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THE ROLE OF SEVERAL KINASES IN MICE TOLERANT TO Δ^9 –TETRAHYDROCANNABINOL[1]

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Running Title Page

Running Title: Kinases involved in THC-induced tolerance

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Nonstandard abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; % MPE, percent maximum possible effect; CB, cannabinoid receptor; bis, Bisindolymaleimide; LMWH, low molecular weight heparin; MAPK, mitogen-activated protein kinase; TK, tyrosine kinase; db, dibutyryl; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PKC, protein kinase C; β-ARK,β-adrenergic receptor kinase; PI3-K, phosphatidylinositol-3 kinase; GPCR, G-protein-coupled receptor; GRK, G-protein receptor kinase; icv., intracerebroventricular; i.t., intrathecal; s.c., subcutaneously; db-cGMP, dibutyryl cyclic GMP; db-cAMP, dibutyryl cyclic AMP.

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ABSTRACT

It has been suggested that the cannabinoid receptor type 1 (CB1), a G-protein-coupled receptor is internalized following agonist binding and activation of the second messenger pathways. It is proposed that phosphorylation enhances the down-regulation of the CB1 receptor, thus contributing to tolerance. Alterations in phosphorylation of proteins in the signal transduction cascade following CB1receptor activation could also alter tolerance to cannabinoids. We addressed our hypothesis by evaluating the role of several kinases in antinociceptive tolerance to Δ^9-THC. We evaluated PKA using KT5720, a PKA inhibitor; PKC using bisindolylmaleimide I, HCl (bis), a PKC inhibitor; PKG using KT5823, a PKG inhibitor; β-ARK using low molecular weight heparin (LMWH), a β-ARK inhibitor; PI3-K using LY294002, a PI3-K inhibitor and PP1, a src family tyrosine kinase inhibitor. The cAMP analog employed was dibutyryl-cAMP and the cGMP analog employed was dibutyryl-cGMP. Our data indicate that selective kinases may be involved in cannabinoid tolerance. Mice and rats were rendered tolerant to Δ^9-tetrahydrocannabinol (THC). The PKG inhibitor, KT5823, the β-ARK inhibitor, LMWH, the PI3-K inhibitor, LY294002 and inhibition of PKC by bis had no effect on tolerance. Bis, at a higher dose, attenuated the antinociceptive effect of Δ^9-THC in non-tolerant mice. PP1, the src family tyrosine kinase inhibitor and KT5720, the PKA inhibitor, reversed THC-induced tolerance. In addition, inhibition of PKA reversed a decrease in dynorphin release shown to accompany THC-tolerance in rats. These data

support a role for PKA and src tyrosine kinase in phosphorylation events in Δ^9-THC-tolerant mice.

INTRODUCTION

Δ^9-THC is the major psychoactive component in marijuana. There are two known cannabinoid receptors: CB1, primarily in the central nervous system (Felder et al., 1993) and its amino-terminal variant, the CB1A receptor (Shire et al., 1995), and the CB2 receptor found on cells of the immune system (Munro et al., 1993). Δ^9-THC produces psychoactive effects through binding to CB1 receptors (Ledent et al., 1999; Zimmer et al., 1999; Buckley et al., 2000) that have been cloned (Matsuda et al., 1990; Gerard et al., 1991; Munro et al., 1993). CB1 and CB2 receptors have specific antagonists (Rinaldi - Carmona et al., 1994; Rinaldi - Carmona et al., 1998).

CB1 and CB2 receptors are G protein-coupled receptors (GCPRs) linked to a $G_{i/o}$ protein which, when activated, inhibits the activity of adenylyl cyclase (Howlett and Fleming, 1984). Upon agonist binding, the $\beta\gamma$ subunit dissociates from the α subunit of the $G_{i/o}$ -protein (Childers and Deadwyler, 1996). The α subunit inhibits adenylyl cyclase, while the $\beta\gamma$ subunit has been linked to activation of other cellular events such as activation of tyrosine kinases (TKs).

Tolerance develops to the in vivo and in vitro pharmacological effects of cannabinoids (Martin, 1985; Dill and Howlett, 1988; Mason et al., 1999). Receptor down-regulation is a possible mechanism of Δ^9-THC tolerance (Oviedo et al., 1993, Rodriquez de Fonseca et al., 1994). Studies indicate that the cannabinoid receptor is rapidly internalized following binding of an agonist (Hsieh, 1999). However, Abood et

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al., (1993), however, found no alterations in cannabinoid receptor mRNA or protein levels in mouse whole brain homogenates following a chronic injection paradigm. Thus, the effects of long-term administration of Δ^9-THC on receptor down-regulation are unclear. It is proposed that phosphorylation enhances the down-regulation of the CB1 receptor. We hypothesized that modification of intracellular phosphorylations with several kinase inhibitors might attenuate the expression of THC-induced tolerance.

PKA. Acute administration of Δ^9-THC decreases cAMP formation by inhibiting adenylyl cyclase and decreases cAMP-dependent protein kinase (PKA) activity. Conversely, chronic cannabinoid exposure enhances the adenylyl cyclase activity, increases cAMP levels and PKA activity in the same areas that CB1 receptor down-regulation is observed (i.e., cerebellum, striatum and cortex) (Rubino et al., 2000). Thus, the adenylyl cyclase cascade appears to become constitutively active during tolerance.

PKG. The cannabinoid, levonantradol, but not dextronantradol, decreases basal and isoniazid-induced increases in cGMP (Leader et al., 1981). Thus, cannabinoids could alter cyclic-GMP formation [and thus PKG activity] in tolerance expression.

PKC. Δ^9-THC increases the activity of brain protein kinase C (PKC) *in vitro* (Hillard and Auchampach, 1994; De Petrocellis et al., 1995). PKC appears to directly affect CB1 receptors. Phosphorylation of the CB1 receptor with PKC suppresses the modulation of calcium channels by cannabinoids (Garcia et al., 1998). Neurotransmitters that activate PKC, restore the neuronal excitability and synaptic activity inhibited by cannabinoids. We hypothesized that PKC inhibitors might reverse Δ^9-THC tolerance.

TKs. CB1 receptor activation of the βγ subunit of G-proteins can stimulate tyrosine kinases (TK). One target of activation by the βγ subunit is Src tyrosine kinase that has been shown to activate Ras, activating MAP kinase. CB1 and CB2 stimulation increases the activation of MAPK (Rinaldi-Carmona et al., 1998) which becomes tyrosine-phosphorylated in cannabinoid-treated cells, an effect blocked by TK inhibitors (Bouaboula et al., 1995). We tested the Src tyrosine kinase inhibitor PP1 (Daub et al., 1997) in mice for its effects on THC-induced antinociception and reversal of tolerance.

PI3-K. PI3-K is an early intermediate of the $G_{\beta\gamma}$-mediated MAPK signaling pathway (Daub et al., 1997). Therefore, we proposed to block PI3-K and reduce the βγ subunit-mediated tyrosine phosphorylation of MAPK.

GRK. The beta adrenergic receptor desensitization involves rapid protein kinase A (PKA) and G-protein-receptor kinase (GRK) phosphorylation. GRK phosphorylation in turn promotes β-arrestin binding and receptor internalization (Seibold et al., 1998). We inhibited β-adrenergic receptor kinase (β-ARK), a type of GRK, with low molecular weight heparin (LMWH) in order to evaluate the chronic affects of Δ^9-THC following inhibition of β-ARK.

Finally, in order to evaluate a biochemical correlate to behavioral tolerance, we evaluated the release of dynorphin in the spinal cord of THC-tolerant and non-tolerant rats. Tolerance to Δ^9-THC induces a decrease in dynorphin release temporally correlated to decreased antinociception (Mason and Welch, 1999). We hypothesized that reversal

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of behavioral tolerance by a kinase inhibitor might also reverse the decrement in dynorphin release observed in THC-tolerant rats.

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METHODS

Animal model of Δ^9-THC tolerance. All studies using the tail-flick test were performed on male ICR mice. The mice were kept on a 12hour/12hour light/dark cycle Δ^9-THC and received food and water *ad libitum*. In the acute studies mice weighed 16 to 25 g, in chronic studies mice weighed 25 to 34 g upon testing. Mice were rendered tolerant to Δ^9-THC over seven days. The mice received twice daily s.c. injections of Δ^9-THC (20mg/kg) for six days and on day seven just received the morning dose. On the morning of day 8 mice were challenged with an ED80 dose (i.t.) of Δ^9-THC for determination of tolerance. Rats were used for the spinal cord release of dynorphin in order to obtain sufficient cerebrospinal fluid for testing. Male Sprague Dawley rats, weighing between 350 and 400 g, obtained from Harlan Laboratories were housed in plastic cages, two rats per cage, and maintained on a fixed 12 - h light cycle at a temperature of 22 $\pm 2^{O}$C. Water and food (Harlan Rat Chow) were provided *ad libitum.* Rats were rendered tolerant to Δ^9-THC using the doses and time course as for mice and were also challenged on day 8 with the ED80 dose of Δ^9-THC (i.t.) for tolerance determination.

Intrathecal (i.t.) injections. I.t. injections were performed in mice following the protocol of Hylden and Wilcox (1983). Unaesthetized mice were injected with 5 ul of drug between the L5 and L6 area of the spinal cord with a 30-gauge needle. In studies using rats, the pentobarbital-anesthetized rats were placed in stereotaxis and an incision

made on the atlanto - occipito membrane to expose the cisterna magna. A catheter of PE - 10 polyethylene tubing was inserted through the exposed cisternal cavity, caudally, into the subarachinoid space of the spinal cord. The catheter contained an artificial cerebrospinal fluid, composed of 125 mM Na+; 2.6 mM K+; 0.9 mM Mg2+; 1.3 mM Ca2+; 122.7 mM Cl-; 21.0 mM HOC-; 2.4 mM HOP2-; 0.5 mg/ml bovine serum albumen, bacitracin (30 mg/ml), 0.01% Triton X and effervesced with 95% O_2 and 5% CO_2. Positioned as such, the catheter extended caudally 8.5 cm passing through the thoracolumbar region to an area just above the sacral enlargement. Following catheter implantation, animals were allowed to acclimate approximately 30 - min on a heating pad. Following acclimation, baseline tail-flick latency was assessed. Only animals exhibiting normal tail-flick response to noxious stimuli, greater than 1.5 -sec, but less than 4 - sec latency, were used to avoid use of any rat with spinal cord damage. Drug or vehicle (1:1:18; ethanol:emulphor:saline) was administered in a 20-µl bolus over 1-min using a peristaltic pump.

Measurement of dynorphin. Measurement of dynorphin A - (1-17) was accomplished using a dynorphin A - (1-17)- specific radioimmunoassay kit obtained from Peninsula Laboratories, Inc. The reconstituted samples were analyzed in duplicate. The manufacturer reports cross - reactivity of dynorphin A- (1-17) antibody as 100% versus dynorphin - (1-24), a parent compound, and less than 2% versus smaller peptide fragments. We found no cross - reactivity of the antibody to dynorphin A - (1-8), dynorphin A - (1-13), dynorphin B, , AEA, or morphine. Only the linear portion of the

radioimmunoassay standard curve, between 0.1 and 64 pg/ml of the standard dynorphin peptide, was used to calculate dynorphin concentration. The CSF from individual rats was analyzed for dynorphin concentrations using at least 6 rats per test group. The rats were evaluated in the tail-flick test prior to the removal of CSF for testing. Thus, the behavioral effects of each rat can be compared to the dynorphin levels in that individual rat's spinal fluid.

The tail-flick test. Mice and rats were tested for antinociception by the tail-flick procedure (D'Amour and Smith, 1941). Reaction times of 1.5 to 4 sec were employed for the control, while a time of 10 sec was used for the cutoff to prevent tissue damage. Antinociception was quantified as the percent maximum possible effect (% MPE) formula:

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

(Harris and Pierson, 1964). % MPE values were calculated for each animal, using at least 6 animals per dose for which mean effect and standard error of the mean (SEM) were calculated for each dose. At least 3 doses of each test drug or combination of drugs were used to generate dose-response curves.

Materials. Doses for all drugs used were predetermined in naïve animals using the maximal dose without toxicity. Time points were determined in naïve animals to ascertain the point at which each drug had its peak effect. 100% dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Company (St. Louis, MO). KT5720 purchased from Calbiochem (La Jolla, CA) was prepared in 100% DMSO and was

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injected i.t. at a dose of 2.7μg/mouse 15 min prior to drug or vehicle (i.t.). The tail-flick test was then conducted 15 min following the second injection. KT5823 purchased from Calbiochem (La Jolla, CA) was prepared in 100% DMSO and injected i.t. at a dose of 2.5μg/mouse 15 min prior to drug or vehicle (i.t.). The tail-flick test was then conducted 15 min following the second injection. Dibutyryl-cAMP (10μg/mouse) and dibutyryl-cGMP (5μg/mouse) were purchased from Calbiochem (La Jolla, CA) and were prepared in distilled water (dH2O) and injected i.t 15 min prior to the i.t. injection of drug or vehicle. Fifteen-min later the tail-flick test was conducted. Δ^9-THC was obtained from the National Institute of Drug Abuse (NIDA) and was prepared in 100% DMSO for acute tests, and 1:1:18 [1 part ethyl alcohol purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY): 1 part emulphor: 18 parts 0.9% normal saline purchased from Baxter (Deerfield, IL)] for tolerance studies. LY294002 was purchased from Biomol (Plymouth Meeting, PA) and was prepared in 100% DMSO and injected i.t. 15 min prior to drug or vehicle, (i.t.). The tail-flick test was then conducted 15 min following the second injection. Bisindolymaleimide I, HCl purchased from Calbiochem (La Jolla, CA) was prepared in dH2O and injected i.t. (5μg/mouse) or (0.5μg/mouse) 15 min prior to drug or vehicle (i.t.). The tail-flick test was then conducted 15 min following the second injection. Low molecular weight heparin (LMWH) was purchased from Sigma Chemical Corporation (St. Louis, MO) and was prepared in dH2O and injected i.t. (30μg/mouse) 15 min preceding the i.t. injection of drug or vehicle. The tail-flick test was then conducted 15 min following the second injection. PP1 purchased

from Alexis was prepared in 100% DMSO and injected i.t. 10 min prior to the i.t. injection of drug or vehicle, with the administration of the tail-flick test 15 min after the second i.t. injection.

Statistical analysis. Analysis of variance (ANOVA) was used to determine significant differences between control and treatment animal groups followed by Dunnett's t-test. These calculations were performed using StatView, version 512+ (BrainPower, Inc. Agoura Hills, CA). P values of less than 0.05 were deemed significant. Parallelism of the dose-response curves was determined by the methods of Tallarida and Murray (1987). Potency ratios were determined using the methods of Colquhoun (1997).

RESULTS

The i.t. administration of KT5720, a protein kinase A (PKA) inhibitor, at a dose of 2.7μg/mouse in 100% DMSO vehicle (i.t.) significantly ($p<0.05$) reversed Δ^9-THC antinociceptive tolerance in a dose-dependent manner, as determined by the tail flick test. There was a leftward shift of the dose response curve. The ED50 in the Δ^9-THC-tolerant mice was shifted from 80μg/mouse (95% confidence limits from 62 to 102) to 8.6 μg/mouse (95% confidence limits from 4.7 to 16) in the KT5720-treated mice. The lines were parallel and the potency ratio was 9.3 (Figure 1).

The protein kinase G (PKG) inhibitor, KT5823, at a dose of 2.5μg/mouse in 100% DMSO vehicle (i.t.) had no effect on Δ^9-THC antinociceptive tolerance. [2% MPE in the tolerant mice compared to 7% in the tolerant animals treated with KT5823 (Figure 2 A)]. A higher dose of KT5823 (5 μg/mouse) had no greater effect. Pretreatment with 10 μg/mouse produced lethality.

Bisindolylmaleimide I, HCl (bis), a protein kinase C (PKC) inhibitor, at a dose of 0.5μg/mouse administered i.t. did not affect the antinociceptive tolerance in mice. The % MPEs in the tolerant groups treated with bis compared to vehicle-treated mice were not significantly different (20 ± 15 vs. 14 ± 6.0, respectively) (Figure 2B). At an increased dose of 5μg/mouse, there was not a significant shift in the ED50 values of the tolerant mice treated with bis compared to tolerant mice treated with vehicle (41 [32 - 51]) versus 80 [50 - 129] (Figure 3), although clearly there was a trend toward a

rightward shift in the dose-effect curve. The dose-effect curves for mice pretreated with bis versus those pretreated with vehicle were not parallel in the tolerant mice. Interestingly, in the non-tolerant mice, there was an attenuation of the antinociceptive effect of Δ^9-THC. There was a significant ($p<0.05$) rightward shift in the dose-response curve of Δ^9-THC. The ED50 was shifted from 7 (5 - 11) in the vehicle-treated non-tolerant animal to 26 (16 - 45) in the bis-treated non-tolerant animal. The dose-effect curves were parallel and the potency ratio was 3.6.

LY294002, a phosphatidylinositol-3-kinase inhibitor, administered i.t. in 100% DMSO vehicle at a dose of 0.1μg/mouse, did not significantly alter Δ^9-THC antinociceptive tolerance in mice (Figure 2C). The LY294002-treated tolerant mice had a % MPE of 10 ± 10 compared to the tolerant vehicle treated mice who had a % MPE of 2 ± 1. At higher doses LY294002 produced an antinociceptive effect that confounded use for tolerance reversal studies.

Low molecular weight heparin (LMWH), which inhibits beta adrenergic receptor kinase (β-ARK), at a dose of 30 μg/mouse administered i.t., did not affect the antinociceptive tolerance in the mice. The % MPE in the Δ^9-THC -tolerant group treated with LMWH (5 ± 2) was not significantly different than the vehicle-treated THC-tolerant group (14 ± 6.0) (Figure 2D).

We also evaluated PP1, a Src family tyrosine kinase inhibitor. Since the βγ subunit of the cannabinoid receptor interacts with tyrosine kinase to activate MAPK, we wanted to determine what would happen if this pathway was disrupted. At a dose of

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0.0001μg/mouse, in 100% DMSO vehicle administered i.t., PP1 significantly ($p<0.05$) reversed Δ^9-THC antinociceptive tolerance in mice. The 0.0001μg/mouse dose was shown to be inactive (% MPE 4 ± 1) in the tail-flick test in naïve mice, and in the non-tolerant group the % MPE [44 ± 20] did not differ from that of vehicle-pretreated mice [% MPE 45 ± 19] (Figure 4). At doses of 0.001μg/mouse and higher, PP1 shows a variable antinociceptive affect that confounded studies at the higher doses.

We also evaluated enhancement of tolerance. Since PKA inhibition reversed tolerance, we evaluated the ability of a cAMP analog to enhance tolerance. The challenge dose of Δ^9-THC was increased to 100μg/mouse i.t. to get approximately 50% MPE in the tolerant mice. Dibutyryl cyclic-GMP at 5μg/mouse administered i.t. did not significantly enhance tolerance, (36 ± 20 % MPE in the tolerant animals compared with 46 ± 23 % MPE in the db-cGMP-treated animals) (data not shown). Higher doses (30 and 50 μg/mouse) of db-cGMP had intrinsic antinociceptive effects. Dibutyryl cyclic-AMP at doses of 10μg/mouse – 50 μg/mouse administered i.t. also did not enhance Δ^9-THC antinociceptive tolerance, (55 ± 14 % MPE in the tolerant animals compared to 46 ± 23 % MPE in the db-cAMP-treated animals for the 10 μg/mouse group). Db-cAMP did not produce intrinsic antinociception at the doses tested.

A study was performed to address the hypothesis that KT5720 reversal of tolerance might restore dynorphin release by 300μg/rat Δ^9-THC to levels observed in non-tolerant rats. Doses and time points for KT5720 administration and tail-flick testing were as those in the mouse. Figure 5 Panel A indicates THC-stimulated dynorphin

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release was significantly depressed in THC-tolerant rats [25 ±7 pg/ml in non-THC-tolerant rats vs. 6 ±3 pg/ml in THC-tolerant rats]. Administration of KT5720 prior to Δ^9-THC did not alter dynorphin release in non-tolerant rats [22 ± 6 pg/ml] compared to the non-tolerant Δ^9-THC only group. However, KT 5720 significantly reversed the Δ^9-THC -stimulated decrease in dynorphin observed in Δ^9-THC -tolerant rats [levels of dynorphin were raised to 32 ±8 pg/ml]. Panel B is the behavioral response (tail-flick test) in the same rats. The rats were tolerant to Δ^9-THC as indicated by "a". KT 5720 significantly reversed tolerance as indicated by "b". Thus, as the behavioral response returned to non-tolerant levels, the release of dynorphin increased to non-tolerant levels.

DISCUSSION

We sought to address the role of various kinases in Δ^9-THC antinociceptive tolerance. We evaluated kinases that were downstream from the cannabinoid receptor (PKA, PI3-K, TK), that may interact directly with the receptor (PKA, β-ARK, PKC) and others that act in different pathways (PKC, PKG). When a ligand binds to a GPCR, as the cannabinoid receptor, there is a decreased affinity between the α and βγ subunits of the G-protein and they separate from one another. In the acute model of Δ^9-THC exposure, the α subunit will produce a decrease in adenylate cyclase, decreases cAMP and decreases PKA activation. There is also an associated opening of low voltage potassium channels leading to an efflux of potassium and a modulation of calcium channels leading to decreased calcium conductance. In animals chronically treated with THC, there is a compensatory increase in adenylate cyclase, cAMP and PKA activation.

The intrathecal administration of the protein kinase A inhibitor, KT5720, reversed the antinociceptive tolerance to Δ^9-THC in both mice and rats. Of significance, inhibition of PKA also reversed a biochemical correlate of tolerance, namely dynorphin release. Thus, our data indicate that protein kinase A plays a role in the mechanism of Δ^9-THC antinociceptive tolerance. The role of PKA in THC-induced tolerance is unknown. However, several possibilities for the site of action of PKA exist. PKA could be responsible for phosphorylating the CB1 receptor upon binding of a ligand to the receptor. PKA also could be increasing potassium conductance through phosphorylation

of the potassium channel, causing the cell to become hyperpolarized. Other possible roles of PKA in THC-mediated tolerance include the possibility that PKA is rapidly and continuously phosphorylating the CB1 receptor if and when it is down-regulated into the cytosol in tolerant animals. The CB1 receptor appears to be rapidly internalized upon exposure to cannabinoids (Hsieh et al., 1999). Once the cell is "tolerant", we propose that there will be a compensatory increase in production of PKA. Higher levels of PKA could be responsible for a continuous phosphorylation of the CB1 receptor while in the cytosol. This continued phosphorylation might facilitate the receptor down-regulation. Upon inhibition of PKA by KT5720, the receptor would in theory no longer remain phosphorylated, and could therefore be recycled to the membrane where it would be capable of binding to the ligand again. If the phosphorylation of the receptor was maintained, and the receptor remained down-regulated, eventually it would be degraded, requiring mRNA for new protein synthesis.

Since PKA inhibition reverses cannabinoid antinociceptive tolerance, we hypothesized that a cAMP analog might enhance tolerance. In a non-tolerant neuron exposed to cannabinoids, cAMP is decreased, but in the presence of forskolin, which increases cAMP, or Cl-cAMP, a cAMP analog, antinociception is attenuated (Cook et al., 1995). However, dibutyryl-cAMP did not enhance tolerance. Perhaps we were not able to increase levels of c-AMP to a level consistent with enhancement of tolerance.

We also evaluated kinases involved in the $G_{\beta\gamma}$-mediated signaling pathway. PI-3 kinase and tyrosine kinase work downstream from the $\beta\gamma$ subunit of the GPCR, these

kinases are generally associated with growth and differentiation. With the membrane destabilizing activity of cannabinoids and release of free arachidonic acid, such kinases might play a role in antinociception. LY294002, (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), is a specific phosphatidylinositol 3-kinase (PI3-K) inhibitor. PI3-K is an enzyme implicated in growth factor signal transduction by associating with receptor and non-receptor tyrosine kinases (Vlahos et al., 1994). Pertussis-sensitive GPCR's and TK's and converge or share a common pathway upstream from the activation of MAPK. Our goal was to determine if by blocking a kinase or kinases in the pathway leading to MAPK activation, we could reverse tolerance. Upon blocking PI3-K, tolerance was not affected. One caveat in our results was that we could not use higher doses of LY294002 due to its intrinsic analgetic effects. Thus, our results with PI3-K blockade are inconclusive. However, the blockade of the Src-family tyrosine kinase with PP1 reversed tolerance. Thus, by blocking a tyrosine kinase, we may be inhibiting downstream actions of the βγ subunit. The βγ subunit may be necessary to maintain a tolerant state by activation of MAPK. Further studies need to be conducted looking the role of the βγ subunit and tyrosine kinases and their role in central cannabinoid effects.

In addition to kinases downstream to the α and βγ subunits, we also tested other kinases involved in a variety of cellular processes. β-ARK is known to phosphorylate the beta2-adrenergic receptor and is a potential candidate for phosphorylation of the cannabinoid receptor prior to internalization. Hsieh et al. (1999) noted that the CB1 receptor is internalized following a pathway grossly similar to the one used by the beta2-

adrenergic receptor. If such were the case, blocking β-ARK with LMWH might be expected to prevent receptor phosphorylation and possibly desensitization or down-regulation and reverse tolerance. Since LMWH did not reverse tolerance, it appears that the cannabinoid receptor is may not be phosphorylated by β-ARK. However, due to solubility and toxicity issues we may not have been able to increase the dose of LMWH to levels needed to block β-ARK.

PKC may act directly on the CB1 receptor and/or downstream from the receptor. It has been shown that cannabinoids increase brain protein kinase C activity in vitro (Hillard and Auchampach, 1994). Our data indicate that using two different doses of the PKC inhibitor did not alter tolerance to cannabinoids. However, we did observe at the higher dose of PKC inhibitor, that the effects of Δ^9-THC in non-tolerant animals were significantly attenuated. Hillard and Auchampach (1994) showed that cannabinoids increase the levels of PKC in rat brain and that these increased levels are responsible for reestablishing neuronal excitability. Since inhibiting PKC attenuates the effects of cannabinoid-induced antinociception, it is likely that increased levels of PKC may be at least partially responsible for cannabinoid-induced antinociception.

In summary, the data presented indicate that by inhibiting PKA and src tyrosine kinase, Δ^9-THC antinociceptive tolerance can be reversed. It seems likely that these two kinases work independently of one another. The other kinase inhibitors for PKG, PKC, PI3-K and β-ARK, did not alter tolerance at the doses testable. One has to take these negative data in the context of the possibility that the drug did not achieve high enough

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levels in the whole animal to inhibit the kinases. Thus, positive data as with PKA and src TK indicate potential sites for Δ^9-THC modulation, while negative data remain rather inconclusive. However, the higher dose of PKC inhibitor was shown to attenuate the antinociceptive effects of Δ^9-THC in the non-tolerant mice which indicates that the inhibitor very likely reaches its site of action at a concentration that is active. However, although negative results must be interpreted with caution, positive results also need to be interpreted with caution as to the site of action of the drugs used. Our data indicate that the PKA and tyrosine kinase inhibition have prominent roles in tolerance to cannabinoids. The drugs used to inhibit such kinases (KT5720 and PP1, respectively) are the most selective drugs available. Such inhibitors likely act on PKA and TK at various sites intracellularly. It is intriguing that reversal of tolerance is a rapid process and occurs using drugs that do not alter the acute effects of THC. A similar effect has been shown for KT5720-induced reversal of tolerance to morphine (Bernstein and Welch, 1998). It takes several days to develop tolerance. The ability to reverse tolerance so rapidly is surprising and opens the door to a plethora of questions to be answered as to the plasticity of the neuronal system during the tolerance process. Certainly, the ability to reverse tolerance to any drug has profound clinical implications. A future direction would be to evaluate the phosphorylation state of the receptor. If PKA were responsible for the initial desensitization or maintaining the down-regulated state of the receptor, we would expect to see the receptor in the phosphorylated state in tolerant animals. We would also expect to see a dephosphorylated receptor in tissue that had been treated with

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the PKA inhibitor immediately prior to harvest. Additionally, and of greater clinical importance, will be to determine the duration of the reversal of tolerance with the kinase inhibitors.

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Footnotes

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LEGENDS FOR FIGURES

Figure 1. The reversal of Δ^9-THC -induced tolerance by the PKA inhibitor, KT5720. The graph shows a significant rightward shift in the Δ^9-THC (i.t.) dose-effect curve indicating the development of tolerance in mice pretreated chronically with Δ^9-THC as described in the "Methods" versus those pretreated chronically with vehicle. Also indicated is a significant leftward shift of the dose-effect curve of Δ^9-THC (i.t.) in mice chronically treated with Δ^9-THC and pretreated with KT5720 (2.7 µg/mouse, icv.) prior to testing. KT5720 did not shift the Δ^9-THC dose-effect curve in chronic vehicle-pretreated (non-tolerant) mice. Each point represents an "n" of at least 8 mice per dose. The effects of icv. administration of 100% DMSO (5 µl/mouse) alone in the tolerant and non-tolerant mice were less than 20% MPE. Non-tolerant mice receiving vehicle (icv DMSO) + Δ^9-THC (i.t.) = "white circles"; non-tolerant mice receiving KT5720 (icv.) + Δ^9-THC (i.t.) = "black circles"; tolerant mice receiving vehicle (icv DMSO) + Δ^9-THC (i.t.) = "white squares"; tolerant mice receiving KT5720 (icv.) + Δ^9-THC (i.t.) = "black squares".

Figure 2. The lack of reversal of Δ^9-THC -induced tolerance by various inhibitors, KT5823, bis, LY,and LMWH. Mice were pretreated chronically with Δ^9-THC or vehicle as described in the "Methods". Mice were evaluated for tolerance to Δ^9-THC via i.t. administration of Δ^9-THC (i.t., 20 µg/mouse) + DMSO (icv.). **Panel A**: Mice were tested for reversal of tolerance via a challenge dose of Δ^9-THC (20 µg/mouse, i.t.) + KT5823 (2.5 µg/mouse, icv.). **Panel B:** Alternatively, mice were tested for reversal of

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tolerance via a challenge dose of Δ^9-THC (20 µg/mouse, i.t.) + bis (0.5 µg/mouse, icv.). **Panel C:** Alternatively, mice were tested for reversal of tolerance via a challenge dose of Δ^9-THC (20 µg/mouse, i.t.) + LY (20 µg/mouse, icv.). **Panel D**: Alternatively, mice were tested for reversal of tolerance via a challenge dose of Δ^9-THC (20 µg/mouse, i.t.) + LMWH (30 µg/mouse, icv.). The effects of icv. administration of 100% DMSO (5 µl/mouse) + veh (i.t.) [veh + veh] alone in the tolerant and non-tolerant mice were less than 20% MPE in all cases (Panels A-D). [Inhibitor (icv.) + veh (i.t.)] produced less than 25% MPE in both tolerant and non-tolerant mice in all cases (Panels A- D). In non-tolerant mice [veh + Δ^9-THC] produced nearly 100% MPE and was significantly reduced to less than 15% MPE in Δ^9-THC-tolerant mice [* <0.01, Panels A- D]. [Inhibitor + Δ^9-THC] in non-tolerant mice was 98% MPE or greater and was reduced to less than 22% MPE in tolerant mice [*p<0.01]. Thus, all the inhibitors in Figure 2 were inactive in that they failed to reverse tolerance to Δ^9-THC. Non-tolerant mice = black bars; Tolerant mice = white bars.

Figure 3. The attenuation of Δ^9-THC -induced antinociception by the PKC inhibitor, bis (5 µg/mouse, icv.). The graph shows a significant rightward shift in the Δ^9-THC (i.t.) dose-effect curve indicating the development of tolerance in mice pretreated chronically with Δ^9-THC as described in the "Methods" versus those pretreated chronically with vehicle. Also indicated is a significant leftward shift of the dose-effect curve of Δ^9-THC (i.t.) in mice chronically treated with vehicle and pretreated with bis (5 µg/mouse, icv.) prior to testing. Bis did not shift the Δ^9-THC dose-effect curve significantly in

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chronically Δ^9-THC -pretreated (tolerant) mice, although there is a trend toward attenuation of the effects of Δ^9-THC. Each point represents an "n" of at least 8 mice per dose. The effects of icv. administration of 100% DMSO (5 µl/mouse) alone in the tolerant and non-tolerant mice were less than 20% MPE. . Non-tolerant mice receiving vehicle (icv DMSO) + Δ^9-THC (i.t.) = "white circles"; non-tolerant mice receiving bis (5 µg/mouse, icv.) + Δ^9-THC (i.t.) = "black circles"; tolerant mice receiving vehicle (icv DMSO) + Δ^9-THC (i.t.) = "white squares"; tolerant mice receiving bis (5 µg/mouse, icv.) + Δ^9-THC (i.t.) = "black squares".

Figure 4. The reversal of Δ^9-THC -induced tolerance by the TK inhibitor, PP1. Mice were pretreated chronically with Δ^9-THC or vehicle as described in the "Methods". Mice were evaluated for tolerance to Δ^9-THC via i.t. administration of Δ^9-THC (20 µg/mouse, i.t) + DMSO (icv.). Alternatively, mice were tested for reversal of tolerance via a challenge dose of Δ^9-THC (20 µg/mouse, i.t.) + PP1 (0.0001 µg/mouse, icv.). Each point represents an "n" of at least 8 mice per dose. The effects of icv. administration of 100% DMSO (5 µl/mouse) + veh (i.t.) [veh + veh] alone in the tolerant and non-tolerant mice were less than 30% MPE. [PP1 (icv.) + veh (i.t.)] produced less than 30% MPE in both tolerant and non-tolerant mice. In non-tolerant mice [veh + Δ^9-THC] produced 100% MPE and was significantly reduced to 2% MPE in Δ^9-THC -tolerant mice [* <0.01]. [PP1 + Δ^9-THC] in non-tolerant mice was 93% MPE and remained 93% MPE in tolerant mice. That is, PP1 reversed tolerance to Δ^9-THC. Non-tolerant mice = black bars; Tolerant mice = white bars.

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Figure 5. Reversal of tolerance to Δ^9-THC by KT5720 in the rat restores dynorphin A release.

A study was performed to address the hypothesis that KT5720 reversal of tolerance might restore dynorphin release by 300µg/rat Δ^9-THC to levels observed in non-tolerant rats. Doses and time points for KT5720 (KT) administration and tail-flick testing were as those in the mouse. Panel A indicates dynorphin release (pg/ml ±S.E.M.). Panel B is the behavioral response (tail-flick test, average % MPE ± S.E.M.) in the same rats. As the behavioral response returned to non-tolerant levels, the release of dynorphin increased to non-tolerant levels. N= 6 rats/group. Panel A: "a" indicates $p<0.05$ from "THC-non-tolerant" group; "b" indicates $p<0.05$ from "THC-tolerant" group. Panel B: "a" indicates $p<0.05$ from "THC-non-tolerant" group; "b" indicates $p<0.05$ from "THC-tolerant" group.

INDEX TERMS

JPET Fast Forward. Published on February 11, 2003 as DOI: 10.1124/jpet.102.044446
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36
Δ^9-THC

PKA

PKC

Tyrosine kinase

Antinociception

Tolerance

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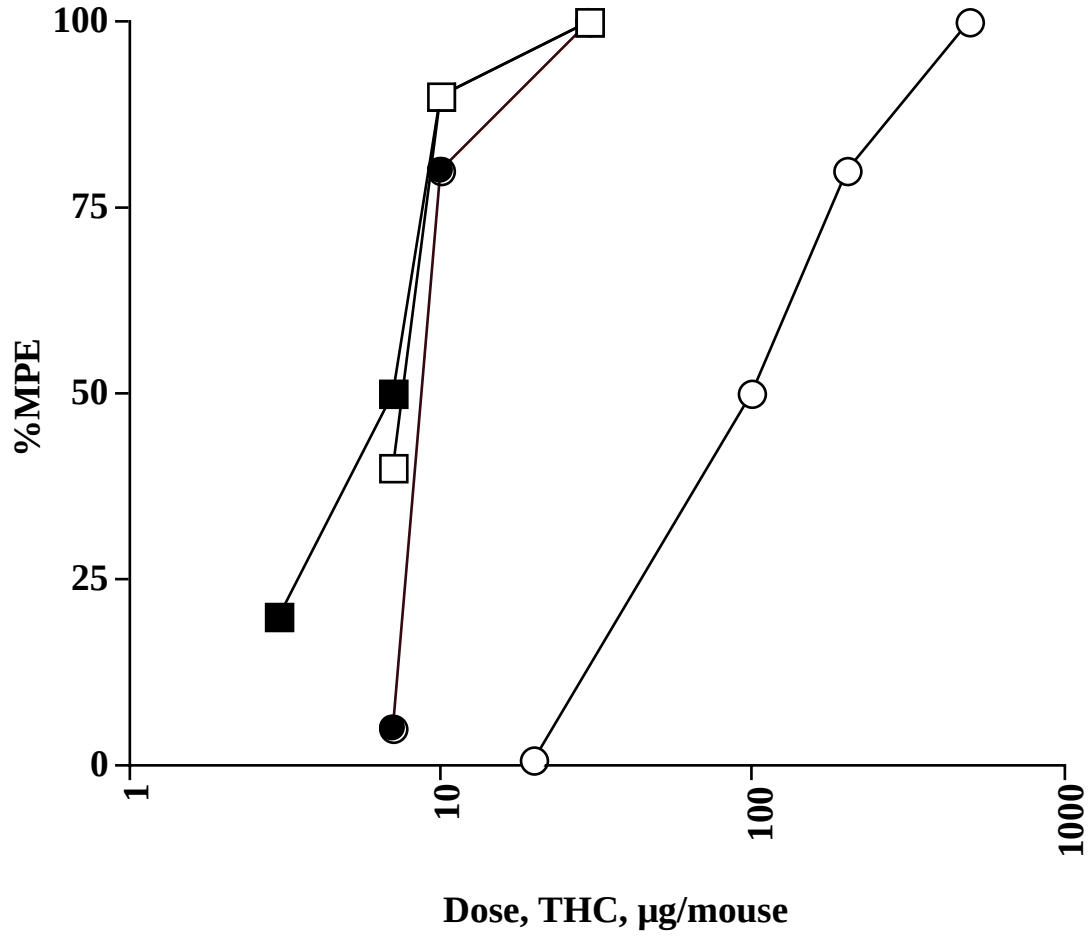

JPET, Figure 1
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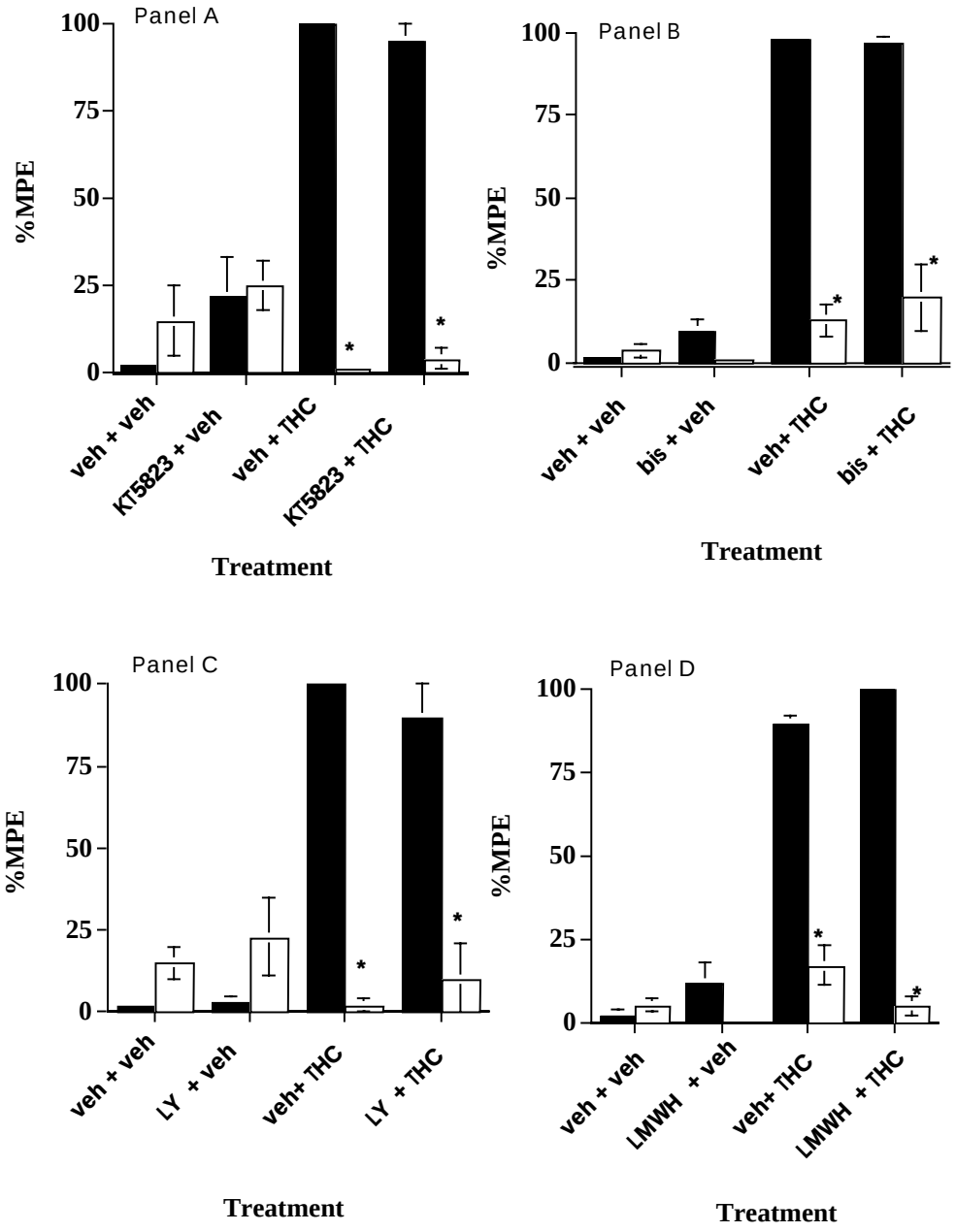

JPET, Figure 2
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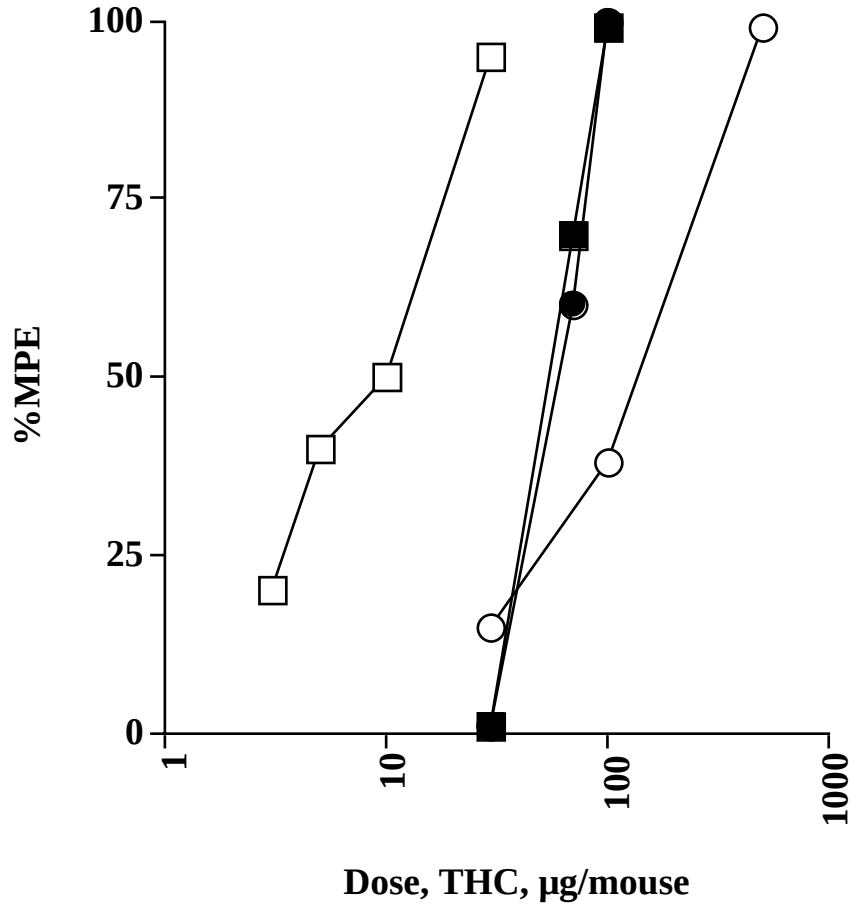

JPET, Figure 3
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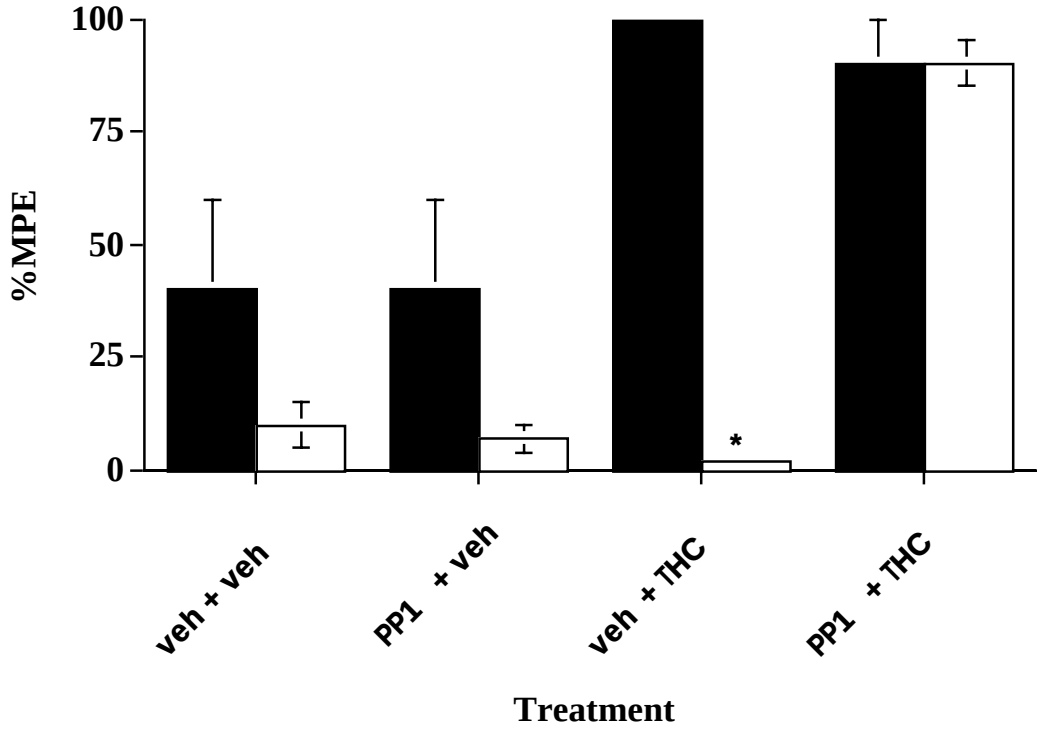

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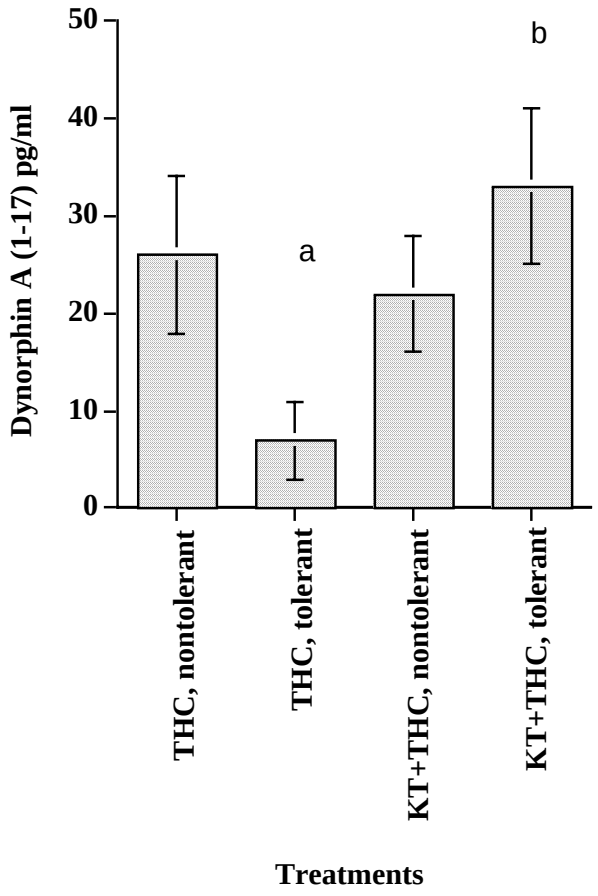
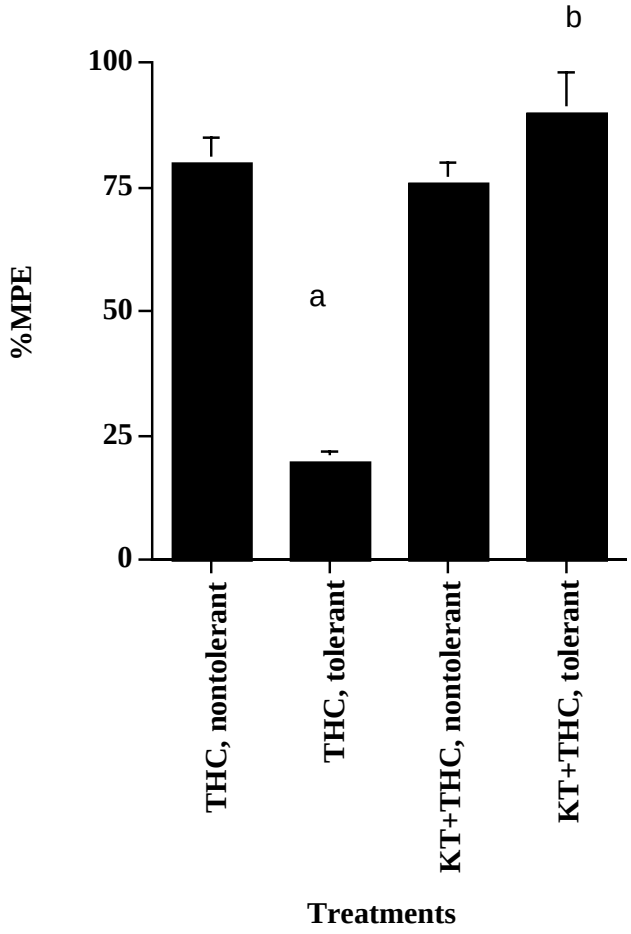
Panel A.
Dynorphin release by 300µg/rat THC (i.t.)



Panel B.
%MPE following THC 300 µg/rat (i.t.)



a = p<0.05 from non-tolerant

b = p<0.05 from tolerant

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