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The Roles of Several Kinases in Mice Tolerant to Delta-9-Tetrahydrocannabinol

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

AA	Arachadonic acid
AC	Adenylyl cyclase
ACTH	adrencorticotropic hormone
ANA	Anandamide
β-AR	Beta-adrenergic receptor
β-ARK	Beta-adrenergic receptor kinase
Bmax	Binding capacity of a receptor
Bis	Bisindolylmaleimede I, HCl or 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide, HCl
CB1	Cannabinoid 1 receptor
CB2	Cannabinoid 2 receptor
cAMP	Cyclic adenosine 3' - 5' monophosphate
DAG	Diacylglycerol
dibutyryl-cAMP	adenonsine 3',5'-cyclic monophosphate, N^6,$O^{2'}$-dibutyryl-,sodium salt
dibutyryl-cGMP	Guanosine 3',5'-cyclic monophosphate, N^2,2'-O-dibutyryl-, sodium salt
DMSO	Dimethyl sulfoxide
ED50	Dose that produces effect in 50% of the population
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
5-HT	5-hydroxytryptamine
IGF	Insulin-like growth factor
inj.	injection
IP3	Inositol-triphosophate
i.t.	Intrathecal
KD	Affinity
kg	Kilogram
LMWH	Low molecular weight heparin
LY294002	[2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]
MAPK	Mitogen-activated protein kinase
μg	Microgram
mg	Milligram
%MPE	Percent maximum possible effect
nal-BZH	Naloxone Benzoylhydrazone
nor-BNI	17, 17'-bis(Cyclopropylmethyl)-6,6',7,7'-tetradehydro-4,5,4',5'-diepoxy-6,6'-(imino)[7,7'-bimorphinan]-3,3',14,14'-tetrol dihydrochloride or nor-Binaltorphimine dihydrochloride
PIP2	Phosphatidylinositol-bisphosphate
PGE1	Prostaglandin E1
PI-3K	Phosphatidylinositol 3-kinase

PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA2	Phospholipase A2
PLC	Phospholipase C
PP1	4-amino-5-(4-methylphhenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PTX	Pertussis toxin
RTK	Receptor tyrosine kinase
SEM	Standard error of the mean
Δ^9-THC	Δ^9-Tetrahydrocannabinol
TK	Tyrosine kinase
tol	Δ^9-THC tolerant group of mice
non-tol	Δ^9-THC non-tolerant group of mice

Abstract

THE ROLE OF SEVERAL KINASES IN MICE TOLERANT TO Δ^9-TETRAHYDROCANNABINOL.

Matthew C. Lee

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Director: Sandra P. Welch, Ph.D., Department of Pharmacology and Toxicology

It has been suggested that the CB1 G-protein-coupled receptor is internalized following agonist binding and activation of the second messenger pathways. The process of desensitization and resensitization is intimately involved with receptor internalization. Phosphorylation alters tolerance to cannabinoids thus contributing to tolerance. It is proposed that phosphorylation enhances the down-regulation of the CB1 receptor. These findings led us to look at which kinase(s) may be involved in cannabinoid tolerance. We therefore hypothesize that by preventing phosphorylation of the CB1 receptor, we may reverse tolerance. We evaluated our hypothesis by testing the role of several kinases in tolerance: protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), Beta Adrenergic Receptor Kinase (β-ARK), Phosphatidylinositol 3-kinase (PI3K) and the src family tyrosine kinase. We also looked at cAMP and cGMP analogs. 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; PI3K using LY294002, a PI3K inhibitor and PP1 a src family tyrosine kinase inhibitor. The cAMP analog was dibutyryl-cAMP and the cGMP analog was dibutyryl-cGMP. ICR mice were rendered tolerant to Δ^9-tetrahydrocannabinol (Δ^9-THC) by administering injections of 20mg/kg Δ^9-THC s.c. every 12 hours for 6.5 days. The mice were subsequently challenged 24 hours later with an ED80 of Δ^9-THC at 20μg/mouse (i.t.). Antinociception was measured by the tail-flick test, %MPE's and ED50's were calculated. The PKG inhibitor, KT5823, showed no significant change in %MPE. The β-ARK inhibitor, LMWH, showed no significant change in the %MPE. The PI3K inhibitor, LY294002, showed no significant change in the %MPE. Inhibition of PKC, by bis had no effect on tolerance, but at a higher dose attenuated the antinociceptive effect of Δ^9-THC in non-tolerant mice. PP1, the src family tyrosine kinase inhibitor, reversed tolerance. KT5720, the PKA inhibitor reversed Δ^9-THC tolerance. These data support a role for PKA and tyrosine kinase in phosphorylation events in THC tolerant mice. (Supported by NIDA grants K02DA00186 and P50DA05274).

Introduction

Background. Cannabis is one of the most controversial drugs of our time, even though it has been used for both recreational and medicinal purposes for centuries. In the United states, ranking only behind alcohol and tobacco, cannabis is one of the most commonly abused drugs. The use of *Cannabis Sativa*, also known as Indian Hemp, dates back over 12,000 years (Abel 1979). Its uses include that of making clothes and rope by the ancient Chinese and Greeks. It was cultivated in Jamestown, Virginia for its fiber early in American history (Grinspoon and Bakalar 1993). Medicinally it has long been used in China, India, the Middle East, South America and South Africa. The earliest references to its medicinal uses date back to 2700 BC (Grinspoon and Bakalar 1993). Uses in ancient China included treatment for constipation, malaria, rheumatic pains and female disorders. Around 2000 to 1400 BC in India the euphoric properties were discovered, and cannabis was recommended for reducing fevers, producing sleep, stimulating the appetite, relieving headaches and curing venereal diseases (Mechoulam and Feigenbaum 1987).

It was not until 1839 that cannabis was introduced into western medicine. During this time cannabis was found to be a very safe drug (Snyder 1971, Lemberger 1984). In further experiments Snyder showed that high doses did not kill animals. He noted the therapeutic effects including anticonvulsant action, analgesia, antianxiety and antiemetic

properties humans. These reports made cannabis an acceptable form of medicine in England and other European countries. The medicinal use of cannabis waned in the United States and Europe at the turn twentieth century, due to the development of synthetic medicines (Adams and Martin 1996).

The rising fear of cannabis use in the United states began in the 1920's, and the use of cannabis was abolished by the enaction of the Marijuana Tax act in 1937 (Musto 1987). Marijuana is a Mexican term that refers to cannabis leaves or other crude plant material. Despite the legal measures in the United States, cannabis was a major drug of abuse during the 1960s with the peak use in the late 1970s and early 1980s. Since then, use declined to a low in 1992, and has seemed to be on the rise since (Johnson et al. 1995).

Cannabinoids are psychoactive compounds that are secreted as a resin from the flowering tops and leaves of the *Cannabis Sativa*, subspecies indica, plant. Cannabinoids are found in the highest concentration in the flowering tops of the plant, followed by the leaves. Small amounts of cannabinoids are found in the stem and roots, but none in the seeds (Adams and Martin 1996). More than 400 compounds are synthesized by the plant, more than 60 of which are related to Δ^9-THC. Δ^9-THC is the prototypical cannabinoid and major psychoactive component in marijuana. It is rapidly metabolized to 11-hydroxy-Δ^9-THC, *in vivo*. Most of the other cannabinoids are inactive or weakly active (Abood et al. 1996). Δ^9-THC is a non-crystalline, waxy-liquid substance at room temperature. The pharmacological activity of Δ^9-THC is stereoselective, with the (-)-trans isomer having

6-100 times more potency than the (+)-trans isomer, depending on the pharmacological test (Dewey et al. 1984).

The Cannabinoid Receptors. It was initially thought that due to the lipophilic nature of Δ^9-THC and the central depressant effects, cannabinoids medicated their actions through the disruption of membrane ordering, similar to the mechanism of general anesthetics (Paton and Pertwee 1972; Lawrence and Gill 1975). Four chemically distinct subclasses of compounds exist with pharmacological and behavioral similarities to Δ^9-THC, including compounds with three rings, such as Δ^9-THC, bicyclic compounds (CP-55940), aminoalkylindoles (Win 55,212) and eicosanoids (anandamide). The enantioselectivity of Δ^9-THC reinforced the notion that some cannabinoid actions may act through a receptor (Mechoulam et al. 1988). Definitive evidence for a specific cannabinoid receptor became apparent when it was cloned (Matsuda et al. 1990). A clone isolated from a rat brain library had homology with other receptors that interacted with G proteins in the cell membrane. None of the traditional agonists of G proteins bound to this receptor clone. An identification breakthrough occurred with the discovery that the mRNA distribution of the receptor clone paralleled that of the cannabinoid receptor. Confirmation of the identity of the clone occurred when adenylyl cyclase was inhibited upon exposure to CP 55,940 and Δ9-THC in cells transfected with this clone. Adenylyl cyclase in non-transfected cells did not respond to cannabinoids. CP 55,940 is a non-classical, bicyclic cannabinoid with a 4-25 times greater potency than Δ^9-THC. Studies show that CP 55,940 cross-generalized in rat and monkey drug discrimination, and cross

tolerance developed between the two compounds (Gold et al. 1992, Pertwee et al. 1993). The human cannabinoid receptor was subsequently cloned and found to have almost identical homology to the rat receptor (Gerard et al. 1991). The cannabinoid receptor is linked to a G_i protein which, when activated by phosphorylation, inhibits the activity of adenylyl cyclase (Howlett and Fleming 1984). Adenylyl cyclase then cannot catalyze the conversion of ATP to the second messenger cyclic AMP (cAMP) (Howlett et al. 1986). It has been shown electrophysiologically that cannabinoids inhibit an omega conotoxin sensitive, high voltage-activated N-type calcium channel (Caufield and Brown 1992, Mackie and Hille 1992). Cannabanoids also have been reported to enhance the low-voltage A-type potassium channels (Deadwyler et al. 1993).

There are two cannabinoid receptors, CB1 which is located primarily in the brain with the highest concentration in the substantia nigra pars reticula, globus palidus, and molecular layer of the cerebellum (Felder et al. 1993) and also to a lesser extent in the periphery in human peripheral blood lymphocytes (Bouaboula et al. 1993) and mouse spleen cells (Kaminski et al. 1993); an amino-terminal variant of CB1 receptor, the CB1A has also been discovered in brain and several peripheral tissues (Shire et al. 1995); and CB2 which has been found on splenic macrophages (Munro et al. 1993). The role or the receptors in the spleen remains elusive. Even though the CB1 and CB2 receptors only share 40% homology, Δ^9-THC and CP 55,940 demonstrate similar binding affinity for both receptor subtypes. It was been demonstrated by Lement et al. (1999) through studying CB1 receptor knockout mice that the main pharmacological responses to Δ^9-THC, as well as the addictive properties of cannabinoids, are mostly mediated by the CB1

receptor. It was also suggested that the CB1 receptors are required for the development of physical dependence or to obtain a complete manifestation of the somatic signs of opiate withdrawal. Since cannabinoids are highly lipid soluble they have been reported to partition into cell membranes and alter the activity of a number of membrane-associated enzymes, providing an alternate non-receptor-mediated mechanism of action (Makriyannis et al.). In CHO cells transfected and not transfected with the CB1 receptor, cannabinoid agonist-activated arachidonic acid release and an increase in intracellular calcium, as well as an inhibition of arachidonic acid uptake, suggest a CB1 receptor independent mode of action (Felder et al. 1992). The receptor-independent actions require higher concentrations of agonist to mediate their effects where as the receptor dependent actions only require nanomolar concentrations of agonist (Felder et al. 1994).

Cannabinoid receptor signaling. The CB1 and CB2 receptors are heterotrimeric G-protein coupled receptors of the inhibitory subtype, composed of α, β and γ subunits. Upon a ligand binding to the receptor, affinity of the α subunit for GTP increases and affinity for GDP decreases, so the α subunit is able to bind and hydrolyze GTP. Also the binding of GTP decreases the affinity for the $\beta\gamma$ subunit, which then disassociates from the α subunit (Childers and Deadwyler 1996). The α subunit acts by inhibiting adenylyl cyclase, which then decreases the amount of cAMP which results in an increase potassium "A" current (Childers and Deadwyler 1996). CB1 and CB2 stimulation induces the inhibition of adenylate cyclase and the activation of MAPK (Rinaldi-Carmona et al. 1998). CB1 activation has recently been shown to induce

immediate-early gene expression such as *Krox 24* through a cAMP-independent pathway (Bouaboula et al. 1995) This central cannabinoid receptor mediated effect was blocked by the CB1 receptor antagonist SR141716A (Rinaldi-Carmona et al. 1996). Since *Krox 24* induction is receptor-mediated, but cAMP-independent there must be another mechanism of CB1 receptor-mediated effects besides through cAMP. From the observation of two independent signaling pathways, several conclusions have emerged: MAPK activation is mediated through the $G_{\beta\gamma}$ subunit and adenylate cyclase responses are mediated through the $G_{\alpha i}$ subunit (Rinaldi-Carmona et al. 1998). The tyrosine kinase inhibitor, herbimycin A, has been shown to inhibit the induction of *Krox 24*, suggesting that a protein TK may be involved in the Gi stimulation of the gene expression (Bouaboula et al. 1995). MAPKs, which become tyrosine phosphorylated in cannabinoid-treated cells, can also be blocked by TK inhibitors, again suggesting that TK may play a role in CB1 receptor-mediated effects (Bouaboula et al. 1995).

MAPKs can be activated in response to both GPCR or receptor-tyrosine kinase (RTK) stimulation. Insulin receptors belong to the RTK family, and their stimulation by insulin has been shown to activate MAPKs in CHO cells (Bouaboula et al. 1997). SR141716A completely inhibited insulin-activated MAPK in CHO cells transfected with the CB1 receptor, giving rise to the fact that CB1 activation is required for insulin activation of MAPK (Bouaboula et al. 1997). This effect could not be extended to other RTKs (Bouaboula et al. 1997). It has also been reported that activation of PI-3K upstream to the MAPKs is required for PTX-sensitive activation of MAPK by GPCR (Bouaboula et al. 1997). Wortmannin, a PI-3K inhibitor, which is an early

intermediate of the $G_{\beta\gamma}$ mediated MAPK signaling pathway, inhibitor resulted in significant inhibition of both CP-55,940 and insulin-mediated MAPK activation (Bouaboula et al. 1997). In contrast to the classical EGF receptor paradigm, stimulation of the MAPK pathway by the IGF1 RTK also requires the participation of $G_{\beta\gamma}$ subunits derived from PTX-sensitive G proteins. Similar to the GPCR pathway, the IGF signal can be blocked by either PTX or by an inhibitor of $G_{\beta\gamma}$ subunit-mediated signaling (Luttrell et al. 1995). Thus upstream to Ras there is a convergence or common pathway of the IGF class of RTK with the GPCR signaling pathway.

There have been other systems of cannabinoid receptor signal transduction pathways proposed. Some studies show that cannabinoids might activate the inositol phospholipid pathway. This pathway involves the receptor activating a G protein that in turn activates phospholipase C. Phospholipase C cleaves phosphatidylinositol-bisphosphate (PIP2) into inositol-triphosophate (IP3)and diacylglycerol (DAG). DAG activates PKC, and IP3 triggers calcium release from cellular stores. (Chaudry et al. 1988). Other studies show no effect on phospholipase C activity, and the generation of IP3 (Felder et al. 1992).

An abundance of evidence is available that shows cannabinoids have effects on the biophysical properties of lipid bilayers and biological membranes. Specifically, the psychoactive cannabinoids, Δ^9-THC and 11-OH-Δ^9-THC, decrease the brain synaptosomal membrane lipid ordering, while the cannabinoids lacking psychoactive properties (cannabidiol and cannabinol) do not decrease ordering. This may play a part in

the mechanism of cannabinoids non-receptor mediated effects, including AA release, increase in intracellular calcium, and inhibition of AA uptake. It is thought the AA release may be due in part to inhibition of free fatty acid reacylation. It is possible PLA2 is being activated while simultaneously acyl-transferase is being inhibited. PLA2 and AA release have been shown to depend on an increase in intracellular calcium levels (Felder et al. 1992).

Endogenous Ligands. The first endogenous ligand for the cannabinoid receptor anandamide (ANA) was isolated in 1992 by Devane et. al. It has a greater affinity for the CB1 receptor than the CB2. It has both similarities and dissimilarities to the classic cannabinoids. Similarities exist in the interaction with a G_i protein, inhibition of adenylyl cyclase and modulation of c-AMP levels in cells (Felder et al. 1993, Vogel et al. 1993) and inhibition of N-type calcium channels (Felder et al. 1993, Mackie et al. 1993). ANA is 4 to 20 times less potent than Δ^9-THC and has a shorter duration of action (Smith et al. 1994). ANA is structurally dissimilar to Δ^9-THC and it also differs from the classic cannabinoids in that it is only a partial agonist at the N-type calcium channels, whereas the cannabinoids are full agonists. ANA appears to display binding and functional properties similar to previously studied cannabinoid agonist, including the ability to induce both receptor mediated and non-receptor mediated signaling (Felder et al. 1993). Anandamide produced similar pharmacological effects to THC, such as antinociception, catalepsy, hypomotility and hypothermia, (Fride and Mechoulam 1993). ANA has also been shown to be cross tolerant to Δ^9-THC (Pertwee et al. 1993; Welch et al. 1995). It is

interesting to note that Δ^9-THC, CP-55,940 and WIN 55,212-2 affected memory in rats where anandamide and cannabidiol did not (Lichtman et al. 1995). Is this due to subtypes of receptors with different specificities or interaction with NMDA receptors? The answers to these questions remain unanswered.

The Cannabinoid Receptor Antagonist and Inverse Agonist. The antagonist SR141716A, has a high affinity for the CB1 receptor, but not the CB2 receptor (Rinaldi-Carmona et. al. 1994). *In vitro*, it antagonized both cannabinoid-induced inhibition of adenylyl cyclase activity in rat brain membranes and mouse vas deferens contractions; *in vivo* it antagonized behavioral effects of cannabinoid agonists (Rinaldi-Carmona et al. 1994). SR141716A also antagonized the discriminative stimulus effects of both Δ^9-THC in rats and rhesus monkeys (Wiley et al. 1995) and CP 55,940 in rats (Wiley et al. 1995). It has also been demonstrated that SR141716A not only functions as an antagonist of cannabinoid mediated effects, but also an inverse agonist (Bouaboula et al. 1997). The inverse agonist property was shown through two different signaling pathways, $G_{\beta\gamma}$-mediated MAPK and $G_{i\alpha}$-mediated AC responses. SR141716A reversed the increase in MAPK activation through the $G_{\beta\gamma}$ pathway. SR141716A also prevented the AC responses through the $G_{i\alpha}$ pathway. In cells transfected with CB1 receptors, SR141716A will block insulin stimulated MAPK induction, but in wild type cells SR141716A had no effect on insulin activated MAPK (Bouaboula et al. 1997). This effect seems only to be related to the PTX-sensitive RTKs including the insulin and IGF1 receptors, not other RTKs.

General Effects of Δ^9-THC. The effects of THC are due to its direct cellular actions on peripheral tissues, and due to its high lipophilicity it is able to cross the blood brain barriers and cause CNS affects. Behavioral effects are characterized at low doses as a mixture of depressant and stimulatory effects and at higher doses as predominantly CNS depression (Dewey 1986). The depressant affects of cannabinoids produce hyperreflexia. Cannabinoids generally cause a reduction in spontaneous locomotor activity (Little et al. 1988) and a decrease in response rates with different reinforcement schedules (Carney et al. 1979). Cannabinoids also impair learning and memory in rodents (Carlini et al., 1970) and non-human primates (Ferraro and Grilly 1973). Other affects that have been shown in the mouse include hypothermia, immobility (catalepsy) and antinociception (Martin 1985).

Cannabinoid-induced antinociception. Several possible transduction mechanisms for cannabinoid-induced spinal antinociception have been proposed, including modulation of adenylate cyclase, calcium, potassium, prostaglandins, and opioids. Cannabinoids have been shown to interact with c-AMP, potassium channels and opioids, but not calcium, in the production of antinociception in the spinal cord (Welch et al. 1995). The administration of pertussis toxin, which prevents G_i proteins from interacting with receptors, significantly attenuates or blocks the antinociception produced by cannabinoids i.t. This action suggests the mechanism for antinociception is through the GPCR. Forskolin pretreatment, which stimulates adenylyl cyclase, there by increasing cAMP levels, significantly reduced the antinociception produced by Δ^9-THC administered i.t. However the administration of dibutyryl cAMP failed to alter the antinociception

produced by cannabinoids i.t., but Cl-cAMP a stable analog of cAMP, significantly blocked the antinociceptive effects produced by i.t. administered cannabinoids. Thus, in the spinal cord, the antinociceptive effects of the cannabinoids seem to be produced *via* a G_i or G_o protein in conjunction with the modulation of cAMP because the antinociceptive effects of i.t. cannabinoids are blocked by pertussis toxin, forskolin and Cl-cAMP (Welch et al. 1995). The potency of numerous cannabinoids to inhibit cAMP formation in the neuroblastoma cells was found to correlate to antinociceptive effects of the drugs in vivo (Howlett et al, 1986). Various calcium modulators were tested in combination with the cannabinoids and failed to produce antinociception or alter the antinociception produced by administration of cannabinoids i.t. Multiple potassium channel modulators administered i.t. failed to alter cannabinoid-induced antinociception with the exception, apamin, a blocker of small (low) conductance calcium-gated potassium channels, which caused a parallel rightward shift in the dose-effect curves of several cannabinoids (Welch et al. 1995). The *kappa* antagonist, nor-BNI (i.t.), and the *kappa*1 receptor antagonist, naloxone benzoylhydrazone (Nal-BZH), but not other opioid antagonists, blocks cannabinoid (i.t.)-induced antinociception. This antinociceptive block does not occur due to direct interaction with the *kappa* receptor, because nor-BNI and nal-BZH fail to displace cannabinoid binding at the cannabinoid receptor. There is evidence to support an indirect interaction between the *kappa* receptor and Δ^9-THC in a study in which the antinociceptive effects of Δ^9-THC are attenuated with antisera to Dynorphin A (1-8) and Dynorphin A (1-17) (Rowen et al. 1998). These data indicate that interaction of Δ^9-THC

with the cannabinoid receptor may cause release of endogenous spinal dynorphins or leucine enkephalin, a metabolite of dynorphin, leading to antinociception. Some possible points of interaction of the opioids and cannabinoids include the modulation of calcium, cAMP and potassium flux (Welch et al. 1994). Data also supports the interaction of cannabinoids with several endogenous compounds that inhibit nociception. Data supports the involvement, in addition to opioids, PGE1, catecholamines, and 5-HT (Adams and Martin 1996).

Tolerance to Cannabinoids. Tolerance develops to the pharmacological effects of cannabinoids in a variety of animal species, including pigeons, rodents, dogs, monkeys and rabbits. Tolerance has occurred to antinociception (Martin 1985), anticonvulsant activity (Colasanti et al. 1982), catalepsy (Pertwee 1974), depression of locomotor activity (Karler et al. 1984), hypothermia (Thompsom et al. 1974), hypotension (Birmingham 1973), corticosteroid release (Miczek and Dihit 1980), ataxia in dogs (Martin et al. 1976) and schedule- controlled behavior (McMillan et al. 1970). Tolerance does not develop to all cannabinoid effects, such as ACTH secretion (Dewey et al. 1970). Tolerance has also developed to cannabinoid-inhibition of adenylyl cyclase activity (Dill and Howlett 1988). It is interesting to note that tolerance developed to cannabinoid-induced stimulation of prostaglandin E2 production and arachidonate release (Burstein et al. 1985), but as was mentioned earlier the arachidonate release is not receptor-mediated. The precise mechanism of the development of tolerance is unknown. Changes at the cannabinoid receptor level following exposure to cannabinoids for a long period of time could result in conformational changes in the receptor which would produce an altered

receptor structure, to which the ligand could not bind. Receptor internalization is another possible mechanism of tolerance. With receptor internalization, receptors are removed into the cytoplasm where they are either degraded or recycled. The number of receptors on the surface is decreased. Therefore, the binding to the receptor is decreased (Oviedo et al. 1993, Rodriquez de Fonseca et al. 1994). It has been proposed (Rinaldi-Carmona et al. 1998) that the rapid receptor internalization process is distinct from the slower process of receptor down-regulation. Evidence has been presented that shows the cannabinoid receptor is rapidly internalized following binding of an agonist. The internalization appears to occur through clathrin coated pits and is rapid and reversible after short treatment (<15 min.), but not after long treatment (>90 min.). Internalization was not blocked by pretreatment with PTX and/or cholera toxin, suggesting activation of PTX- or CTX-sensitive G proteins was not required for internalization. This pathway is similar to the beta2-adrenergic receptor (Mackie 1998). There is little evidence that chronic administration of cannabinoids alters disposition or metabolism of cannabinoids in the brain or periphery (Oviedo et al. 1993), suggesting that tolerance is pharmacodynamic in nature rather than a consequence of reduced bioavailability. In autoradiographic studies it was shown that binding to the CB receptor was decreased, with no apparent regional selectivity, suggesting a lack of involvement of neural circuitry, second messengers, or other intervening variables that might lead to differential effects. The reductions appear to be receptor-mediated. In chronically treated animals the changes were the result in loss of binding capacity (Bmax) rather than a change in affinity (KD) (Oviedo et al. 1993).

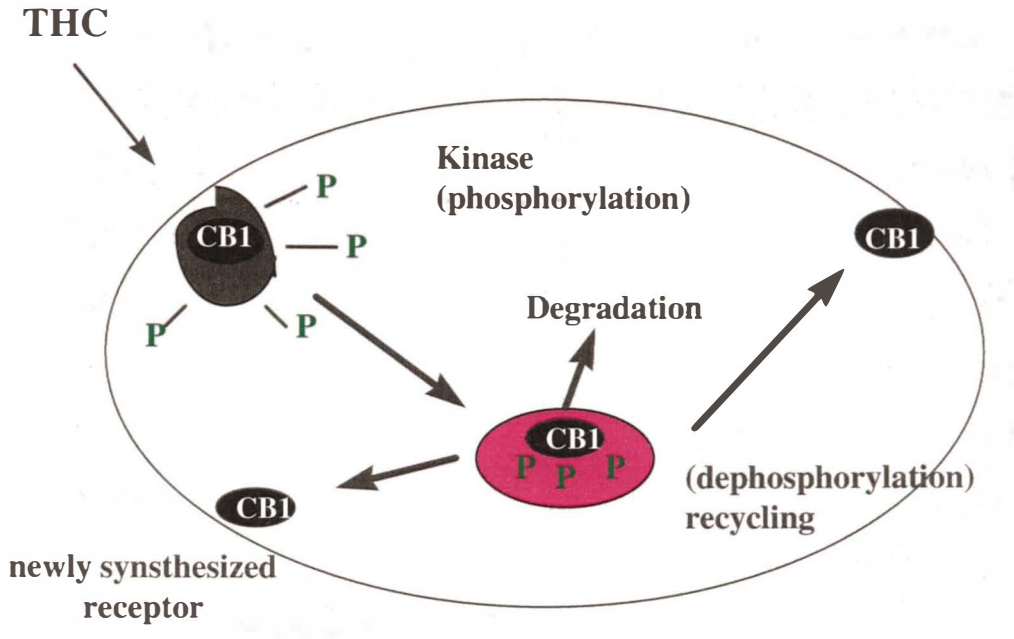

Figure 1. Down-regulation of the cannabinoid receptor. When THC or a CB receptor ligand binds to the cannabinoid receptor, the receptor is thought to be phosphorylated and rapidly internalized through clathrin-coated pits. Once in the cytosol several processes may occur: the receptor can be degraded, which would then require protein synthesis for a new receptor; or it can be dephosphorylated and recycled to the membrane.

This rules out the theory that there may be a conformational change in the receptor in chronically treated animals.

It is thought that the desensitization of the CB receptor may be similar to that of the beta adrenergic receptor (β-AR). The decreased responsiveness of the β-AR after stimulation with a near saturating concentration of ligand appears to be caused by rapid PKA and GRK phosphorylation. GRK phosphorylation in turn promotes β-arrestin binding and receptor internalization (Seibold et al. 1998). In the following study, we inhibit β-ARK, with low molecular weight heparin. If β-ARK plays a role in receptor activation upon ligand binding or role in receptor desensitization, then we may see an effect upon of acute or chronic affects of Δ^9-THC upon inhibition of β-ARK.

There is a bi-directional cross tolerance noted between the kappa opioids and Δ^9-THC which implies a common mechanism of tolerance may underlie both classes of drugs (Rowen et al. 1988). Kappa1 antisense administration blocks the antinociceptive effects of Δ^9-THC administered i.t. The antinociceptive effects, but not the hypothermic, hypoactive or cataleptic are blocked by Dynorphin A (1-8) and Dynorphin (1-17) antisera. Another mechanism of tolerance that must not be discounted is the role of the G protein subunit, $G_{i2\alpha}$, which might be involved in opioid-induced tolerance expression. Antisense specific for the $G_{i2\alpha}$ subunit blocks morphine-induced antinociception and to different degrees also blocks the effects of different mu agonists. Therefore we may postulate that alterations in G proteins, or an uncoupling at the receptor, could account for

cannabinoid-induced tolerance via the interaction of the cannabinoids with kappa-opioids. (Rowen et al. 1998).

The Role of Protein Kinase C in Cannabinoid effects. It has been shown that cannabinoids increases the phosphorylating activity of brain PKC *in vitro* (Hillard and Auchampach 1994). Activation of PKC attenuates the modulation of N- and P/Q-type calcium currents and the Kir currents by G-protein coupled receptors. Fast modulation of all three channels is mediated by direct binding of G-protein βγ subunits to the channel itself (Zamponi et al. 1997). Phosphorylating the CB1 receptor with PKC strongly suppressed the modulation of P/Q type calcium channels and the Kir current by cannabinoids (Garcia et al. 1998). Therefore, neurotransmitters coupled to PKC restore neuronal excitability and synaptic activity reversing the effects of cannabinoids. Since PKC disrupts actions of cannabinoids by phosphorylating the CB1 receptor, we evaluated the antinociceptive effects PKC in tolerant mice. In a tolerant mouse the cells function as if no Δ^9-THC is present, basically the cells have compensated and is able to function normally even though Δ^9-THC is present. Since PKC disrupts the actions of cannabinoids by phosphorylating the receptor, we also evaluated the effects of inhibition of PKC on acute antinociceptive effects of Δ^9-THC.

MAP Kinase and its Role in Cannabinoid Effects. In addition to cannabinoid-induced signal transduction through cAMP accumulation and blockade of N-type calcium channels, several actions of cannabinoids may result from the increased level of tissue eicosaniods that occur in humans and animals treated with cannabinoids. Included in

these effects are the effect on time estimation, heart rate acceleration, and the subjective 'high' in humans; catalepsy induction in mice; and hypotension in dogs. The ability of cannabinoids to mobilize arachidonic acid from cellular phospholipid storage sites by activation of PLA2 is thought to be the reason for the increase eicosanoid level. Anandamide activates the MAP kinase signal transduction pathway in mouse peritoneal cells, and can be blocked by pertussis toxin, suggesting the effect is receptor-mediated (Wartmann et al. 1995). PLA2 is activated by the MAP kinase signal transduction pathway, which then causes and increase in arachidonic acid (Lin et al.1993).

Phosphotidalinositol-3 kinase. It is unclear how PI3-K activity might contribute to activation of the MAPK pathway. It is known that PI3-K is an early intermediate of the $G_{\beta\gamma}$-mediated MAPK signaling pathway. It is interesting to note that phosphatidylinositol-3,4,5,-trisphosphate (PIP3) can directly interact with SH2 domains and might contribute to their recruitment. Partial reduction of SHC tyrosine phosphorylation following GPCR stimulation was detected upon inhibition of PI3-K (Daub et al. 1997). Therefore, we proposed to block PI3-K and reduce the SHC tyrosine phosphorylation. Such inhibition of PI3-K should block MAPK and cPLA2 release further down the pathway. If this block altered tolerance, then the action of PI3-K, or the results of activating the $\beta\gamma$ subunit could play a role in tolerance or antinociception.

MAPK activation and interactions. MAPKs become activated in response to growth factors either through RTK or GPCR triggered signals. Transmission of these signals requires the formation of a complex between the Grb2 adapter protein with the guanine nucleotide exchange factor Sos, which upon recruitment to the plasma membrane

allows activation of the small G protein Ras, resulting in subsequent activation of the MAPK pathway (Daub et al, 1997). Stimulation of various GPCRs rapidly induce tyrosine phosphorylation of the adapter protein SHC and SHC-Grb2 complex formation, steps that couple both GPCRs and RTKs to Ras. GPCR-mediated activation of the Ras/MAPK pathway has been reported to involve src function, leading to the idea that RTKs and Src family kinase might be required to initiate intracellular signaling cascades (Daub et al. 1997). A dominant negative mutant EGFR cell was evaluated and MAPK activation through a Gi coupled receptor was abolished (Daub et al. 1997). Give such data indicating that GPCRs interact with RTKs in intracellular signaling involving MAPK, we asked how the GPCR cannabinoid receptor and RTKs, interact and affect intracellular processes. An increase in SHC tyrosine phosphorylation and MAPK stimulation through both G_q- and G_i-coupled receptors was reduced greatly upon selective inhibition of EGFR function (Daub et al 1997). Using PP1, a potent inhibitor of the src family of tyrosine kinases, we may be able to inhibit MAPK stimulation. Inhibition of MAPK is proposed to address the possibility of antinociception or tolerance working through MAPK.

PP1 has been shown to suppress tyrosine phosphorylation of various Src substrates, which moderately increased EGF-induced EGFR tyrosine phosphorylation, while GPCR transactivation was inhibited somewhat. PP1 targets essential signaling elements upstream of Ras and downstream of the EGFR (Daub et al. 1997). Stimulation of the CB1 receptor with agonist leads to the activation of *krox-24* and immediate early growth related gene. *Krox-24* activation, which is blocked by PTX treatment cannot be

ascribed to known PTX-sensitive G-protein pathways: adenylate cyclase, PLC and ion channel modulation. It has been shown, that herbimycin A, a tyrosine kinase inhibitor, inhibited the induction of *krox-24*, suggesting a tyrosine kinase may lie on the route between Gi and *krox-24* (Bouaboula 1995). They also showed that MAPKs became tyrosine phosphorylated in cannabinoid-treated cells. Thus we had reason to evaluate PP1 in cannabinoid tolerant animals.

With data suggesting that there are at least two, if not three pathways (α subunit, $\beta\gamma$ subunit, and possibly ion channel modulation) activated by an agonist binding to the cannabinoid receptor, and several potential kinases which are activated by those pathways, we evaluated the role of inhibition of kinases down stream from the Gi-protein cannabinoid receptor. Our aim was to determine if tolerance and/or antinociception was altered upon kinase inhibition. We were, thus, addressing the following HYPOTHESIS:

> Delta-9 THC induced antinociceptive tolerance is a function of the altered phosphorylation state of either the CB1 receptor or proteins involved in the signal transduction of the CB1 receptor. Our goal is to address this hypothesis by the use of inhibitors of candidate kinases using drugs selective for such kinases.

Methods

Animal model of Δ^9-THC tolerance. All studies were performed on male ICR mice. The mice were kept on a 12hour/12hour light/dark cycle 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. They received twice daily s.c. injections of Δ9-THC (20mg/kg) for six days and on day seven they just received the morning dose. On the morning of day 8 mice were challenged with an ED-80 of Δ^9-THC.

Intrathecal (i.t.) injections. I.t. injections were performed 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, ½-inch needle.

The tail flick test. Mice were tested for antinociception by the tail flick procedure (D'Amour and Smith 1941). Reaction times of 2 to 4 seconds were employed for the control, while a time of 10 seconds was used for the cutoff to prevent tissue damage. Antinociception was quantified as the percent maximum possible effect (%MPE) formula:

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

(Harris and Pierson, 1964). Values were calculated for each mouse, using at least 4 mice 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 generated dose-response curves.

Materials. Doses for all drugs used were determined in naïve animals using the maximal dose without toxicity. Time points were determined in naïve animals to where the drug had its peak effect. 100% dimethyl sulfoxide was purchased from Sigma Chemical Company (St. Louis, MO). KT5720 purchased from Calbiochem (La Jolla, CA) was prepared in 100% DMSO and was injected i.t. at a dose of 2.7μg/mouse 15 minutes prior to drug or vehicle (i.t.). The tail flick test was then conducted 15 minutes 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 minutes prior to drug or vehicle (i.t.). The tail flick test was then conducted 15 minutes following the second injection. Dibutyryl-cAMP (10μg/mouse) and dibutyryl-cGMP (5μg/mouse) were purchased from Calbiochem (La Jolla, CA) and were prepared in dH2O and injected i.t 15 minutes prior to the i.t. injection of drug or vehicle, fifteen minutes later the tail flick test was conducted. Δ^9-THC 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 EL-620:18 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 minutes prior to drug or vehicle, also injected i.t. the tail flick test was then conducted 15 minutes following the second injection. Bisindolymaleimide I, HCL purchased from Calbiochem (La Jolla, CA) was prepared in dH2O and injected i.t. (5μg/mouse) and (0.5μg/mouse) 15 minutes prior to drug or vehicle (i.t.). The tail flick test was then conducted 15 minutes 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 minutes preceding the i.t. injection of drug or vehicle, the tail flick test was then conducted 15 minutes following the second injection. PP1 purchased from Alexis was prepared in 100% DMSO and injected i.t. 10 minutes prior to the i.t. injection drug or vehicle, with the administration of the tail flick test 15 minutes 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 Coluhoun (1997).

First i.t. inj.	Drug action	Dose (μg/mouse)	Time	Second i.t. inj.	Time	Test
KT5720	PKA inhibitor	2.7	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
KT5823	PKG inhibitor	2.5	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
Bis	PKC inhibitor	0.5 and 5.0	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
LMWH	β-ARK inhibitor	30	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
LY294002	PI-3K inhibitor	1	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
PP1	src TK inhibitor	0.0001	10 minutes	Δ^9-THC or veh	15 minutes	Tail flick
d-cAMP	cAMP analog	10	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick
d-cGMP	cGMP analog	5	15 minutes	Δ^9-THC or veh	15 minutes	Tail flick

Figure 2. Summary of drugs, actions, times between injections and test performed.

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 Δ9-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 79.63 (95% confidence limits from 62.10 to 102.12) to 8.62 μg/mouse (95% confidence limits from 4.65 to 15.99) in the KT5720 treated mice. The lines were parallel and had a potency ratio of 8.32 with 95% confidence limits. (Figure 3).

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. [2.4 %MPE in the tolerant mice compared to 6.7% in the tolerant animals treated with KT58. (Figure 4).]

Bisindolylmaleimide I, HCL (bis), a protein kinase C (PKC) inhibitor, at a dose of 0.5μg/mouse administered i.t. in water vehicle did not affect the antinociceptive tolerance in mice. The %MPEs in the tolerant groups treated with bis compared to vehicle treated were not significantly different (20.1 ± 15 vs. 14.1 ± 6.0, respectively) (Figure 5). At an increased dose of 5μg/mouse there was not a significant shift in the ED50 values of the

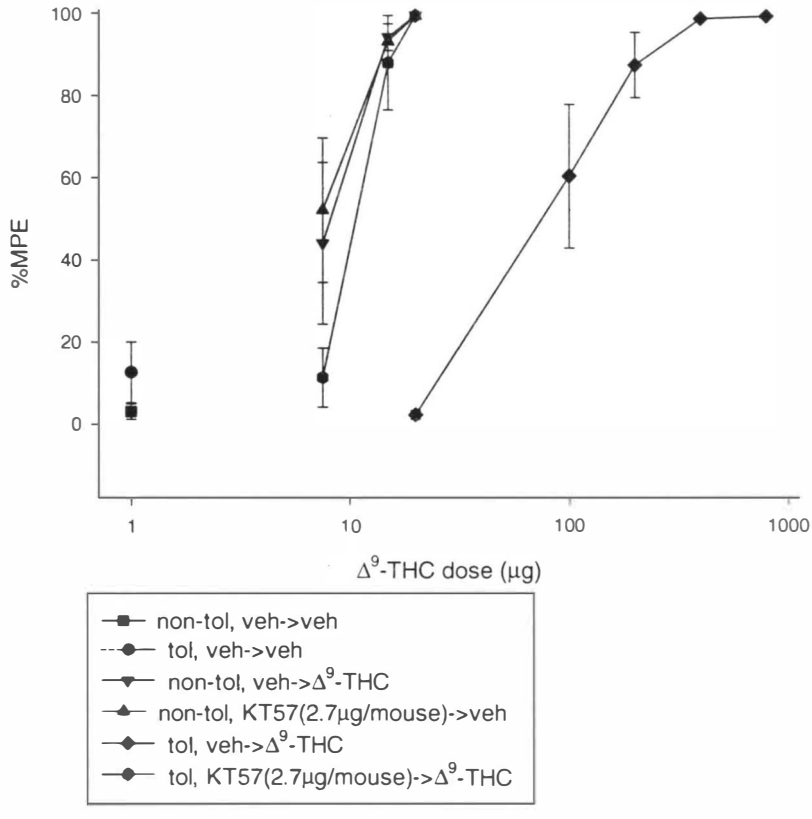

Figure 3. The reversal of Δ^9-THC by a PKA inhibitor (KT5720). The graph shows a significant (*) leftward shift of the curve representing the tolerant mice treated with KT5720 compared to the tolerant mice treated with vehicle. The effect of the PKA inhibitor on mice tolerant to Δ^9-THC is the reversal of tolerance.

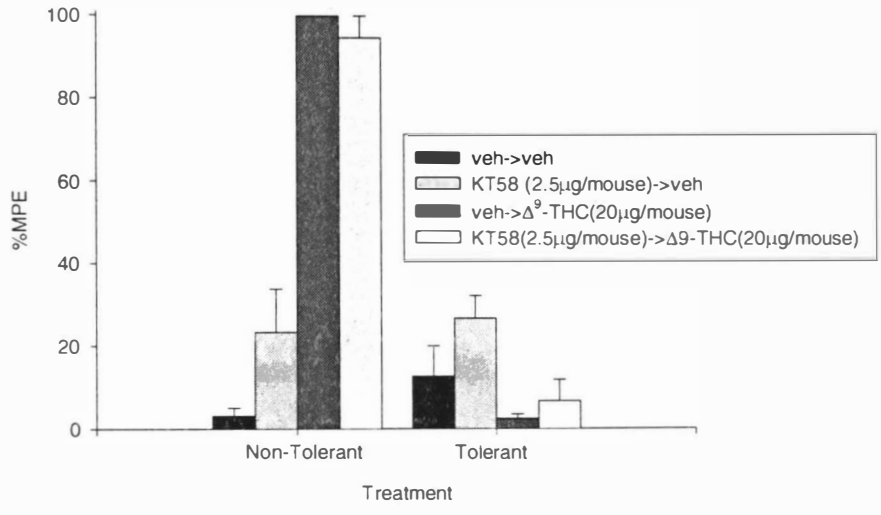

Figure 4. A PKG inhibitor (KT5823) does not alter Δ^9-THC antinociceptive tolerance. This is shown by the fact that there was not a significant change in tolerance in mice treated with KT5823 or vehicle.

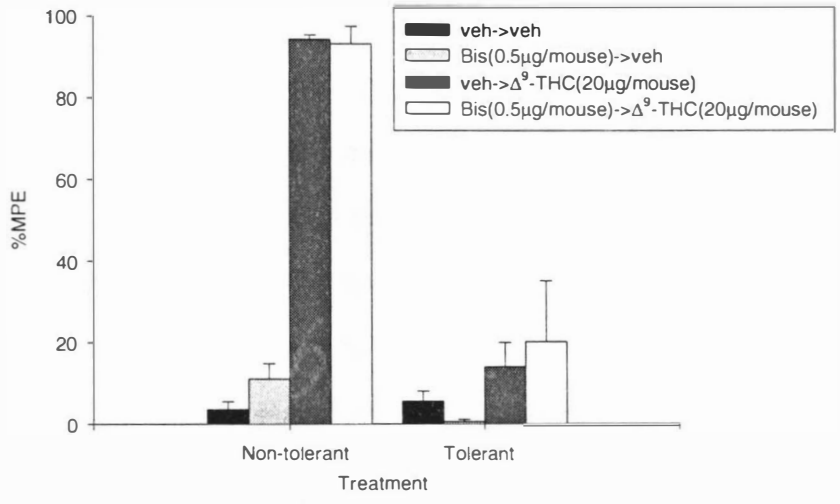

Figure 5. The PKC inhibitor, bis, at a dose of 0.5µg/mouse did not alter Δ^9-THC antinociceptive tolerance. As can be seen from the graph, there is not a significant difference between the tolerant groups of mice treated with bis or vehicle.

tolerant mice treated with bis compared to tolerant mice treated with vehicle (40.5 with 95% confidence limits of 31.9 to 51.4 vs. 80.1 with 95% confidence limits of 49.7 to 128.98, respectively) (Figure 6). The lines were not parallel in the tolerant mice and the potency ratio was 2.04. Interesting though, 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. The ED50 was shifted from 7.1 (95% confidence limits from 4.5 to 11.2) in the vehicle-treated non-tolerant animal to 26.3 (95% confidence limits from 15.5 to 44.8) in the bis treated non-tolerant animal. The lines on the graph are parallel and the potency ratio is 3.6.

Low molecular weight heparin (LMWH), which inhibits beta adrenergic receptor kinase (β-ARK), at a dose of 30 μg/mouse in water vehicle administered i.t. did not affect the antinociceptive tolerance in the mice. The %MPE in the tolerant group treated with LMWH (5.2 ± 1.8) was not significantly different than the vehicle treated tolerant group (14.1 ± 6.0), (Figure 7).

LY294002, the a phosphatidylinositol-3-kinase inhibitor, administered i.t. in 100% DMSO vehicle at a dose of 0.1μg/mouse did not significantly alter Δ9-THC antinociceptive tolerance in mice, (Figure 8). The LY294002-treated tolerant mice had a

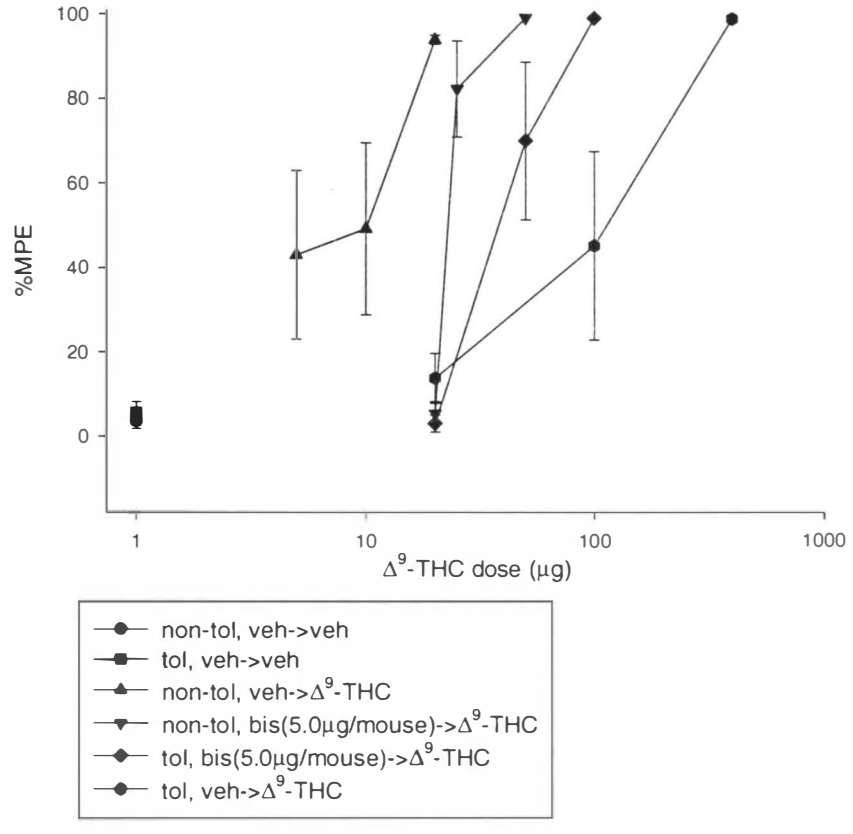

Figure 6. The PKC inhibitor bis, at a dose of 5µg/mouse attenuated the effects of Δ^9-THC in non-tolerant mice. As can be seen from the graph, there was a significant (*) rightward shift of the curve representing non-tolerant treated mice.

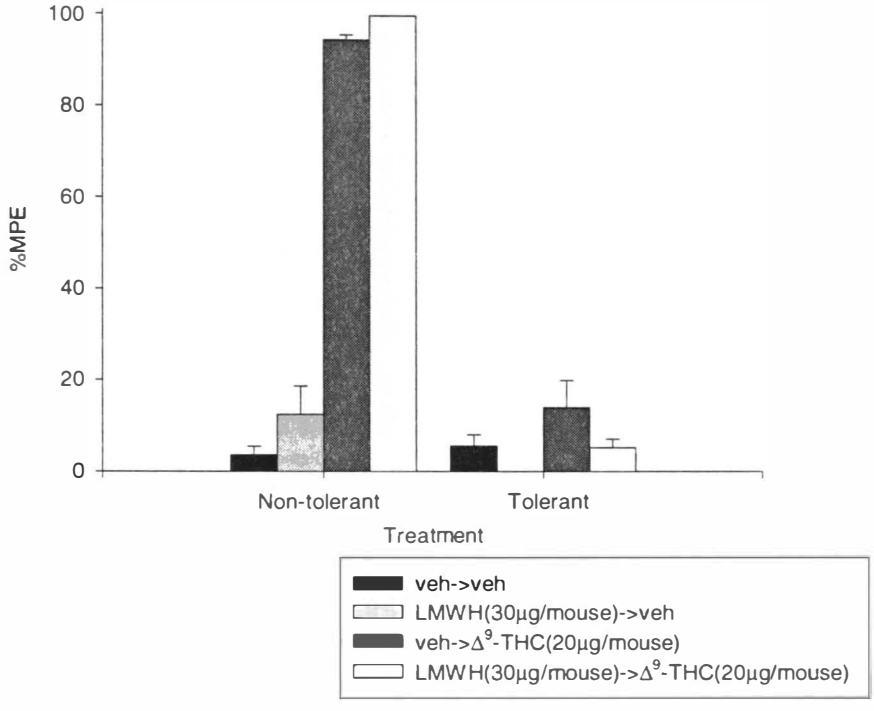

Figure 7. The β-ARK inhibitor, LMWH, did not significantly alter Δ^9-THC antinociceptive tolerance. As can be seen from the graph there is not much change between the vehicle and LMWH treated tolerant mice.

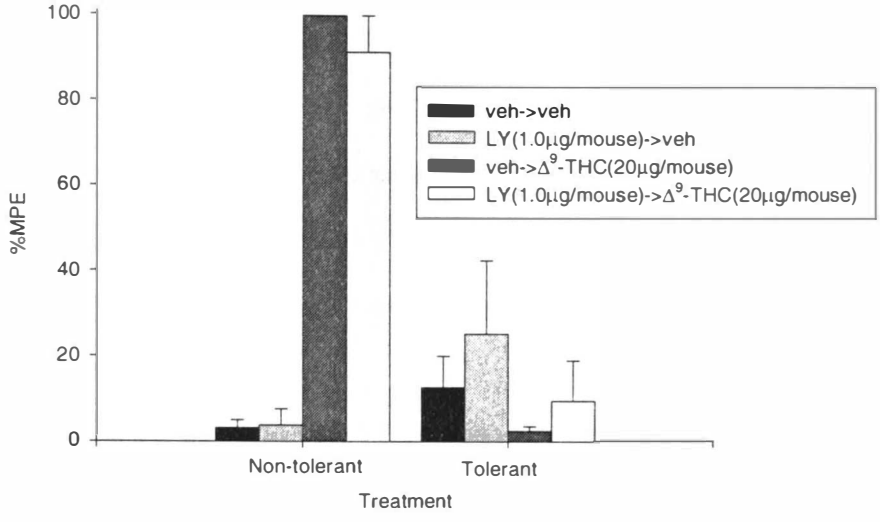

Figure 8. The PI3-K inhibitor, LY294002, did not significantly affect antinociceptive tolerance. This can be seen from the graph in that there is not a significant difference between the LY294002 and vehicle treated tolerant animals.

%MPE of 9.5±9.5 compared to the tolerant vehicle treated mice who had a %MPE of 2.4±1.1.

We also looked at PP1 which is a src family tyrosine kinase. Since the βγ subunit of the cannabinoid receptor interacts with tyrosine kinase to activate MAPK, we wanted to look at what would happen if this pathway was disrupted. At a dose of 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 3.7±0.8) in the tail flick test in naïve mice, but in the non-tolerant group in had a %MPE of 43.7±19.6. The non-tolerant vehicle treated group also had a high %MPE (45.3±19.2). (Figure 9). At doses of 0.001μg/mouse and higher PP1 shows a variable antinociceptive affect

The next step was to look at potentiation of tolerance. If PKA inhibition reversed tolerance, what would happen if a cAMP analog was given? In order to tell whether of not there was a potentiation, the dose of Δ^9-THC had to be raised to 100μg/mouse to get around a 50% MPE in the tolerant mice. Dibutyryl cyclic-GMP at 5μg/mouse in water vehicle administered i.t. did not significantly potentiate tolerance, a 35.5±20.4 %MPE in the tolerant animals compared with a 45.8±22.5 %MPE in the drug-treated animals

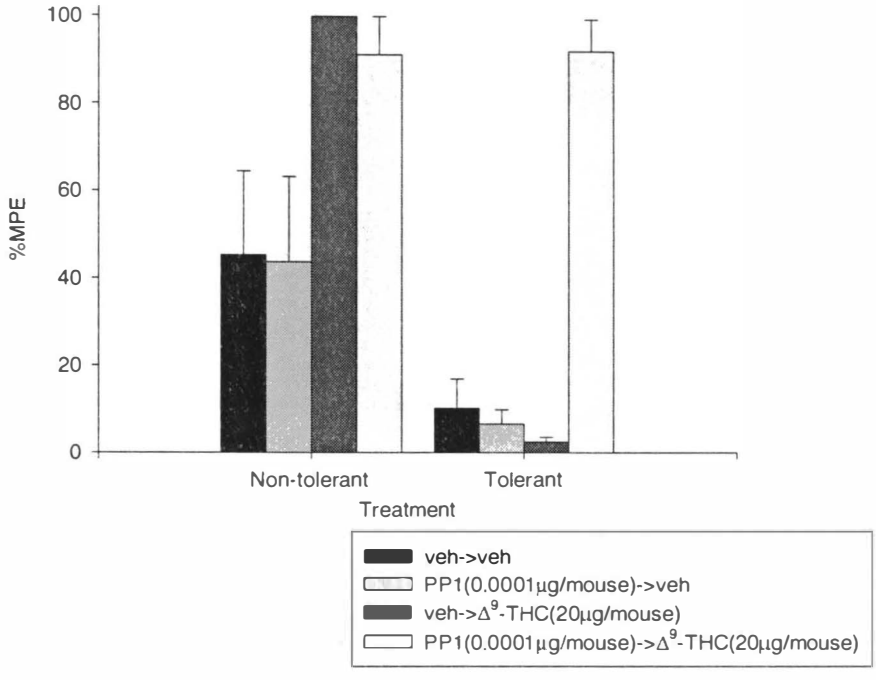

Figure 9. The src family tyrosine kinase, PP1, reversed Δ^9-THC antinociceptive tolerance. It is shown in the graph that there was a significant reversal of Δ^9-THC antinociceptive tolerance in tolerant mice treated with PP1. However the graph also shows a PP1 effect alone and a vehicle effect in the non-tolerant group.

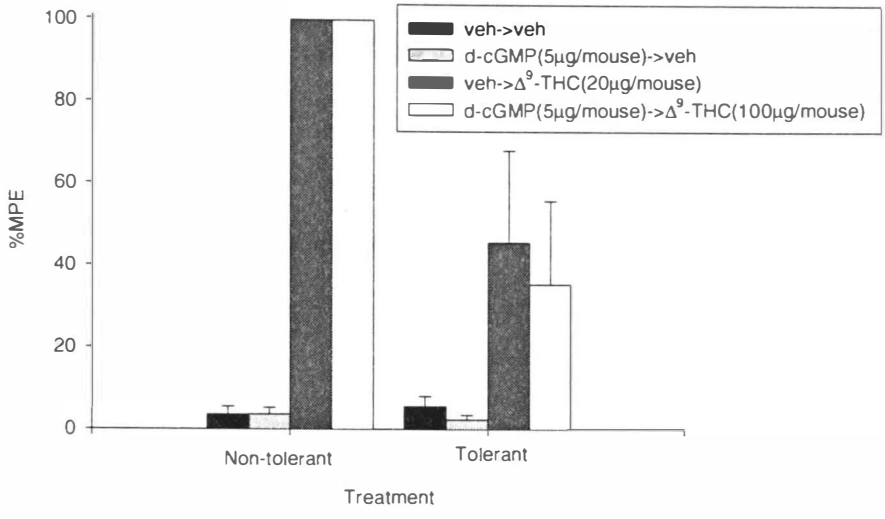

Figure 10. Dibutyryl-cGMP does not enhance Δ^9-THC antinociceptive tolerance. The graph shows no significant change between the dibutyryl-cGMP treated tolerant mice and the vehicle treated tolerant mice.

(Figure 10). Dibutyryl cyclic-AMP at a dose of 10µg/mouse in water vehicle administered i.t. also did not potentiate Δ^9-THC antinociceptive tolerance, a 54.7$\pm$13.8 %MPE in the tolerant animals compared to 45.8$\pm$22.5 %MPE in the drug treated animals.

Even though it was not significant dibutyryl-cAMP seemed to attenuate the effects of Δ^9-THC in the non-tolerant animals (Figure 12).

Kinase	Inhibitor	Effect on tolerance
PKA	KT5720	Reversal
PKG	KT5823	No change
PKC	Bisindolylmaleimide I, HCl	No change
PI3-K	LY294002	No change
Tyrosine kinase	PP1	Reversal
β-ARK	Low molecular weight heparin	No change

Figure 11. Summary of the kinases putatively inhibited, inhibitor and the effect on tolerance.

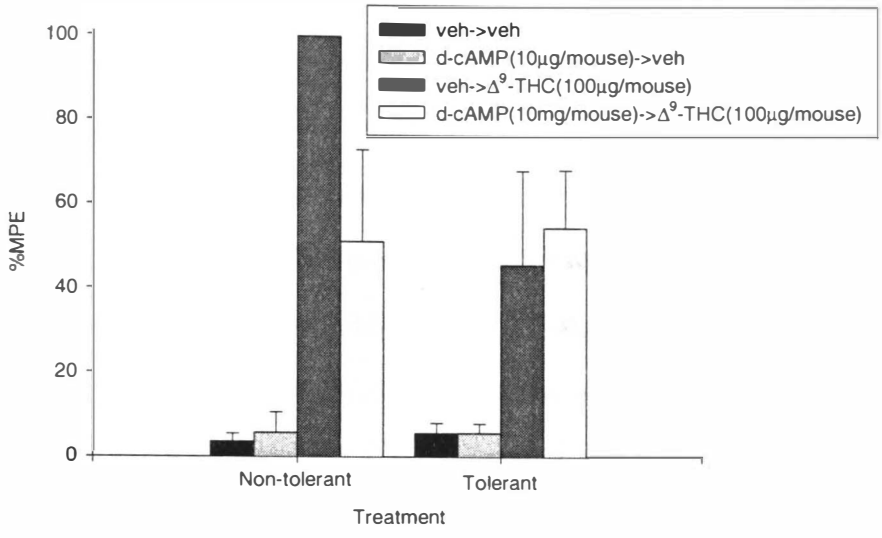

Figure 12. Dibutyryl-cAMP does not enhance Δ^9-THC antinociceptive tolerance. Although not significant it does appear to attenuate the antinociceptive effects in non-tolerant mice. It can be seen in the graph that enhancement does not occur because there is no significant change in %MPE in the tolerant animals treated with dibutyryl-cAMP or vehicle.

Discussion

The question I sought to address in these studies was: what role do various kinases play 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). This discussion begins with kinases downstream from the α subunit, then goes to kinases downstream to the βγ subunit, and ends with the "others" group.

When a ligand binds to a GPCR, as the cannabinoid receptor, there is a decreased affinity between the α and βγ subunits and they differentiate from one another. In the acute model of Δ^9-THC exposure the α subunit will cause a decrease in adenylate cyclase which then decreases cAMP followed by a decrease in PKA. 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 the chronically treated Δ^9-THC model downstream from the α subunit there is a compensatory increase in adenylate cyclase, cAMP and PKA. The is no longer a Δ^9-THC induced modulation of potassium and calcium channels.

The intra-thecal administration of the protein kinase A inhibitor KT5720 reversed the antinociceptive tolerance of Δ^9-THC. This indicates that protein kinase A plays a role

in the mechanism of Δ^9-THC antinociceptive tolerance whether it is direct or indirect is yet to be determined. PKA could be responsible for phosphorylating the CB1 receptor upon binding of the ligand to the receptor, this activation could result in a chain of events one of which the end result is antinociception. PKA also could be increasing potassium conductance through phosphorylation of the potassium channel, causing the cell to be hyperpolarized and unable to fire. Other possible roles of PKA in Δ^9-THC mediated tolerance includes the possibility that PKA is rapidly and continuously phosphorylating the CB1 receptor when it is down-regulated into the cytosol in tolerant animals. Our lab has shown that receptor density does not change in mice tolerant to Δ^9-THC, the down-regulated receptors are not being degraded and new receptors are not being synthesized, or both processes are going at the same rate, the former is more plausible. The receptors are rapidly internalized upon exposure to cannabinoids (Mackie et al. 1998). It has been suggested that the rapid internalization process of the cannabinoid receptor is distinct from the slower process of receptor down-regulation. As the cell becomes tolerant the internalization of the receptor may get longer and longer as the cell undergoes compensation. Once the cell is tolerant there will be an increased production of PKA to make up for decrease caused by Δ^9-THC. These higher levels of PKA, compared to initial exposure could be responsible for a continuous phosphorylation of the CB1 receptor while in the cytosol. This continued phosphorylation might be what keeps the receptor down-regulated in the cytosol. Upon inhibition of PKA the receptor will no longer be phosphorylated and could therefore be recycled to the membrane where it will

be active and capable of binding to the ligand again. If the phosphorylation is not halted, and the receptor is kept in the cytosol, eventually it will be degraded, requiring mRNA for new protein synthesis.

PKA could be responsible for the phosphorylation of the N-type calcium channels and potassium channels giving rise to the hyper-polarized state of a naïve cell and resulting in antinociception. Upon inhibiting PKA in the tolerant animal, the phosphorylated channels become dephosphorylated and are therefore inactive again (potassium conductance is increased and calcium conductance is decreased), giving rise to the antinociceptive effects of Δ^9-THC. We know, apamin, a blocker of small (low) conductance calcium-gated potassium channels blocked the antinociceptive effects of i.t. administered cannabinoids (Welch 1995). However, i.t. administration of cannabinoids are not sensitive to calcium modulation and thus may not directly involve calcium modulation (Welch 1995). This leads me to believe the spinal mediated antinociception does not involve calcium channels.

If PKA reverses cannabinoid antinociceptive tolerance, what would happen if we gave a cAMP analog? We would think that a cAMP analog would enhance tolerance. In a non-tolerant cell 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). But, in our case dibutyryl-cAMP did not enhance tolerance, though it did, but not significantly attenuate antinociception in the non-tolerant mice. It would be interesting to see what happens to cannabinoid tolerance in forskolin treated animals or with a different cAMP analog that is more potent.

Unsure as to whether or not the $G_{\alpha i}$ mediated signaling pathway through cAMP was responsible for antinociception, we also wanted to look at kinases involved in the $G_{\beta\gamma}$-mediated signaling pathway. PI-3 kinase and tyrosine kinase work downstream from the βγ 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 they might play a role in antinociception. The first of these being LY294002, a specific phosphatidylinositol 3-kinase 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). PTX-sensitive RTKs and GPCRs converge or share a common pathway upstream from ras, which leads to the activation of MAPK. Our goal was to see if by blocking a kinase or kinases in the pathway, would we affect tolerance. In the case of blocking PI-3K tolerance was not affected. However the blockade of the src family tyrosine kinase reversed tolerance. Was it a true reversal? At higher doses the inhibitor, PP1 is variably antinociceptive. In the non-tolerant groups PP1 had a small antinociceptive effect as well as the vehicle. What is a possible explanation of a reversal of tolerance? By blocking a tyrosine kinase we may be inhibiting downstream actions of the βγ subunit, that may be necessary to maintain a tolerant state or we may be bypassing the traditional route of antinociception, the mice are still tolerant to Δ^9-THC, but by inhibiting tyrosine kinase in the presence of Δ^9-THC antinociception is the result. Possibly by inhibiting MAPK and/or PLA2 in the tolerant animal reverses tolerance. We would think, that since PP1 reversed tolerance, LY294002

would also. Looking at figures 13 and 14, PI3-K is necessary for ras formation. PI3-K is also necessary to produce PI345P3 which is required for the complex to form. By inhibiting either one of these steps LY294002 should reverse tolerance if PP1 is working through this same path to reverse tolerance, but it doesn't. Two, of the many possible explanations are: 1)PP1 is not working at the complex formation, or LY294002 is not getting to the site of action. It would be interesting to look at other TK inhibitors or if tolerance can be enhanced through a tyrosine kinase analog, this would help clarify how this kinase may be working in tolerance reversal. 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 looked at other kinases. β-ARK is known to phosphorylate the β-AR and is a potential candidate for phosphorylating the cannabinoid receptor so it can be internalized in a clathrin coated pit and it is thus desensitized. If this were correct, then by blocking β-ARK with LMWH we could prevent receptor phosphorylation and possibly desensitization or down-regulation. Since LMWH was inactive it appears that the cannabinoid receptor is not phosphorylated by β-ARK and this kinase is not responsible for the desensitization of the receptor. Another possible kinase that could activate the CB1 receptor is β-ARK, of the GRK family of kinases. β-ARK also known as GRK2, is thought to possibly to be involved in the desensitization of the beta adrenergic receptor. Mackie et al. in 1998 noted that the CB1 receptor is internalized following a pathway grossly similar to the one

used by the beta2-adrenergic receptor. Our data indicate that β-ARK does not play a role in receptor down regulation in mice tolerant to Δ^9-THC, since LMWH does not affect the tolerance. If β-ARK plays a role in cannabinoid induced antinociception it is yet to be determined.

PKG is known to interact with cGMP and nitric oxide. The inhibition of PKG did not reverse tolerance. The cGMP analog we looked at, dibutyryl-cGMP did not enhance tolerance.

PKC may act directly on the 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). So what happens if we inhibit PKC in tolerant animals? We showed at two different doses that tolerance to cannabinoids was not affected by inhibiting PKC. It was shown at the higher dose that inhibiting PKC attenuated the effects of Δ^9-THC in non-tolerant animals. Hillard et al. in 1993 showed that cannabinoids increase the levels of PKC in rat brain and that these increased levels are responsible for reestablishing neuronal excitability. Therefore we would expect the inhibition of PKC to prolong the effects Δ^9-THC, but this is not what we saw. If inhibiting PKC attenuates the effects of cannabinoid-induced antinociception, it might be likely that these increased levels of PKC may be at least partially responsible for cannabinoid-induced antinociception.

In summary, the data presented in this thesis demonstrates that by inhibiting PKA and tyrosine kinase Δ^9-THC antinociceptive tolerance can be reversed. It seems likely

that these two kinases work independent of one another. The other kinases that were inhibited including: PKG, PKC, PI3-K and β-ARK did not alter tolerance. However, the higher dose of PKC was shown to attenuate the effects of Δ9-THC in the non-tolerant mice. The cAMP and cGMP analogs had no significant effects on tolerance enhancement.

Even though a kinase or analog did not affect tolerance we must not discount its possible effects or assume it does not alter tolerance. It is possible the drug may not reach the site of action, it is degraded to rapidly to exert its affect or inappropriate time points and doses were chosen, even though attempts were made to optimize both.

A future direction would be to evaluate phosphorylation state of the receptor. If PKA is 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 spinal cord tissue that had been treated with the PKA inhibitor immediately prior to harvest.

In order to produce new and more specific drugs to treat humans, we must first understand their mechanism of action. The goal of this research was to evaluate the underlying mechanisms for Δ^9-THC antinociceptive tolerance. In an attempt to one day prevent tolerance development in clinical situations.

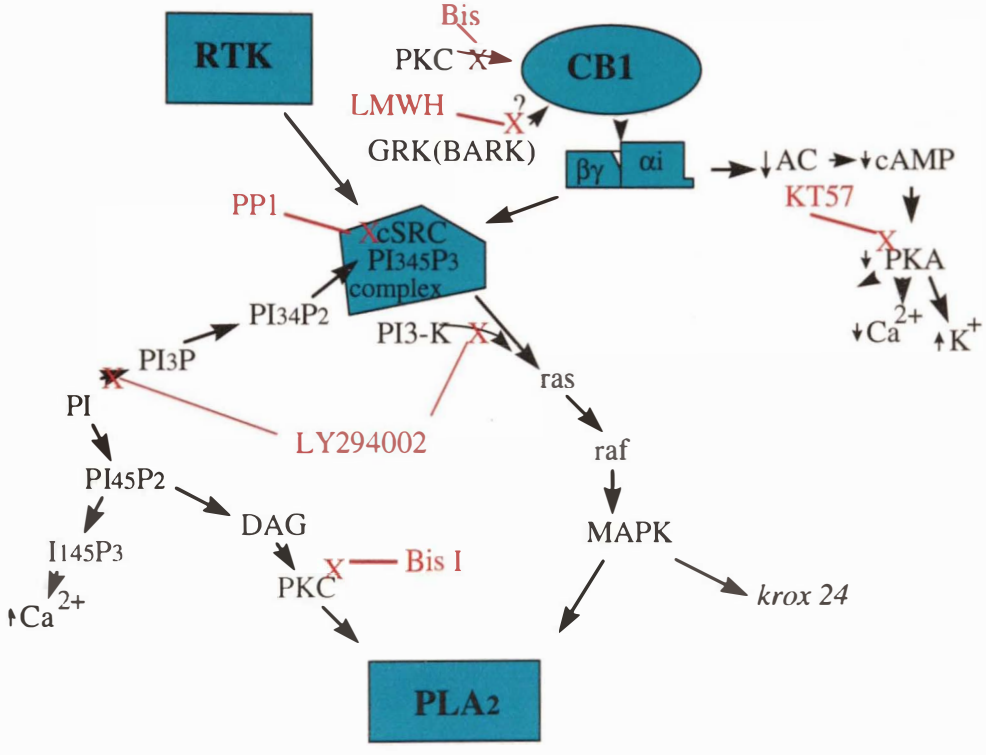

Figure 13. Putative pathway, in the acute model of Δ^9-THC exposure, of second messengers, kinases and where the kinase inhibitors may work. This figure shows that the αi subunit disassociates from the βγ subunit. The α subunit decreases cAMP, adenylyl cyclase and PKA. The decrease in PKA could the be responsible for the: decrease in calcium conductance and increase in potassium conductance. The βγ subunit

goes the other way allowing a complex to be formed with second messengers downstream from the receptor tyrosine kinase (RTK), this convergence of pathways is upstream from ras. The PI345P3 is also necessary for the complex formation. The end result is a modulation of phospholipase A2.

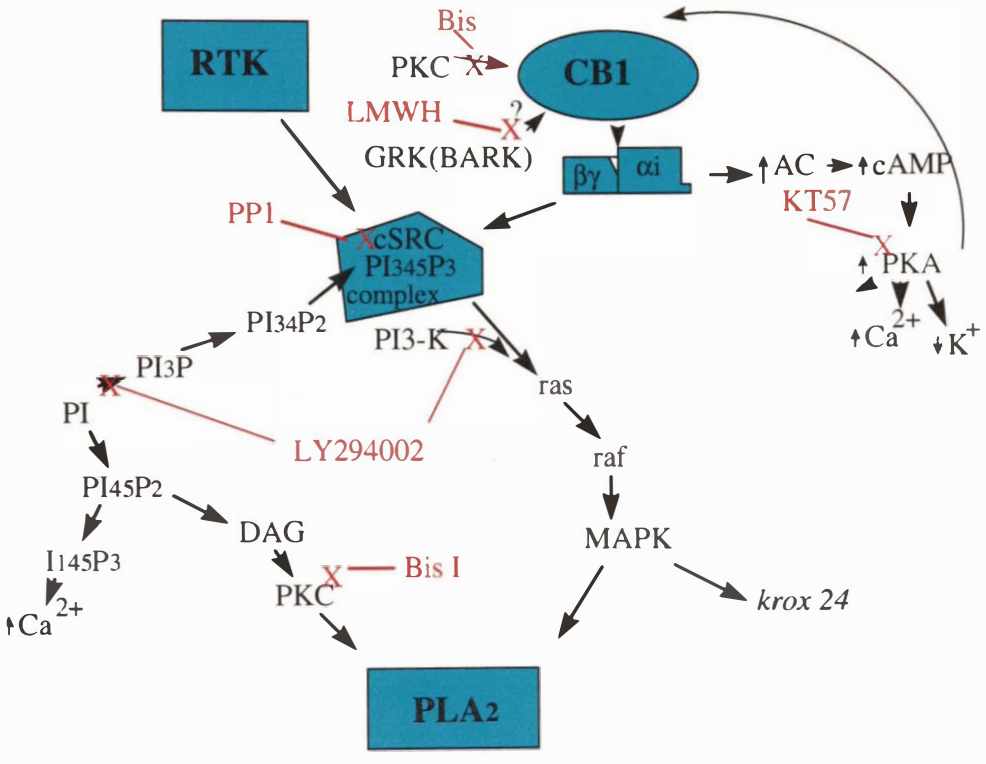

Figure 14. Putative pathway of second messengers and possible places of action on kinase inhibitors in the Δ^9-THC tolerant model. Downstream from the α subunit there is a compensatory increase in AC, cAMP and PKA. Potassium efflux decreases and calcium conductance increases. Inhibiting PKA and the src TK reverses tolerance. Since

inhibiting the src TK reverses tolerance it would seem that inhibiting PI3-K would also reverse tolerance, but it doesn't.

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