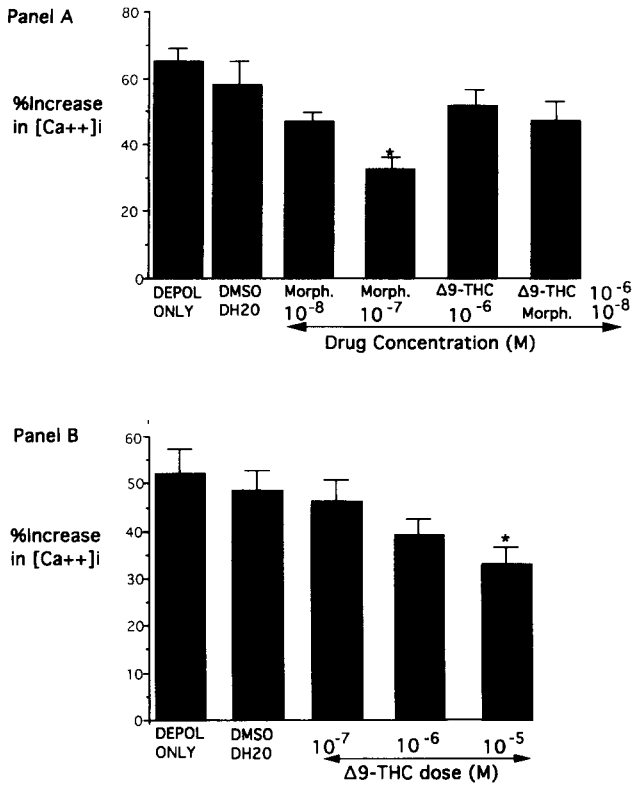

FIG. 2. Data showing the effects of the combination of Δ9-THC and morphine on $[Ca^{++}]_i$ in the brain and the Δ9-THC concentration-response curve in the spinal cord. Panel A: Concentrations of both Δ9-THC and morphine that were inactive in attenuating KCl-stimulated rises in $[Ca^{++}]_i$ were taken from concentration response data (Fig. 1, panels A and B). Δ9-THC was added to brain synaptosomes 2 min before morphine and then depolarzied with 50 mM KCl. The percent change in $[Ca^{++}]_i$ resulting from the Δ9-THC/morphine combination was compared with the percent change in $[Ca^{++}]_i$ produced by vehicle controls, dimethyl sulfoxide and DH20. $n = 5$. $^*p < 0.05$. Panel B: Δ9-THC was added to synaptosomes and then depolarized with 50 mM KCl. Samples treated with Δ9-THC were compared with samples treated with vehicles dimethyl sulfoxide and DH20. $n = 6$. $^*p < 0.05$

of both drugs. The inactive concentrations of CP55,940 and morphine were used in our cannabinoid-opiate interaction studies to evaluate whether this combination resulted in a greater-than-additive decrease in $[Ca^{++}]_i$. In the brain, depolarization with KCl resulted in a 65% (± 6.3) increase in $[Ca^{++}]_i$, which was significantly attenuated by CP55,940 at concentrations ≥ 10^{-8} M. Morphine inhibited KCl-induced rises in $[Ca^{++}]_i$ in brain synaptosomes at drug concentrations ≥ 10^{-7} M (Fig. 1, panel B). In the cannabinoid-opiate interaction studies, the addition of subactive concentrations of the combination of CP55,940 (10^{-10} M) and morphine (10^{-10} M) produced a greater-than-additive decrease in free intracellular calcium in the brain (Fig. 4, panel A). The inhibition produced by this combination was partially reversed in the presence of naloxone. KCl was able to stimulate a 30% (± 4.7) increase in $[Ca^{++}]_i$ in the presence of the combination of CP55,940 and morphine in the brain. The percent change in $[Ca^{++}]_i$ was statistically different from vehicle control treated groups, which showed a 59% (± 5.3) increase in $[Ca^{++}]_i$ in the presence of KCl. Thus, our data showed that neither CP55,940 nor morphine administered alone at subactive concentrations attenuated KCl-induced rises in $[Ca^{++}]_i$. However, when these inactive concentrations are administered in combination, the result is a greater-than-additive effect in blocking KCl-induced rises in $[Ca^{++}]_i$ in the brain.

In spinal cord synaptosomes, depolarization with KCl produced an approximate 50% increase in $[Ca^{++}]_i$, which was significantly attenuated by CP55,940 at concentrations ≥ 10^{-8} M. Morphine blocked KCl-induced rises in $[Ca^{++}]_i$ at concentrations ≥ 10^{-7} M in the spinal cord (Fig. 3, panel A). In the cannabinoid-/opiate interaction study, the addition of the combination of CP55,940 (10^{-10} M) and morphine (10^{-8} M) at subactive concentrations significantly blocked depolarized-induced rises in $[Ca^{++}]_i$ (Fig. 4, panel B). Depolarization with KCl resulted in a 22% (± 4.2) increase in $[Ca^{++}]_i$ in the presence of the combination of CP55,940 and morphine in the spinal cord. This percent change is statistically different from

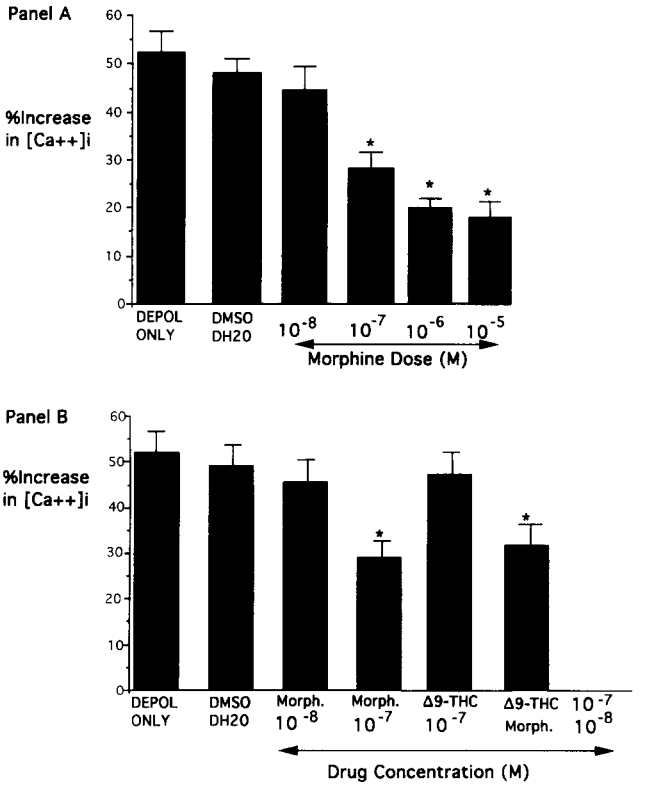

FIG. 3. Data showing the morphine concentration-response curve in the spinal cord and the effects of the Δ9-THC–morphine combination on $[Ca^{++}]_i$ in the spinal cord. Panel A: Morphine was added to synaptosomes and then deplarized with 50 mM KCl. Samples treated with morphine were compared with samples treated with vehicles dimethyl sulfoxide and DH20. $n = 7$. $^*p < 0.05$. Panel B: Concentrations of both Δ9-THC and morphine that were inactive in attenuating KCl-stimulated rises in $[Ca^{++}]_i$ in the spinal cord were taken from concentration response data (Fig. 2, panel B; Fig. 3, Panel A). Δ9-THC was added to spinal cord synaptosomes 2 min before morphine and then depolarized with 50 mM KCl. The percent change in $[Ca^{++}]_i$ resulting from the Δ9-THC–morphine combination was compared with the percent change in $[Ca^{++}]_i$ produced by vehicle controls, dimethyl sulfoxide and DH20. $n = 7$. $^*p < 0.05$.

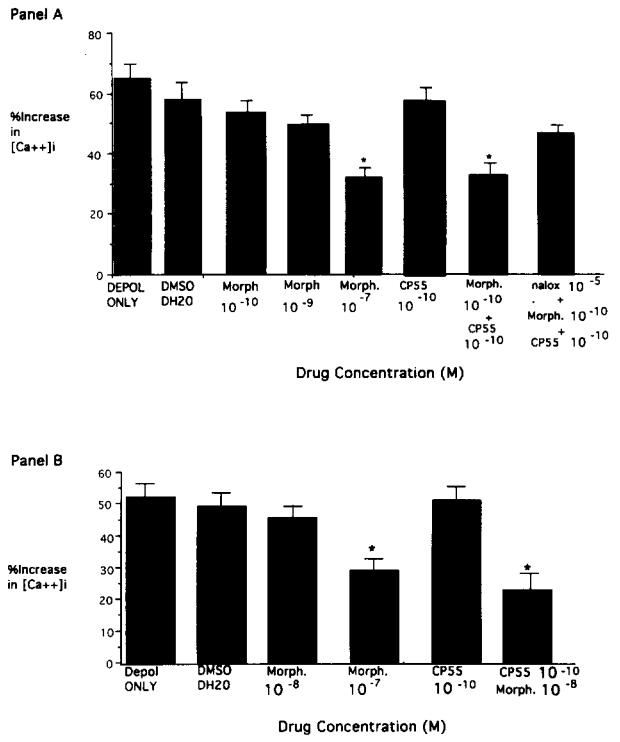

FIG. 4. Effects of the combination of CP55,940 and morphine on KCl-stimulated rises in $[Ca^{++}]_i$ in the brain and spinal cord. Panel A: Concentrations of both CP 55,940 and morphine that were inactive in blocking KCl-stimulated rises in $[Ca^{++}]_i$ alone were added in combination to brain synaptomes. CP55,940 was added 2 min before morphine and then depolarized with 50 mM KCl. $n = 9$. $*p < 0.05$. Panel B: Concentrations of both CP55,940 and morphine that were inactive in blocking KCl-stimulated rises in $[Ca^{++}]_i$ alone were added in combination to spinal cord synaptosomes. CP55,940 was added 2 min before morphine and then depolarized with 50 mM KCl. $n = 12$. $*p < 0.05$.

vehicle control groups that showed a 47% (± 3.3) increase in $[Ca^{++}]_i$ in the presence of KCl.

Effects of the Combination of Δ9-THC and Morphine on cAMP Accumulation in the Brain and Spinal Cord

Basal cAMP levels in brain synaptosomes were approximately 159 pmol/mg per min. Forskolin (10^{-5} M) stimulated a 100% increase in cAMP accumulation in brain synaptosomes, resulting in a change in cAMP levels to approximately 310 pmol/mg per min. Addition of Δ9-THC (10^{-6} M) to brain synaptosomes produced a 10% (± 3.7) decrease over forskolin-stimulated adenylate cyclase (from 310 to 279 pmol/mg per min) (Fig. 5, panel A). Morphine alone (10^{-6} M) produced a 13% (± 4.1) decrease over forskolin-stimulated adenylate cyclase (from 310 to 269.7 pmol/mg per min). The combination of Δ9-THC and morphine (both 10^{-6} M) resulted in a 27% (± 5.3) decrease in cAMP accumulation (Fig. 5, panel A). Thus, the combination of Δ9-THC and morphine produced an additive effect in inhibiting adenylate cyclase in brain synaptosomes. Similar findings were obtained using nanomolar concentrations of Δ9-THC and morphine. Δ9-THC alone (10^{-7} M) produced a 13% (± 5.2) decrease in cAMP accumulation. In the Δ9-THC–morphine interaction studies, our results showed that the effect of the combination of Δ9-THC (10^{-7} M) and morphine (10^{-7} M) on cAMP accumulation in brain synaptosomes was additive (Fig. 5, panel A).

In the spinal cord, basal cAMP levels were approximately 56 pmol/mg per min. Forskolin (10^{-5} M) increased cAMP levels to 102 pmol/mg per min, which was an 82% change in cAMP accumulation. In brain synaptosomes, our results showed that Δ9-THC consistently decreased forskolin-stimulated increases in cAMP accumulation; however, in spinal cord synaptosomes, the effects of Δ9-THC on cAMP accumulation varied depending on the drug concentration used. Δ9-THC (10^{-6} M) produced a slight increase in spinal cord accumulation. Morphine (10^{-6} M) inhibited forskolin-stimulated increases in cAMP accumulation by 15% (± 3.9) in spinal cord synaptosomes (Fig. 5, panel B). The combina-

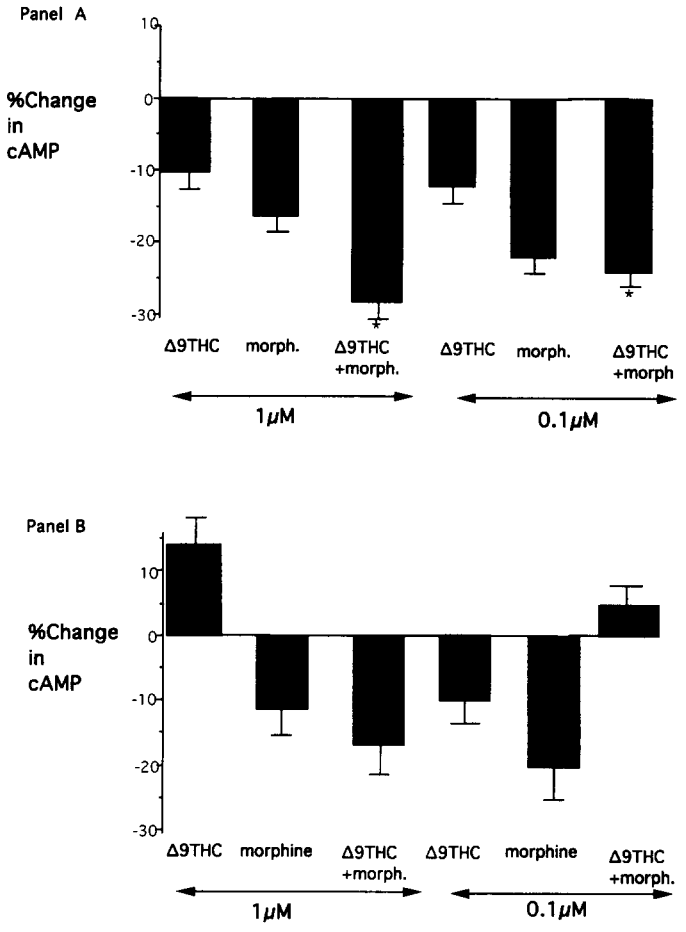

FIG. 5. Effects of the combination of Δ9-THC alone and in combination with morphine on cAMP accumulation in the brain and spinal cord. Panel A: Δ9-THC and morphine were added to brain synaptosomes as described in METHOD. Brain adenylate cyclase was stimulated with forskolin (10^{-5} M). The percent change in cAMP accumulation after administration of the combination of drugs was determined. Panel B: Δ9-THC and morphine were added to spinal cord synaptosomes as described in METHOD. Spinal cord adenylate cyclase was stimulated with forskolin (10^{-5} M). The percent change in cAMP accumulation after administration of the combination of drugs was determined.

tion of both Δ9-THC and morphine (both 10^{-6} M) did not produce a greater-than-additive decrease in cAMP accumulation in the spinal cord (Fig. 5, panel B). Δ9-THC (10^{-7} M) produced a 10% (± 4.5) decrease in cAMP accumulation, and morphine (10^{-7} M) alone produced a 22% (± 5.1) decrease in cAMP accumulation in spinal cord synaptosomes. The combination of both Δ9-THC and morphine did not result in a greater-than-additive decrease in cAMP accumulation (Fig. 5, panel B). The effects of CP55,940 on cAMP accumulation was determined in initial experiments; however, our data showed a lack of correlation between the effects produced by the combination of CP55,940 and morphine in vitro and the greater-than-additive antinociceptive effects observed in vivo.

DISCUSSION

Calcium has an important physiologic role as an intracellular second messenger, as well as a role in modulating pain, either through direct actions or by modulating the activity of endogenous substances. Because both the cannabinoids and opioids alter $[Ca^{++}]_i$, we hypothesize that the greater-than-additive antinociceptive effect seen after IT administration of Δ9-THC and morphine is due to concurrent activity of both drugs on calcium channels enhancing the decrease in $[Ca^{++}]_i$ in the spinal cord. It has been shown that the antinociceptive effects of cannabinoids administered IT were not altered by the IT administration of calcium chloride or a series of calcium modulators of calcium (34). These data indicate that the antinociception produced by the IT administration of the cannabinoids is not sensitive to calcium modulation, and thus may not directly involve calcium modulation. Although calcium does not appear to be directly involved in the antinociceptive effects of the IT-administered cannabinoids, calcium modulation by the cannabinoids may alter opiate antinociception, which may result in an enhanced antinociceptive effect.

In the spinal cord we investigated the effects of the interaction of morphine with Δ9-THC or CP55,940 on $[Ca^{++}]_i$. We were particularly interested in whether the changes in $[Ca^{++}]_i$ correlate with the greater-than-additive antinociceptive effects observed in vivo. Our data showed that concentrations of Δ9-THC and morphine that were inactive in blocking KCl-induced rises in $[Ca^{++}]_i$ when applied alone to spinal cord synaptosomes produced a greater-than-additive effect in blocking KCl-stimulated rises in $[Ca^{++}]_i$ when these same drug concentrations were used in combination. Therefore, we can correlate this finding of an enhanced decrease in $[Ca^{++}]_i$ in spinal cord synaptosomes with our in vivo studies, in which the antinociception of morphine (IT) was enhanced by intrathecal administration of Δ9-THC, 11-OH Δ9-THC, and levonantradol (32). Thus, it appears that calcium plays an important role in modulating the antinociception of the combination of Δ9-THC and morphine in the spinal cord.

The synthetic cannabinoid, CP55,940 has been previously shown to differ from Δ9-THC in that CP55,940 (IT) did not enhance the antinociceptive effects of morphine (IT) (32). Because IT administration of the combination of CP55,940 and morphine does not result in an enhanced antinociceptive effect, we did not expect to see an enhanced decrease in $[Ca^{++}]_i$ in the spinal cord. Our results showed that by using CP55,940 (10^{-10} M) in combination with morphine (10^{-8} M), we were able to significantly attenuate depolarized-induced rises in $[Ca^{++}]_i$ in the spinal cord. Thus, even though the combinations of CP55,940–morphine and Δ9-THC–morphine appear to interact in the modulation of intracellular calcium in the spinal cord, such calcium modulation does not appear to result in an enhanced antinociceptive effects of either drug. Thus, in the spinal cord, interaction of the cannabinoids and the opiates in the modulation of intracellular calcium in vitro is not a predictor of the in vivo antinociceptive effects of the drugs in combination. We can only hypothesize that such a difference in the ability of IT Δ9-THC vs. CP55,940 to modulate IT morphine-induced antinociception may indicate different cannabinoid receptor subtypes or cannabinoid receptor localization within the spinal cord.

It has been shown that increasing $[Ca^{++}]_i$ in the spinal cord produces antinociception, and enhances and extends the duration of action of morphine (18). However, in our experiments we hypothesized that the combination of Δ9-THC and morphine enhanced antinociception by decreasing $[Ca^{++}]_i$. These opposing findings could be due to the neuronal circuitry that is altered by the cannabinoids and opiates. It is possible that the inhibitory actions of both the cannabinoids and morphine are on neurons that contain neuromodulators such as endogenous opioids, which also inhibit neurotransmission. Because calcium is important in neurotransmitter release, we further hypothesized that the inhibition of inhibitory neurons by the cannabinoid-opiate combination elevates $[Ca^{++}]_i$, thereby facilitating the neuronal release of endogenous opioid peptides. It has previously been shown that the increase in calcium in the spinal cord results in antinociception, which is blocked by opiate antagonists, and is therefore hypothesized to result from the release of opioid peptides (33). It has been shown that calcium-induced antinociception in the spinal cord is blocked with *met-enkephalin antisera*, which suggests that calcium (IT) releases endogenous opioids (27).

Intracerebroventricular (ICV) administration of calcium antagonizes the antinociception of morphine (ICV), whereas ICV administration of calcium channel blockers such as verapamil and diltiazem potentiates the antinociception of morphine (ICV) (18,23). Also, the calcium chelator, EGTA, produces antinociceptive effects when administered ICV (26). Therefore, agents that decrease brain $[Ca^{++}]_i$ produce an antinociceptive effect. Thus, we hypothesized that the combination of cannabinoids and morphine produced a greater-than-additive antinociceptive effect in brain by decreasing brain $[Ca^{++}]_i$ levels. This hypothesis is consistent with the finding that calcium (ICV) blocks the antinociceptive effects of cannabinoids (ICV) and that calcium-induced blockade of cannabinoid antinociception appears to result from calcium entry through the "N"-type calcium channel (34). These results are also consistent with reported effects of the cannabinoids observed in vitro (11,19). Our results showed that in the brain, inactive concentrations of CP55,940 and morphine in combination, but not Δ9-THC and morphine in combination, produced a greater-than-additive effect in blocking KCl-stimulated rises in $[Ca^{++}]_i$. Consistent with these observations is the finding that the antinociceptive effect of morphine (ICV) is enhanced by CP55,940 (ICV), but not Δ9-THC (ICV) (32). Thus, in brain, the modulation of calcium by cannabinoids in combination with morphine appears to correlate to the modulation of intracellular calcium by the drugs in combination. Thus, there appears to be a difference in the modulation of the opiates by the cannabinoids depending on whether the interaction is in the brain or spinal cord. As previously discussed, such a difference may represent different cannabinoid subtypes or a localization of cannabinoid-binding sites in the brain vs. the spinal cord. Thus, if different receptor subtypes exist in the brain and spinal cord, then different second-messenger systems could be involved at each site.

We evaluated whether the modulation of cAMP was re-

sponsible for the greater-than-additive antinociceptive effects produced by cannabinoid-morphine interaction in the spinal cord and the brain. In vitro studies using NG108-15 cells have shown that the cannabinoids inhibit adenylate cyclase (14,15) through the guanine nucleotide-binding protein Gi (16). Morphine has also been shown to act via pertussis toxin-sensitive Gi or Go proteins to decrease cellular levels of cAMP (1). Our results using brain or spinal cord synaptosomes show that the combination of Δ9-THC or CP55,940 and morphine do not produce changes in cAMP that are predictive of greater-than-additive effects of the drugs in vivo. It has recently been shown that the antinociceptive effects of the cannabinoids (ICV) are not blocked by agents that increase cAMP levels in the brain, but that conversely, the antinociceptive effects of the cannabinoids IT are blocked by those drugs that increase cAMP in the spinal cord (34). Thus, a differential regulation of cAMP by the cannabinoids in the production of antinociception appears to occur in the brain vs. in spinal cord. However, as previously stated, the antinociceptive effects of the cannabinoids are presumed to occur via their interaction with the cannabinoid receptor. Cannabinoid-induced enhancement of opiate antinociception may occur at a point other than at the cannabinoid receptor. Based on our findings, we conclude that a direct involvement of cAMP is probably not the point of interaction by which the cannabinoids and opiates produce an enhanced antinociceptive effect in the brain or the spinal cord. An indirect involvement of cAMP cannot be ruled out. It is possible that the combination of cannabinoids and morphine produces antinociception by modulating brain cAMP levels, which results in changes in the phosphorylation of calcium channels, thus altering $[Ca^{++}]_i$ levels (25).

In summary, we have attempted to determine the point of interaction between the cannabinoids and the opiates, which results in greater-than-additive antinociceptive effects of the drugs. Our data indicate that in the spinal cord or in the brain, changes in cAMP levels alone cannot account for the greater-than-additive effects of the cannabinoids and morphine. In addition, although calcium modulation in the brain could account for the interaction of the cannabinoids and morphine ICV, spinal calcium modulation does not appear to account for the interaction of the drugs. It is possible that the greater-than-additive interaction of the cannabinoids and opiates is due to an interplay of cAMP with calcium. Alternatively, it is likely that other mechanisms are involved in the cannabinoid-opiate interaction. Some possibilities under investigation include the modulation of potassium channels, which has been documented for both the cannabinoids and opiates (24,31) or the interaction of the drugs at the κ-opiate receptor resulting from the blockade of cannabinoid-induced antinociception by nor-BNI, a κ-antagonist (29). Recently, a second type of cannabinoid receptor has been cloned in the spleen (3). Given that two cannabinoid receptors now exist, it is possible that a distinct receptor subtype exists in spinal cord and differs from that previously shown in the brain.

ACKNOWLEDGEMENTS

This work was supported by National Institute of Drug Abuse Grants DA05274 and DA03672.

REFERENCES

1. Bidaut-Russell, M.; Howlett, A. Opioid and cannabinoid analgetics both inhibit cyclic AMP production in the rat striatum. In: Hamon, M.; Cros, I.; Meunier, J., ed. Advances in the biosciences: Proceedings of the International Narcotics Research Conference. Oxford, England: Pergamon Press; 1988.
2. Bloom, A. S.; Dewey, W. L. A comparison of some pharmacological actions of morphine and Δ9-tetrahydrocannabinol in the mouse. Psychopharmacology 577:243-248; 1978.
3. Bouaboula, M.; Rinaldi, M.; Carayon, P.; Carillon, C.; Delpech, B. Cannabinoid receptor expression in human leukocytes. Eur. J. Biochem. 214:173-180; 1993.
4. Buxbaum, D. M. Analgesic activity of Δ9-tetrahydrocannabinol in the rat and mouse. Psychopharmacology 25:275-280; 1972.
5. Cardenas, H. L.; Ross, D. H. Calcium depletion of synaptosomes after morphine treatment. Br. J. Pharmacol. 57:521-526; 1976.
6. Chesher, G. B.; Dahl, C. J.; Everingham, M.; Jackson, D. M.; Merchant-Williams, H.; Starmer, G. A. The effect of cannabinoids on intestinal motility and their antinociceptive effect in mice. Br. J. Pharmacol. 49:588-594; 1973.
7. Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946-1949; 1992.
8. Fride, E.; Mechoulam, R. Pharmacological activity of the cannabinoid receptor agonist anandamide, a brain constituent. Eur. J. Pharmacol. 231:313-314; 1993.
9. Gamse, R.; Holzer, P.; Lembeck, F. Indirect evidence for presynaptic location of opiate receptors in chemosensitive primary sensory neurones. Arch. Pharmacol. 308:281-285; 1979.
10. Harris, L. S.; Dewey, W. L.; Razdan, R. K. Cannabis: Its chemistry, pharmacology, and toxicology. In: Martin, W. R., ed. Handbook of experimental pharmacology, New York: Springer-Verlag; 1977:371-429.
11. Harris, R. A.; Stokes, J. A. Cannabinoids inhibit calcium uptake by brain synaptosomes. J. Neurochem. 2:443-447; 1982.
12. Herkenham, M.; Lynn, A. B.; Little, M. D.; Johnson, M. R.; Melvin, L. S.; DeCosta, B. R.; Rice, K. C. Cannabinoid receptor localization in the brain. Proc. Natl. Acad. Sci. USA 87:1932-1936; 1990.
13. Hine, B.; Friedman, E.; Torellio, M.; Gershon, S. Morphine-dependent rats: Blockade of precipitated abstinence by tetrahydrocannabinol. Science 187:443-445; 1975.
14. Howlett, A. C. Inhibition of neuroblastoma adenylate cyclase by cannabinoid and nantradol compounds. Life Sci. 35:1803-10; 1984.
15. Howlett, A. C. Cannabinoid inhibition of adenylate cyclase. Biochemistry of the response in neuroblastoma cell membranes. Mol. Pharmacol. 27:429-436; 1985.
16. Howlett, A. C.; Qualy, J. M.; Khachatrian, L. L. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol. Pharmacol. 29:307-313; 1986.
17. Lichtman, A. H.; Martin, B. R. Spinal and supraspinal mechanism of cannabinoid-induced antinociception. J. Pharmacol. Exp. Ther. 258:517-523; 1991.
18. Lux, F.; Welch, S. P.; Brase, D. A.; Dewey, W. L. Interaction of morphine with intrathecally administered calcium and calcium antagonists: Evidence for supraspinal endogenous opioid mediation of calcium-induced antinociception in mice. J. Pharmacol. Exp. Ther. 246:500-507; 1988.
19. Mackie, K.; Hille, B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. USA 89:3825-3829; 1992.
20. Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561-564; 1990.
21. McGovern, S; Maguire, M. E.; Gurd, R. S.; Mahler, H. R.; Moore, W. J. Separation of adrenergic and cholinergic synaptosmes from immature rat brain. FEBS Lett. 31:193-198; 1973.
22. Mechoulam, R. The pharmacohistory of *Cannabis sativa*. In Mechoulam, R., ed., Cannabinoids as Therapeutic Agents. Boca Raton, FL: CRC Press; 1986:1-19.

23. Miranda, H. F.; Pelissier, T.; Sierralta, F. Analgesic efffects of intracerebroven-tricular administration of calcium channel blockers in mice. Gen. Pharmacol. 24: 201-224; 1993.
24. Miura, M.; Belvisi, M. G.; Stretton, C. D.; Yacoub, M. H.; Barnes, P. J. Role of K^+ channels in the modulation of cholinergic neural responses in guinea-pig and human airways. J. Physiol. (Lond) 455:1-15; 1992.
25. Okada, M.; Mine, K.; Fujiwara, M. Relationship of calcium and adenylate cyclase messenger systems in rat brain synaptosomes. Brain Res 501:23-31; 1989.
26. Schmidt, W. K.; Way, E. L. Hyperalgesic effects of divalent cations and antinociceptive effects of a calcium chelator in naive and morphine-dependent mice. J. Pharmacol. Exp. Ther. 212:22-27; 1980.
27. Smith, F. L.; Dewey, W. L. Evidence that endogenous opioids mediate the antinociceptive effects of intrathecally administered calcium in mice. J. Pharmacol. Exp. Ther. 262:995-1003; 1992.
28. Smith, P. B.; Martin, B. R. Spinal mechanisms of Δ9-tetrahydrocannabinol-induced analgesia. Brain Res. 578:8-12; 1992.
29. Smith, P. B. Welch, S. P.; Martin, B. R. Nor-binaltorphimine specifically inhibits Δ9-tetrahydrocannabinol induced antinociception in mice without altering other pharmacological effects. J. Pharmacol. Exp. Ther. (in press).
30. Vaysse, P. J.; Gardner, E. L.; Zukin, R. S. Modulation of rat brain opioid receptors by cannabinoids. J. Pharmacol. Exp. Ther. 241:534-539; 1987.
31. Welch, S. P. Dunlow, D. L. Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: A common mechanism of action? J. Pharmacol. Exp. Ther. 267:390-399; 1993.
32. Welch, S. P.; Sevens, D. L. Antinociceptive activity of intrathecally administered cannabinoids alone, and in combination with morphine, in mice. J. Pharmacol. Exp. Ther. 262:10-18; 1992.
33. Welch, S. P.; Stevens, D. L.; Dewey, W. L. A proposed mechanism of action of the antinociceptive effects of intrathecally administered calcium in the mouse. J. Pharmacol. Exp. Ther. 260: 117-127; 1992.
34. Welch, S. P.; Thomas, C.; Patrick, G. S. Modulation of cannabinoid-induced antinociception following intracerebroventricular vs. intrathecal administration to mice: Possible mechanisms for interaction with morphine. J. Pharmacol. Exp. Ther. (in press).
35. Wilson, R. S.; May, E. L. Analgesic properties of the tetrahydrocannabinols, their metabolites, and analogs. J. Med. Chem. 18: 700;703; 1975.
36. Yamamoto, H.; Harris, R. A.; Loh, H. H.; Way, E. L. Effects of acute and chronic morphine treatments on calcium localization and binding in brain. J. Pharmacol. Exp. Ther. 205:255-264; 1978.