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Reversal of Δ^9-tetrahydrocannabinol-induced tolerance by specific kinase inhibitors

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Abstract

Tolerance develops to the pharmacological effects of Δ^9-tetrahydrocannabinoid (THC) following repetitive administration. Adaptations in signaling pathways involved in tolerance to THC-induced behaviors are not understood. The objective of our study was the evaluation of kinase involvement in the expression of tolerance to the above four THC-induced behaviors. Kinase inhibitors that specifically inhibit cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinase (PKG), calmodulin-dependent protein kinase (PKC) and src tyrosine kinase were tested for reversal of tolerance to THC's effects. PKG and PKC inhibitors did not reverse tolerance in any behavioral measure. Src tyrosine kinase inhibition reversed tolerance to only the hypoactive effects of THC. PKA inhibition reversed tolerance to all measures, although the doses of inhibitor and time-course of inhibition varied among behaviors. Thus, our data suggest that PKA activity plays a major role in THC-induced tolerance, and that THC produces its multiple effects through different signaling pathways.
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Keywords: Cannabinoid; Kinase; Antinociception; Tolerance

1. Introduction

Cannabinioids such as Δ^9-tetrahydrocannabinoid (THC) produce a unique profile of effects in rodents that includes antinociception, hypothermia, catalepsy and inhibition of spontaneous activity (Martin et al., 1991). Through the use of a receptor-selective antagonist and receptor deletion in mice, several laboratories have demonstrated that these THC effects are mediated through the cannabinoid CB_1 receptor (Rinaldi-Carmona et al., 1994; Compton et al., 1996; Zimmer et al., 1999; Di Marzo et al., 2000). While there is evidence to suggest that other cannabinoid receptor subtypes may exist in the central nervous system (Breivogel et al., 2001), it is clear that multiple centrally mediated effects of THC can be attributable to the cannabinoid CB_1 receptor. The question then arises as to how a multitude of behavioral effects can be mediated though a single receptor subtype. There is ample evidence that the cannabinoid CB_1 receptor activates numerous signaling pathways that include multiple G-proteins, potassium and N-type calcium channels, as well as numerous kinases (Howlett et al., 2002). Of course, multiple behavioral effects of THC could arise from activation of a single receptor subtype, such as the cannabinoid CB_1 receptor, and a single signaling pathway, the difference being that the receptor and signaling pathway are located in different brain areas which regulate different physiological functions. On the other hand, it is possible that unique signaling pathways in different brain areas are responsible for each pharmacological effect. Regardless of the mechanism, linking a particular signaling pathway to a specific pharmacological effect would provide valuable insight.

Considerable insight can be gained in receptor-signaling pathways by examining the adaptive changes that occur in the development of tolerance. A remarkable degree of tolerance can be induced to multiple pharmacological effects of THC upon repeated administration (Fan et al., 1994; Sim-Selley and Martin, 2002). Comparison of max-

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imal tolerance induced by a partial agonist (THC) and the full agonist *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate] (WIN 55,212-2) revealed differing degrees of cannabinoid CB_1 receptor down-regulation throughout brain (Sim-Selley and Martin, 2002). In addition, the time-course through which tolerance develops and recovers for THC-induced hypoactivity and antinociception differs (Bass and Martin, 2000). These findings further support the notion that different mechanisms may underlie the expression of tolerance to the different effects of cannabinoids.

Despite a thorough in vivo characterization of cannabinoid tolerance, the cellular events responsible for these adaptive changes remain unclear. It has been shown that numerous alterations in cannabinoid receptor number and affinity, as well as signal transduction mechanisms occur in tolerant animals (Oviedo et al., 1993; Rinaldi-Carmona et al., 1998; Rubino et al., 2000). Tolerance is accompanied by an enhanced activation of the cAMP pathway represented by an increase in adenylyl cyclase activity, basal cAMP production and an increase in cyclic AMP-dependent protein kinase (PKA) activity (Rubino et al., 2000). Kinases play a crucial role in adaptive events in several neuronal systems. Tolerance to opioids is often accompanied by an up-regulation in the cAMP pathway that is thought to involve changes in both adenylyl cyclase and PKA (Punch et al., 1997). It has also been shown that opioid tolerance is associated with a supersensitization of adenylyl cyclase, possibly due to phosphorylation by calmodulin-dependent protein kinase (PKC) or by the up-regulation of different adenylyl cyclase isoforms (Liu and Anand, 2001). Chronic opioid treatment appears to be associated with an increase in PKA concentration and activation (Liu and Anand, 2001). The down-regulation of another G-protein coupled receptor (GPCR), the β_2-adrenoceptor receptor, occurs via PKA-mediated phosphorylation (Daaka et al., 1997).

The mere observation that kinases are altered in tolerant states does not distinguish between cause and effect. However, studies have been conducted in which tolerance to the antinociceptive effects of THC are reversed by the intrathecal administration of a specific PKA inhibitor and a src tyrosine kinase inhibitor (Lee et al., 2003). The reversal of the tolerant state by such inhibitors suggests that PKA and src tyrosine kinase are involved in the expression of tolerance to spinally mediated analgesia rather than a consequence of tolerance. It is presently unknown whether these kinases are also altered in the expression of tolerance to other cannabinoid-mediated effects. Therefore, studies were carried out in order to determine the contribution of candidate kinases to THC-induced tolerance expression for each of the measures of the mouse tetrad. Experiments were conducted in which inhibitors of PKA, PKC, cyclic GMP-dependent protein kinase (PKG) and src tyrosine kinase were administered centrally prior to an i.v. challenge with THC in tolerant mice. The ability of these inhibitors to reverse tolerance in each of the four measures of the mouse tetrad was determined.

2. Materials and methods

2.1. Materials

Male ICR mice (Harlan Laboratories, Indianapolis, IN) weighing between 24 and 30 g were used in all experiments. Mice were maintained on a 14:10-h light/dark cycle with food and water available ad lib. All test groups consisted of 6–12 mice. THC was obtained from NIDA and dissolved in a 1:1:18 solution of ethanol, emulphor and saline, respectively. (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (KT5720), (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo-(*a*,*g*)-cycloocta-(*c*,*d*,*e*)-trinden-1-one] (KT5823) and bisindolylmaleimide I, HCl were purchased from Calbiochem (La Jolla, CA). 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) was purchased from Alexis (San Diego, CA). KT5720 is a specific PKA inhibitor while KT5823, bisindolylmaleimide I, HCl and PP1 inhibit PKG, PKC and src tyrosine kinase, respectively. All inhibitors were administered by intracerebroventricular (i.c.v.) injection in a volume of 5 μl/mouse in ether-anaesthetized mice. Mice were pretreated with KT5720, KT5823 and bisindolylmaleimide 15 min prior to the i.v. injection of THC. PP1 was administered 10 min prior to the i.v. injection of THC. KT5720, KT5823 and PP1 were dissolved in 13% DMSO, while bisindolylmaleimide I, HCl was dissolved in dH_2O.

2.2. Determination of maximal ineffective dose of kinase inhibitors

To ensure that an ineffective dose of the various inhibitors were used in the interaction studies, naïve mice were injected with different doses of the inhibitor, followed by an i.v. injection of 1:1:18 vehicle, and subsequent behavioral evaluations. A dose that did not produce an effect on any of the four behavioral measures was used in subsequent tolerance-reversal experiments.

2.3. Effects of kinase inhibitors on acute THC activity

The ability of the kinase inhibitors to alter the acute behavioral effects of THC was determined. Naïve mice received an i.c.v. injection of kinase inhibitor followed by an i.v. injection of vehicle or THC (3 mg/kg). This dose of THC was chosen because it has less than maximal effects in all four pharmacological measures in ICR mice (Martin et al., 1991; Smith et al., 1993; Bass and Martin, 2000). The

mice were then tested in the four behavioral measures of the mouse tetrad.

2.4. Tolerance protocol

The protocol to produce tolerance to THC has been described previously (Bass and Martin, 2000). Briefly, the mice were injected subcutaneously with THC (10 mg/kg) or vehicle twice per day (approximately 9 AM and 4 PM) for 6 days. On the seventh day, the mice received the 9 AM injection only. Thus, each mouse received a total of 13 injections. Twenty-four hours following the final injection, the mice were administered either vehicle or a kinase inhibitor (i.c.v.) prior to being challenged with a single i.v. dose of THC (3 mg/kg). The mice were then tested in four behavioral assays to assess tolerance.

2.5. Behavioral evaluations

All animals were allowed to acclimate to the observation room overnight. Behavioral tests were conducted to measure tolerance to antinociception, catalepsy, hypothermia and hypomobility. All four measures were determined sequentially in the same animal by ''blind'' observers. The baselines for tail-flick latency (2–4 s) and rectal temperature were determined prior to i.c.v. injections. Rectal temperatures were measured using a telethermometer and a thermometer probe inserted to 25 mm (Yellow Springs Instrument, Yellow Springs, OH). The mice were then given an i.c.v. injection of kinase inhibitor followed by an i.v. injection of THC (3 mg/kg). The mice were placed in individual photocell activity chambers 5 min later. Spontaneous activity was monitored for 10 min in a Digiscan Animal Activity Monitor (Omnitech Electronice, Columbus, OH) as measured by the number of interruptions of 16 photocell beams per chamber. The total number of beam interruptions during the 10-min period was determined and presented as total counts. The mice were then assessed at 20 min following i.v. injection for antinociception using the tail-flick reaction time to a heat stimulus. A 10-s maximum latency was used in order to avoid tail injury. The results are presented as % MPE (maximum possible effect) and are calculated as follows:

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

Rectal temperature was measured 30 min post-i.v. injection. The change in rectal temperature (Δ °C) following THC administration was calculated for each animal. Catalepsy was measured 40 min post-i.v. injection by the ring-immobility test. Mice were placed on a ring 5.5 cm in diameter attached to a stand at a height of 16 cm. The amount of time the mice spent motionless on the ring during the 5-min procedure was measured, with the criteria of immobility being defined as the absence of all voluntary movements, including whisker movement, but excluding respiration. The percent immobility was calculated as:

$$\%\ \text{immobility} = [\text{time immobile (s)}] / [\text{length of session (s)}] \times 100$$

Mice that fell from the ring or actively jumped were allowed five attempts. After the fifth escape, these mice were removed from the ring and not included in the calculations. Data was collected from 6 to 12 mice for each condition tested.

All studies were carried out in accordance with the Declaration of Helsinki and Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

2.6. Data analysis

Means and standard error (S.E.) were calculated for % MPE, number of photocell disruptions, % ring immobility and Δ °C. Analysis of variance (ANOVA) was used to determine significant differences between control and treatment groups followed by Dunnett's *t* test post hoc analysis. Statistical analysis was performed using StatView, version 5.0 (SAS Institute, Cary, NC). Significance was defined as a $p < 0.05$. An inhibitor was determined to have an effect on the acute properties of THC if the combined effect of the inhibitor plus THC injection produced a significant change from the veh/THC combination. Veh/veh and inhibitor/veh control groups were evaluated in acute experiments to ensure that the dose of inhibitor (i.c.v.) did not alter the behavioral effects being quantified. Drug combinations in the reversal studies are designated as follows: chronic treatment/i.c.v treatment/i.v. challenge dose. For example, the veh/veh/THC group received 13 vehicle treatments over 6.5 days, an i.c.v. injection of vehicle on day 8, followed by a challenge dose of THC (3 mg/kg, i.v.). Four planned comparisons were analyzed where all groups were compared to the mice that received veh/veh/THC treatment using the Dunnett's *t* test. The ability of an inhibitor to reverse tolerance was defined as the lack of significance between the THC/inhibitor/THC group and the veh/veh/THC group. The veh/veh/THC group demonstrated the level of effect that THC (3 mg/kg i.v.) elicited by itself. The behavioral quantification of the THC/veh/THC group indicates the level of tolerance produced with chronic THC treatment. The veh/inhibitor/THC group served as the control to determine if the inhibitor produced an effect on the i.v. challenge dose of Δ^9-THC in non-tolerant animals. The THC/inhibitor/THC group results indicate the effect the inhibitor had on tolerance. If mice remained tolerant after the inhibitor was administered, then we would expect that this group would be significantly different from the veh/veh/THC group. If the expression of tolerance were reversed in the behavioral tetrad tests, then we would observe no

significant differences between the veh/veh/THC group and the THC/inhibitor/THC group.

3. Results

3.1. Evaluation of the PKG inhibitor KT5823 on acute and chronic THC effects

Initial studies were conducted to determine whether i.c.v. administration of KT5823 alone produced any pharmacological effects. Doses of 1, 3 and 5.6 μg/mouse were evaluated, and none produced any statistically significant effects on spontaneous activity, tail-flick response, immobility and rectal temperature. The 5.6 μg/mouse dose produced a slight but non-signficant antinociceptive effect. Therefore, a dose of 3.0 μg/mouse was chosen for further study. This dose was also used in a previous study with THC (Lee et al., 2003). Naïve mice were then injected i.c.v with 3 μg KT5823/mouse followed 15 min later by an i.v. injection of THC. KT5823 did not attenuate the effects of an acute injection of THC in measures for antinociception, catalepsy, hypothermia or hypoactivity (data not shown). Moreover, KT5823 did not alter the effects of a challenge dose of THC in THC-tolerant mice. KT5823 clearly did not reverse the expression of tolerance produced by chronic administration of THC in three measures of the mouse tetrad (Fig. 1). Unfortunately, a significant level of tolerance was not produced by chronic administration of THC in the test for THC-induced hypoactivity. The large standard error for the THC/veh/THC group indicates that the variability was too great to produce a significant level of tolerance for this measure. It is apparent, however, that KT5823 did not reverse tolerance for this measure since the THC/KT5823/THC group exhibited a significant level of tolerance.

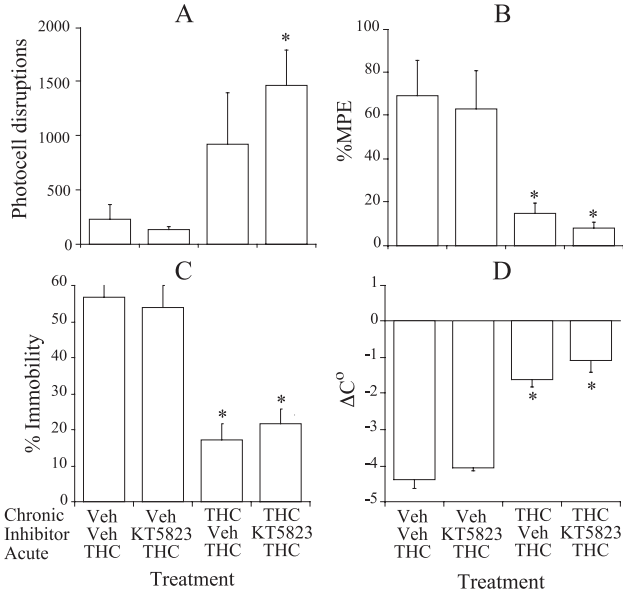

Fig. 1. Effect of the PKG inhibitor KT5823 on THC tolerance. KT5823 (3 μg/mouse) or 13% DMSO vehicle was administered i.c.v. 15 min prior to an i.v. injection of 3 mg/kg THC to mice treated chronically with vehicle or Δ^9-THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean effect (plus S.E.) of 6–12 mice. *Significantly different from the veh/veh/THC group at $p < 0.05$ by Dunnett's t test.

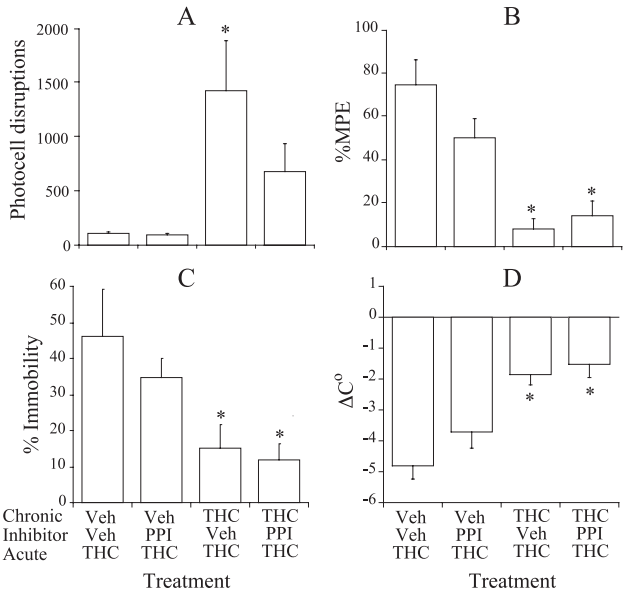

Fig. 2. Effect of src tyrosine kinase inhibitor PP1 on THC tolerance. PP1 (0.01 μg/mouse) or DMSO vehicle was administered i.c.v. 10 min prior to an i.v. injection of 3 mg/kg Δ^9-THC to mice treated chronically with vehicle or Δ^9-THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean (plus S.E.) of 6–12 mice. *Significantly different from the veh/veh/THC group at $p < 0.05$ by Dunnett's t test.

3.2. Evaluation of the src tyrosine kinase inhibitor PP1 on acute and chronic THC effects

Initial studies were carried out to establish a dose of PP1 that did not have an affect on baseline activity of the mice. Even at the highest dose tested (0.01 μg, i.c.v.), PP1 failed to produce any effects in the tetrad. The dose used (0.01 μg/mouse) is approximately 100-fold greater than that used in an earlier study (Lee et al., 2003) to reverse tolerance to THC-induced spinal analgesia and was therefore used in subsequent experiments. PP1 did not reverse THC tolerance for the measures of hypothermia, antinociception and catalepsy (Fig. 2, panels B, C and D). In all cases, the THC/veh/THC and THC/PP1/THC groups are significantly different from the veh/veh/THC group. However, PP1 did significantly reverse tolerance for the measure of hypoactivity (Fig. 2, panel A). Significant tolerance developed to THC's effects on spontaneous activity as evidenced by a significant difference between the veh/veh/THC and THC/veh/THC groups. However, the THC/PP1/THC group was not significantly different from the veh/veh/THC group. It is impor-

tant to note that while PP1 reversed THC tolerance to hypoactivity, it did not appear to fully restore THC's effects.

3.3. Evaluation of the PKC inhibitor bisindolylmaleimide on acute and chronic THC effects

Doses of 0.5, 1.25, 2.5, 5 and 10 μg bisindolylmaleimide/mouse were adiministered i.c.v. to determine their pharmacological effects in naïve mice. The dose of 10 μg/mouse produced significant antinociception, immobility and hypothermia. Therefore, subsequent experiments were performed with 5 μg/mouse, a dose that did not produce significant effects in any behavioral measure. Bisindolylmaleimide did not appear to alter any of the acute effects of THC because in no instance did the veh/THC group significantly differ from the bisindolylmaleimide/THC group (Fig. 3). However, bisindolylmaleimide produced a pronounced but nonsignificant decrease in spontaneous activity and a trend toward an increase in immobility (Fig. 3, panels A and C, respectively) that nevertheless represent possible confounds. In addition, the degree of catalepsy produced by the veh/THC group in this particular experiment was uncharacteristically low. Bisindolylmaleimide did not reverse THC tolerance for the measures of antinociception and hypothermia in that the THC/veh/THC and THC/bisindolylmaleimide/THC groups are both significantly different from the veh/veh/THC group in panels B and D (Fig. 4). However, bisindolylmaleimide significantly reversed tolerance to the hypoactive effects of THC. The veh/veh/THC and THC/bisindolylmaleimide/

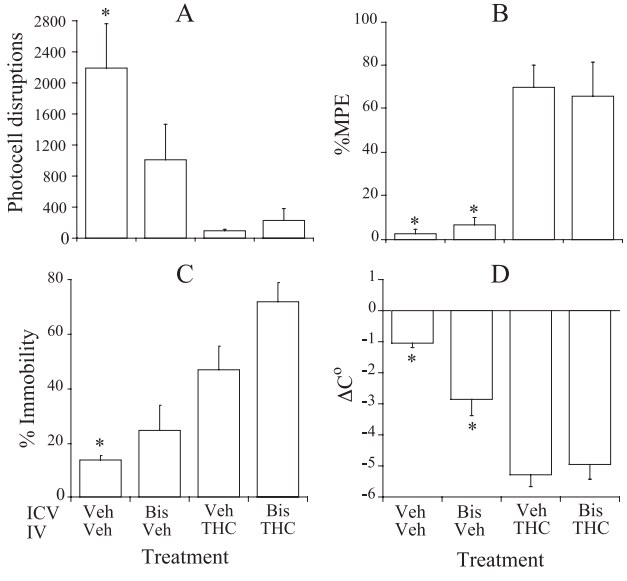

Fig. 3. Effect of PKC inhibitor bisindolylmaleimide on THC activity in näive mice. Bisindolylmaleimide was administered i.c.v to naïve mice at a dose of 5 μg/mouse followed 15 min later by an i.v. injection of THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean (plus S.E.) of 6–12 mice. *Significantly different from the veh/THC group at $p<0.05$ by Dunnett's t test.

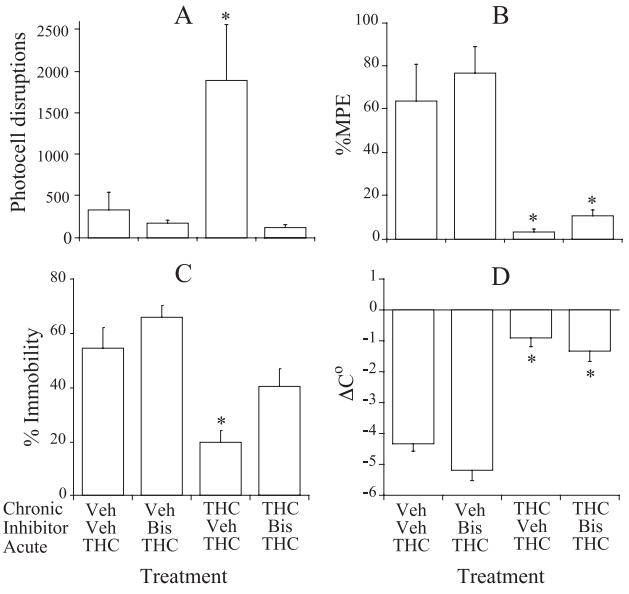

Fig. 4. Effect of the PKC inhibitor bisindolylmaleimide on THC tolerance. Bisindolylmaleimide (5 μg/mouse) or distilled water was administered i.c.v. 15 min prior to an i.v. injection of 3 mg/kg THC to mice treated chronically with vehicle or Δ^9-THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean effect (± S.E.) of 6–12 mice. *Significantly different from the veh/veh/THC group at $p<0.05$ by Dunnett's t test.

THC groups in panel A are not significantly different, whereas the veh/veh/THC and THC/veh/THC groups are significantly different. However, we cannot rule out the possibility that bisindolylmaleimide itself might have contributed to the hypoactivity in the THC/bisindolylmaleimide/THC group thereby making it difficult to distinguish between partial and complete reversal. Bisindolylmaleimide attenuated tolerance to the cataleptic effects of THC in that the veh/veh/THC and THC/bisindolylmaleimide/THC groups are not significantly different (Fig. 4, panel D). It appears that bisindolylmaleimide did not completely restore THC's cataleptic effects to control levels.

3.4. Evaluation of the PKA inhibitor KT5720 on acute and chronic THC effects

Administration of KT5720 (1, 3 and 5.6 μg/mouse, i.c.v.) to naïve mice did not produce any alterations in baseline activity in any of the four behavioral measures at any dose tested. Therefore, a dose of 3 μg/mouse was evaluated for its potential effects on the acute administration of THC in naïve mice which is comparable to that used by Lee et al. (2003). This dose did not alter the acute effects of THC in measures of hypoactivity, antinociception, and hypothermia (Fig. 5A, B and D, respectively). However, this dose of KT5720 does appear to significantly decrease the acute cataleptic effect of THC, because the veh/THC and KT5720/THC groups are significantly different (Fig. 5C). Subsequent experiments demonstrated that this dose of KT5720 reversed tolerance to

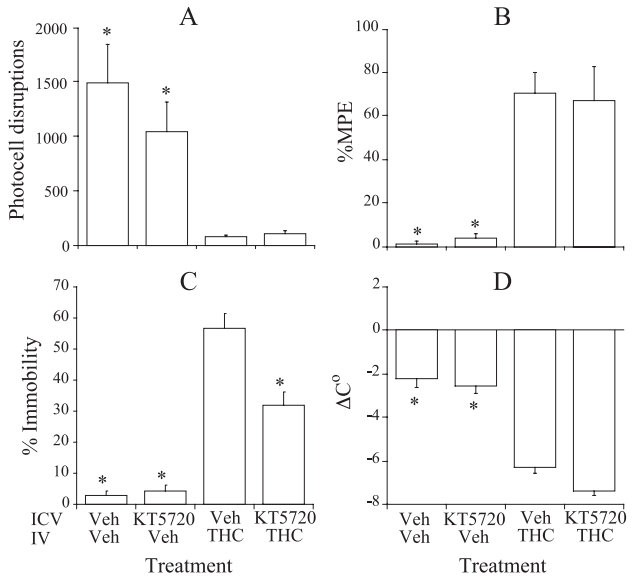

Fig. 5. Effect of the PKA inhibitor KT5720 on THC activity in näive mice. KT5720 was administered i.c.v. to naïve mice at a dose of 3 μg/mouse followed 15 min later by an i.v. injection of Δ^9-THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean effect (± S.E.) of 6–12 mice. *Significantly different from the veh/THC group at $p < 0.05$ by Dunnett's t test.

the hypoactive, antinociceptive, and cataleptic effects of THC (Fig. 6A, B and C, respectively) as indicated by the lack of significance between the THC/KT5720/THC group and the vehicle/vehicle/THC group. Despite the fact that KT5720 attenuated THC-induced catalepsy in naïve mice, KT5720 was still able to reverse tolerance to THC-induced cataleptic effects. However, KT5720 did not reverse tolerance to the hypothermic effects of THC (Fig. 6D). Unfortunately, KT5720 attenuated THC's hypothermic effects in the vehicle-treated mice as indicated by the significant difference between the veh/KT5720/THC and the veh/veh/THC groups. This decrease is puzzling given that KT5720 did not produce any effects on THC in naïve mice as shown in Fig. 5D. Due to a possible confound from KT5720's effects on THC hypothermia in the vehicle-treated mice, a subsequent study was conducted with a lower dose of KT5720 (2 μg/mouse). This dose of KT5720 did not produce any behavioral effect in mice treated chronically with vehicle, and it was unable to reverse the tolerance to THC-induced hypothermia (data not shown).

While the reversal of tolerance with KT5720 was complete for the measures of antinociception and catalepsy, the reversal of tolerance to the THC-induced suppression of spontaneous activity was not complete. Although treatment with KT5720 (3 μg/mouse) did result in a significant decrease in tolerance for this measure, as indicated by decreases in activity, the level of effect did not return to that of mice chronically treated with vehicle (Fig. 6A). Subsequent experiments were conducted with 3 and 5.6 μg/mouse of KT5720 in an effort to determine if KT5720 could produce a complete reversal of tolerance for the measure of hypoactivity (Fig. 7). KT5720 (3 μg/mouse) produced a significant reduction in tolerance, but the effects of THC were not completely restored. However, a higher dose (5.6 μg/mouse) fully reversed tolerance. It can therefore be concluded that the PKA inhibitor KT5720 can completely reverse tolerance to THC for the measures of

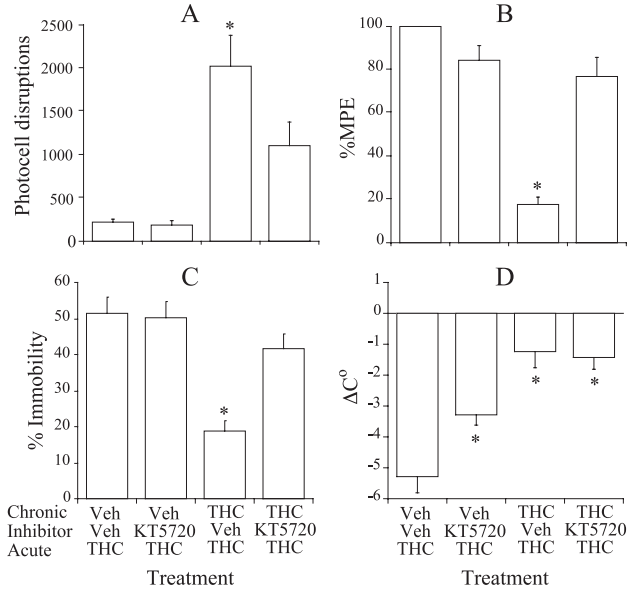

Fig. 6. Effect of the PKA inhibitor KT5720 treatment on THC tolerance. KT5720 (3 μg/mouse) or 13% DMSO vehicle was administered i.c.v. 15 min prior to an i.v. injection of 3 mg/kg THC to mice treated chronically with vehicle or Δ^9-THC. The animals were evaluated for spontaneous activity (A), tail-flick response (B), immobility (C) and rectal temperature (D) as described in the Materials and methods. Each bar represents the mean effect (± S.E.) of 6–12 mice. *Significantly different from the vehicle/vehicle/THC group at $p < 0.05$ by Dunnett's t test.

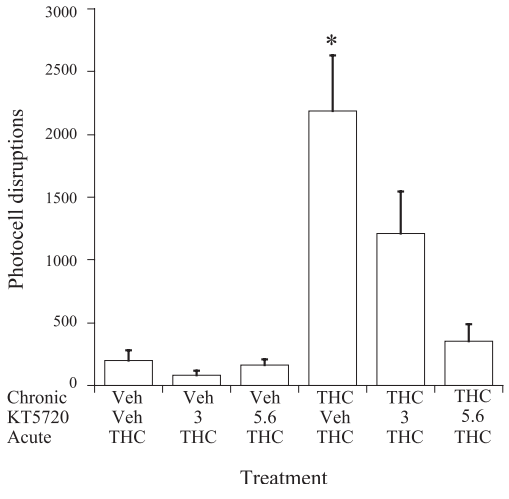

Fig. 7. Effect of different doses of KT5720 on tolerance to THC-induced hypoactivity. KT5720 (3 or 5.6 μg/mouse) or DMSO vehicle was administered i.c.v. 15 min prior to an i.v. injection of 3 mg/kg THC to mice treated chronically with vehicle or Δ^9-THC. Each bar represents the mean effect (plus S.E.) of 6–12 mice. *Significantly different from the veh/veh/THC group at $p < 0.05$ by Dunnett's t test.

antinociception, catalepsy and hypoactivity with greater potency in the former two measures. There are several possible interpretations of KT5720's inability to reverse tolerance to the hypothermic effects of THC. It may be that PKA does not play a role in tolerance to THC-induced hypothermia. Alternatively, a lower concentration of cannabinoid CB_1 receptors in the hypothalamus may dictate different levels of tolerance.

3.5. Time-course studies for KT5720 reversal of THC tolerance

To more fully characterize the role of PKA in THC tolerance, studies were conducted to characterize the participation of PKA in the development of tolerance. The data for antinociception and hypoactivity are presented in Fig. 8. Each panel represents a different group of mice that were chronically treated with either vehicle or THC for either 1.5, 3.5 or 6.5 days. The ability of KT5720 to reverse tolerance at each time point was then assessed. KT5720 did not reverse tolerance for the measure of antinociception after 1.5 or 3.5 days of chronic treatment with Δ^9-THC at either the 3 or 5.6 μg/mouse dose. However, after 6.5 days of dosing, a complete reversal of tolerance occurred with 3 μg/mouse of KT5720. A completely different pattern was observed for the measure of hypoactivity. Reversal of tolerance by KT5720 was observed after 1.5, 3.5 and 6.5 days of dosing with THC for this measure. The reversal was observed with the 3 μg/mouse dose of KT5720; however, the 5.6 μg/mouse dose was needed to return the mice to a level nearly equal that of the non-tolerant mice. Previous time-course studies utilizing dose–response curves revealed that tolerance develops for both measures within 1.5 days of dosing and increases with 3.5 days of dosing (Bass and Martin, 2000). The degree of tolerance remains the same after 6.5 days of dosing for the measure of antinociception, and actually decreases for hypomobility. It is apparent that the participation of PKA in tolerance to the antinociceptive effects of THC does not begin until 6.5 days of dosing. However, PKA does appear to be involved at a much earlier time point for the measure of hypomobility.

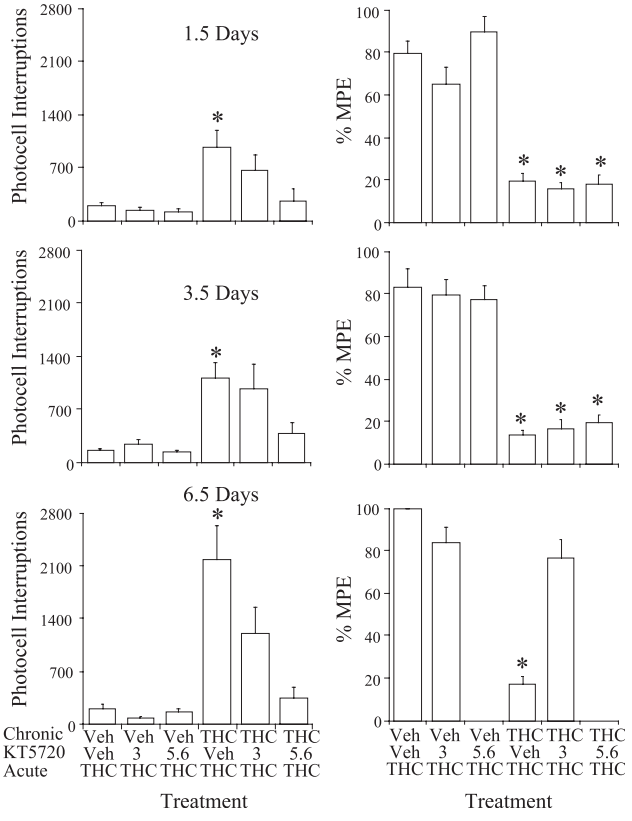

Fig. 8. Effect of KT5720 on the time-course of tolerance to the hypoactivity and antinociceptive effects of THC. Mice were treated chronically with THC for either 1.5, 3.5 or 6.5 days. Mice received an i.c.v. injection of 3 or 5.6 μg/mouse KT5720 15 min prior to an i.v. challenge with 3 mg/kg Δ^9-THC, with the exception of day 6.5 when the animals were treated with only the 3 μg/mouse dose for antinociceptive testing. Tolerance to the hypoactive effects of THC are presented in the three panels on the left and to analgesia on the right. *Significantly different from the veh/veh/THC group at $p<0.05$ by Dunnett's t test.

4. Discussion

It is well established that considerable tolerance develops to cannabinoids, particularly when rigorous treatment regimens are employed (Fan et al., 1994; Sim-Selley and Martin, 2002). The mechanisms underlying tolerance to cannabinoids remain to be elucidated. Studies of tolerance have addressed numerous potential mechanisms. Studies investigating the relationship between cannabinoid receptor down-regulation and behavioral tolerance to THC have been contradictory. Chronic administration of cannabinoids has been shown to produce receptor down-regulation and a concomitant reduction in the regulation of second messenger systems (Oviedo et al., 1993; Rinaldi-Carmona et al., 1998; Rubino et al., 2000). There have been conflicting reports of increased receptor binding (Romero et al., 1995) or no change in receptor binding in whole brain homogenates (Abood et al., 1993) during the development of tolerance.

Studies of G-protein activation have been used to demonstrate cannabinoid receptor desensitization following chronic THC treatment (Sim et al., 1996). It has been shown that cannabinoid CB1 receptor desensitization is dependent upon the efficacy of the cannabinoid used and the brain area studied (Fan et al., 1994; Sim-Selley and Martin, 2002). Homologous desensitization to the inhibition of cAMP accumulation occurs during chronic cannabinoid exposure (Dill and Howlett, 1988) leading to a significant increase in cAMP levels and PKA activity in the same areas that cannabinoid CB_1 receptor down-regulation is observed (Rubino et al., 2000). It has also been established that PKA is involved in spinally mediated cannabinoid analgesia (Lee et al., 2003).

The effects of cannabinoids on multiple families of kinases indicate the importance of alterations in protein phosphorylation in the mechanism of action of cannabinoids. THC increases the activity of brain protein kinase C (PKC) in vitro. Neurotransmitters that activate PKC restore the neuronal excitability and synaptic activity inhibited by cannabinoids (Hillard and Auchampach, 1994). Inhibition of PKC activity blocks the antinociceptive effect of THC (Lee et al., 2003). In addition, cannabinoid CB_1 receptor activation of the $\beta\gamma$ subunit of G-proteins stimulate src tyrosine kinases that in turn activate mitogen-activated protein kinase via phosphorylation (Bouaboula et al., 1997). The src tyrosine kinase inhibitor, PP1, reverses THC antinociceptive tolerance in the spinal cord (Lee et al., 2003). Thus, the question arises as to whether tyrosine kinases play a role in the development or expression of tolerance to cannabinoid effects other than spinally mediated analgesia. Therefore, the present study examined the role of PKA, PKG, PKC and src tyrosine kinase in supraspinally mediated behavioral effects of cannabinoids.

An important observation from our study is that the role of PKA is not confined to cannabinoid-induced analgesia. Tolerance to THC-induced antinociception and catalepsy is readily and completely reversed by KT5720. Higher doses of KT5720 were required to reverse tolerance to THC-induced hypoactivity. Tolerance to THC-induced hypothermia was not reversed by KT5720. These findings are intriguing given that all four of the cannabinoid pharmacological effects are mediated through the cannabinoid CB1 receptor. The differential effects of KT5720 on tolerance indicate that multiple signaling pathways are involved in diverse cannabinoid effects.

The results with KT5823 suggest that PKG is not involved in tolerance to any THC effect that we measured. Similarly, PP1 treatment did not reverse tolerance to THC-induced antinociception, catalepsy or hypothermia. Furthermore, the reversal of tolerance to THC-induced hypoactivity was not complete suggesting that src tyrosine kinase does not play a major role in this effect. Failure of PPI to reverse tolerance to THC's antinociceptive effects was unexpected given the previous finding that it reversed antinociceptive tolerance when administered spinally (Lee et al., 2003). These differences are even more striking considering that the i.c.v. doses used in the present study were 100 times greater than those administered spinally. A role for src tyrosine kinase in only the spinal components of tolerance underscores the point that multiple signaling pathways are likely to be involved the development of tolerance throughout the central nervous system. While PKA may be an important contributor to the expression of tolerance, it is likely that tolerance is the result of many converging mechanisms. The ability of one kinase to totally reverse tolerance for any particular measure suggests that either that kinase is the only contributing factor or that other mechanisms promoting tolerance converge on the PKA pathway. Although the origin of the adaptive process might lie upstream in cannabinoid CB_1 receptor number or affinity, the consequences of PKA inhibition down-stream would be the reversal of tolerance.

The ability of KT5720 to completely reverse tolerance suggests that attenuation of PKA activity alone accounts for tolerance. However, time-course studies suggest that PKA is not totally responsible for expression of tolerance, particularly that involving antinociceptive tolerance. It is apparent that tolerance is not reversed until the 6.5 day time point for the measure of antinociception, but is reversed as early as the 1.5 day time point and through the 3.5 and 6.5 day time points for hypoactivity. The differences in the time-courses between the two measures indicate a difference in the mechanisms responsible for their development with PKA modulation being a critical component. It is also important to note that PKA inhibition of tolerance to the hypoactive, cataleptic and antinociceptive effects of THC was not only time-dependent, but also dose-dependent. A higher dose of the inhibitor was needed to completely reverse tolerance to the hypoactive effects of THC. PKA may play a more important role in tolerance during earlier time points for the measure of hypoactivity, but the fact that a higher dose is needed throughout to reverse tolerance for this measure indicates that it is not the only mechanism at work. Our studies do not completely rule out a role for PKC and src tyrosine kinases. It is certainly reasonable to conclude that if they contribute at all, their contribution is much less than that of PKA. Determining whether PKA is responsible for, rather than a consequence of, tolerance is also not readily discernable from our studies. It is also important to point out that the PKA inhibitor KT5720 could have other unknown mechanisms when administered in vivo that accounts for the reversal of tolerance. It will be important in future studies to directly measure the activity of PKA in the development and reversal of tolerance. Regardless of the mechanism, it is remarkable that KT5720 has such dramatic effects on cannabinoid tolerance.

While there is still much to learn about how the cannabinoid system is regulated through phenomena such as tolerance, it is possible to obtain some indication of the underlying processes by examining GPCR systems that have been more thoroughly studied. Opioids provide a relevant comparison to the cannabinoid system in that opioid receptors are coupled to many of the same effector systems as cannabinoids. Opiates also produce a marked degree of tolerance. Bi-directional cross-tolerance between kappa opioid agonists and THC suggests a significant portion of the cannabinoid effect is kappa-opioid receptor-mediated (Smith et al., 1993). Tolerance to opiates is accompanied by an up-regulation in the cAMP pathway that is thought to involve changes in both adenylyl cyclase and PKA (Punch et al., 1997). Evidence also suggests that opioid receptors can stimulate the production of cAMP during tolerance by directly stimulating adenylyl cyclase via $G\alpha_s$ proteins. Chronic opioid treatment is associated

with an increase in PKA concentration and activation (Batkai et al., 2001). It has been reported that up-regulation of PKA activity occurs with chronically, but not acutely, administered morphine in the locus coeruleus (Nestler and Tallman, 1988). Reversal of morphine tolerance with the PKA inhibitor, KT5720, has been observed (Bernstein and Welch, 1997). Reversal of dependence to morphine has also been observed after injection of a PKA inhibitor (Punch et al., 1997). In addition, the ability of selective PKC inhibitors to reverse morphine tolerance implicates the phosphatidylinositol system as well (Smith et al., 1999; Smith et al., 2002).

There are several caveats to take into account when conducting experiments of this nature. It is critical to use doses of inhibitors that are devoid of effects themselves. Studies with agents such as bisindolylmaleimide demonstrate the confusion that can result from the use of an inhibitor that produces an effect by itself in the behavioral model. Evaluation of time-courses for reversal of tolerance is also critical as evidenced by the KT5720 study. In addition, the efficacy of the tolerance-inducing agent is likely to play a role in the activation of kinase pathways in tolerance expression. Although we cannot discern the nuances of certain contributory mechanisms, our studies provide evidence for kinase involvement when an inhibitor reverses tolerance. Therefore, while PKG and src tyrosine kinase did not appear to have a role in tolerance as defined by the endpoints of this study, the data do not necessarily rule out a role in tolerance altogether.

In summary, the evidence presented supports the theory that activation of the cAMP pathway plays a role in tolerance to THC. The exact nature of this up-regulation is beyond the scope of this study and remains undetermined. Further studies will need to verify the role of PKA in tolerance and how it is integrated with other mechanisms of tolerance.

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