

# Reversal of Morphine Antinociceptive Tolerance and Dependence by the Acute Supraspinal Inhibition of $\text{Ca}^{2+}$ /Calmodulin-Dependent Protein Kinase II<sup>[S]</sup>

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## ABSTRACT

Previous studies have suggested that  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) can modulate opioid tolerance and dependence via its action on learning and memory. In this study, we examined whether CaMKII could directly regulate opioid tolerance and dependence. CaMKII activity was increased after the treatment with morphine (100 mg/kg s.c. or 75 mg s.c. of morphine/pellet/mouse); the effect exhibited a temporal correction with the development of opioid tolerance and dependence. In mice treated with morphine (100 mg/kg s.c.), morphine tolerance and dependence developed in 2 to 6 h. An acute supraspinal administration of KN93 [2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine], a CaMKII inhibitor, was able to dose-dependently reverse the already-established antinociceptive tolerance to morphine ( $p < 0.001$  for 15–30 nmol; not significant for 5 nmol). KN92 [2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine] (30

nmol i.c.v.), a kinase-inactive analog of KN93, did not affect opioid tolerance. Neither KN92 nor KN93 affected basal nociception or acute morphine antinociception (1–10 nmol i.c.v.). Likewise, dependence on morphine was abolished by the acute administration of KN93, but not KN92, in a dose-dependent manner. Pretreatment of mice with KN93 also prevented the development of morphine tolerance and dependence. The effect of acute CaMKII inhibition was not limited to the particular experimental model, because KN93 also acutely reversed the established opioid tolerance and dependence in mice treated with morphine (75 mg/pellet/mouse s.c.) for 6 days. Taken together, these data strongly support the hypothesis that CaMKII can act as a key and direct factor in promoting opioid tolerance and dependence. Identifying such a direct mechanism may be useful for designing pharmacological treatments for these conditions.

Opioids, such as morphine, are clinically used primarily as analgesics. Drug tolerance and dependence are two of the major problems associated with these drugs, which greatly limit their effectiveness and usage. The molecular mechanisms underlying opioid tolerance and dependence are not entirely understood. The current study aims to test the hypothesis that  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) can directly regulate opioid tolerance and dependence.

CaMKII is a multifunctional  $\text{Ca}^{2+}$ /calmodulin-activated protein kinase, whose  $\alpha$  and  $\beta$  isoforms are abundant in the central nervous system (Hudmon and Schulman, 2002). A vast amount of information is available for the interaction of CaMKII  $\alpha$  isoform and N-methyl-D-aspartate (NMDA) receptors in generating long-term potentiation in hippocampal neurons, which is critical for learning and memory (e.g., Mayford et al., 1996). Inhibition or disruption of CaMKII impairs spatial learning (Silva et al., 1992). Interestingly, antagonists of the NMDA receptor effectively inhibit the development of opioid tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991). It also has been reported that chronic microinjection of CaMKII inhibitors into hippocampus was able to prevent the development of opioid tolerance (Fan et al., 1999). The same CaMKII inhibitors were ineffective when chronically

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**ABBREVIATIONS:** CaMKII,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; CREB, cAMP-response element-binding protein; KN93, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; KN92, 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; MK801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; NMDA, N-methyl-D-aspartate;  $\mu\text{OR}$ ,  $\mu$  opioid receptor; MPE, maximum possible effect; pCaMKII, phosphorylated CaMKII; MS, morphine.

microinjected into striatum. Furthermore, acute administration of these CaMKII inhibitors into hippocampus did not affect opioid tolerance. These data supported the importance of learning and memory pathways in opioid tolerance (Fan et al., 1999).

However, these studies did not address the possibility that CaMKII could directly (i.e., not necessarily relying on learning and memory) modulate opioid tolerance and dependence. Identifying such a direct mechanism for CaMKII is not only important to our understanding but will also have a profound impact on designing therapeutic interventions for opioid tolerance and dependence. Studies have suggested that the NMDA system may be directly involved in promoting opioid tolerance and dependence (Gutstein and Trujillo, 1993). Similar direct mechanisms have not been proposed for CaMKII.

In cellular models of opioid tolerance,  $\mu$  opioid receptor ( $\mu$ OR) desensitization was enhanced when a constitutively active form of CaMKII was also expressed in *Xenopus* oocytes (Mestek et al., 1995; Koch et al., 1997). The effect was absent if the native receptor was replaced with a mutated receptor lacking the consensus CaMKII phosphorylation sites (Koch et al., 1997). On the other hand, intracellular  $\text{Ca}^{2+}$ , calmodulin, and CaMKII can all be regulated by opioids. Cytosolic free  $\text{Ca}^{2+}$  was increased after the treatment with opioids (Fields and Sarne, 1997; Smart et al., 1997; Spencer et al., 1997; Quillan et al., 2002). Likewise, chronic treatments with opioids have been found to increase calmodulin activity (Nehmad et al., 1982) and mRNA levels (Niu et al., 2000). Indeed, CaMKII activity was increased in opioid tolerance in vivo (Lou et al., 1999; Wang et al., 2003; Liang et al., 2004). Anatomically, CaMKII and  $\mu$ OR were found to be colocalized in dorsal root ganglia sensory neurons and superficial layers of spinal cord dorsal horn (Bruggemann et al., 2000). Moreover, upon activation,  $\mu$ OR was found to internalize to intracellular locations where CaMKII proteins were located (Bruggemann et al., 2000). Therefore, cellular and biochemical evidence support the possibility that CaMKII and opioid system can directly interact with each other, leading to cellular opioid desensitization. In a preliminary study, we found that tolerance to morphine was abolished by an acute spinal treatment with a single dose of KN93 [2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine], a CaMKII inhibitor (Niki et al., 1993), consistent with the hypothesis that CaMKII can directly modulate opioid tolerance (Wang et al., 2003). However, we caution that multiple factors can confound this interpretation. The current study extends our previous findings by carefully applying a different degree of CaMKII inhibition using multiple doses of KN93 and a negative control, KN92 [2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine] (Tombes et al., 1995), in two rodent models of opioid tolerance. In addition, in this study, we tested whether opioid dependence can be directly regulated by CaMKII.

## Materials and Methods

**Materials.** Morphine and placebo pellets were obtained from the National Institute on Drug Abuse (Rockville, MD). Morphine sulfate was from Abbott Laboratories (North Chicago, IL).

KN93 and KN92 were purchased from Calbiochem (San Diego,

CA). Naloxone and all other chemicals were from Sigma (St. Louis, MO). ICR mice (20–25 g; Harlan Laboratories, Indianapolis, IN) were maintained on a 12-h light/12-h dark cycle and provided with food and water ad libitum before experimental procedures. All experiments were performed in accordance with the National Institutes of Health guidelines and after approval by the Animal Care and Use Committee of the University of Illinois at Chicago.

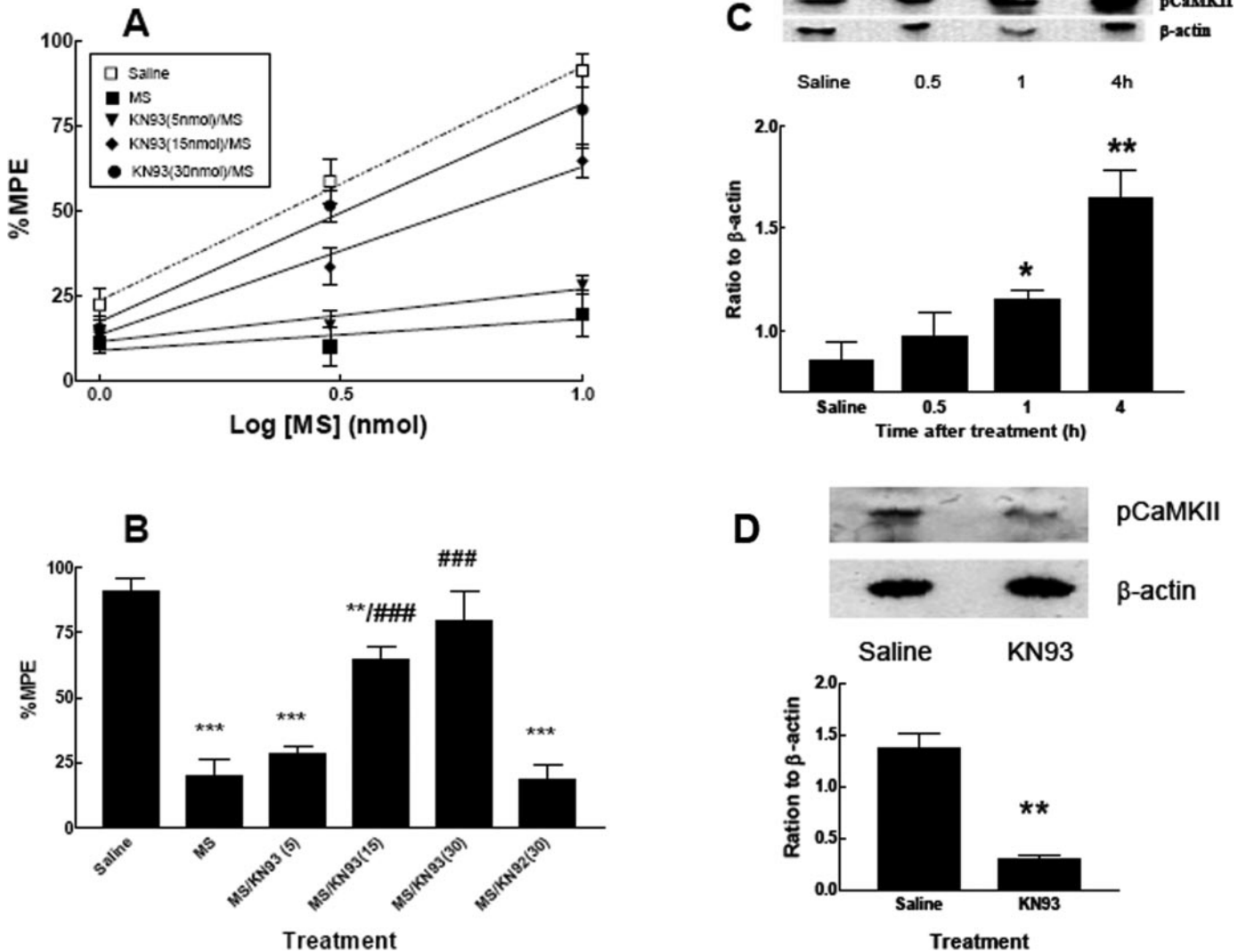
**Drug Administration.** Intracerebroventricular injections, under light ether anesthesia, were made into the left lateral ventricles as described previously (Bilsky et al., 1996; Wang et al., 2001). Standard procedures were used for i.p. and s.c. injections. Placebo and morphine pellets were implanted in the s.c. space as described previously (Way et al., 1969; Patrick et al., 1975).

**Tests for Antinociception.** Basal nociception and morphine-induced antinociception were evaluated using the tail-immersion test as described previously (Wang et al., 2001). In brief, the test was performed by dipping the distal 1/3 of the tail into a water bath maintained at 52°C and recording the latency to a rapid tail-flick response. Morphine-induced (i.c.v.) antinociception was tested at the time of peak drug response after the injection of morphine and expressed as the percentage of maximal possible effect (MPE) according to the following formula: %MPE =  $100 \times (\text{postdrug latency} - \text{predrug latency}) / (\text{cut-off} - \text{predrug latency})$ . A 12-s cut-off was applied to prevent tissue injury. The time of peak drug response was determined to be 20 min in our studies and was not altered by opioid tolerance (see Supplemental Data).

**Acute Opioid Tolerance and Dependence.** Separate groups of eight ICR mice (20–25 g) were made acutely tolerant to and dependent on opioids by the administration of a large dose of morphine (100 mg/kg s.c.) (Wang et al., 1994; Bilsky et al., 1996). We have reported that maximal morphine tolerance and dependence developed over 2 to 6 h (Bilsky et al., 1996). Control mice received the same volume of saline. Tolerance to opioids was studied in these mice 4.5 h later by measuring the antinociceptive effect exhibited by a test dose of morphine (1–10 nmol i.c.v.). A significant reduction of antinociceptive effect signified the presence of tolerance to morphine. To assess dependence, mice were given naloxone (1–10 mg/kg i.p.) 5 h after the administration of morphine (100 mg/kg s.c.) and were immediately placed inside glass cylinders. Vertical jumps were recorded for 15 min. To determine the effect of CaMKII inhibition, separate groups of mice were given the CaMKII inhibitor KN93 (5–30 nmol i.c.v.) (Niki et al., 1993) or its kinase-inactive structural analog, KN92 (30 nmol i.c.v.) (Tombes et al., 1995), 15 min before naloxone or the test dose of morphine. To test whether KN93 or KN92 prevented the development of tolerance and dependence, KN93 or KN92 was coadministered with morphine (100 mg/kg s.c.).

**Effect of KN93 and KN92 on Basal Nociception and Morphine Antinociception.** To determine whether CaMKII inhibitor KN93 or its inactive structural analog, KN92, affected tail-flick latency and interfered with morphine antinociception, groups of eight mice were given KN93 (30 nmol/5  $\mu$ l i.c.v.), KN92 (30 nmol/5  $\mu$ l i.c.v.), or saline (5  $\mu$ l i.c.v.) 15 min before the administration of morphine (1–10 nmol i.c.v.) or saline (5  $\mu$ l i.c.v.). Latencies to tail-flick responses were determined 20 min later.

**Chronic Model of Opioid Tolerance and Dependence.** On day 0, separate groups of six male ICR mice (20–25 g) were implanted subcutaneously with morphine pellets (1 pellet/mouse; each pellet contains 75 mg of morphine base) to induce opioid tolerance and dependence. Control mice received placebo pellets (1 pellet/mouse; a placebo pellet is made of the same excipients but contains no morphine). Tolerance and dependence developed over the course of 2 to 6 days (Ho et al., 1975; Patrick et al., 1975). To determine morphine tolerance, mice were injected with a test dose of morphine (10 nmol i.c.v.). A significant reduction of morphine-antinociceptive effect signified the presence of tolerance to morphine. The presence of opioid dependence was revealed by challenging morphine-treated mice with naloxone (10 mg/kg i.p.) on



**Fig. 1.** Effect of KN93 on morphine-antinoceptive tolerance and CaMKII activity. **A**, the dose-response curve of KN93 in reversing the established opioid tolerance. Groups of eight male ICR mice received morphine or an equal volume of saline (Saline). Four hours later, KN93 (5, 15, or 30 nmol, dissolved in 5  $\mu$ l of saline i.c.v.) was given to several groups of morphine-treated mice. The remaining groups of mice received an i.c.v. injection of saline (5  $\mu$ l). Fifteen minutes later, morphine (1, 3, or 10 nmol i.c.v.) was administered to mice to determine its antinoception by the tail-flick assay. Data are expressed in %MPE (mean  $\pm$  S.E.M.). **B**, comparison of the effects of KN93 and KN92 on opioid antinoceptive tolerance. In morphine-treated mice (100 mg/kg s.c.), tolerance to morphine (10 nmol i.c.v.) was reversed by the acute treatments with KN93 (15 and 30 nmol i.c.v.) but not by the lowest dose of KN93 (5 nmol i.c.v.) or KN92 (30 nmol i.c.v.). **C**, the time course of the activation of CaMKII by morphine. Groups of three mice were injected s.c. with saline or morphine (100 mg/kg). Brain samples were taken at the indicated time points to determine supraspinal CaMKII activity. The activated CaMKII was determined by the Western blotting method using an antibody specific for the Thr286-pCaMKII. Histogram data, expressed as mean  $\pm$  S.E.M., were constructed from the representative figure shown and three other experiments. **D**, inhibition of CaMKII activation by KN93. Groups of three morphine-treated mice (100 mg/kg s.c. for 4 h) were injected i.c.v. with either saline (5  $\mu$ l) or KN93 (30 nmol in 5  $\mu$ l of saline). Fifteen minutes later, brain samples were taken for the analyses of supraspinal CaMKII activity as described above. Histogram data, expressed as mean  $\pm$  S.E.M., were constructed from the representative figure shown and three other experiments. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  compared with the Saline group; ###,  $p < 0.001$  compared with the MS group.

Day 6. Immediately after the administration of naloxone, mice were placed inside glass cylinders, and the number of withdrawal jumps was recorded for 15 min. To determine the effect of acute CaMKII inhibition on tolerance or dependence, separate groups of mice were given KN93 (5–45 nmol i.c.v.) 15 min before naloxone or the test dose of morphine on day 6.

**Western Blot Analysis.** Western blotting procedures were performed as described previously (Wang et al., 2001). In brief, brain cortices were dissected and quickly frozen on dry ice. Tissues from three mice of the same experimental group were pooled and homogenized with a glass homogenizer in a buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 5 mM EDTA in phosphate-buffered saline, pH 7.4] (3 ml/g wet tissue) in the presence of protease inhibitors (0.05 mg/ml bestatin,

0.05 mg/ml leupeptin, 0.05 mg/ml pepstatin, and 0.1 mg/ml phenylmethylsulfonyl fluoride). The homogenates were incubated on a rotator at 4°C for 2 h, and the soluble fraction was collected after separation by centrifugation (45,000g, 60 min). Protein content was determined by a modified Bradford method (Pierce Biotechnology, Rockford, IL). Samples (15  $\mu$ g of protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane. The membrane was pre-blocked in 5% nonfat milk in 20 mM Tris-buffer saline (pH 7.6) with 0.1% Tween 20 and probed with rabbit antibodies recognizing CaMKII (1/1,000; Santa Cruz Biotechnology, Santa Cruz, CA), the activated form of CaMKII [antiphosphorylated CaMKII (pCaMKII) antibody, 1/1000; Promega, Madison, WI], or the activated form of cAMP-response element-binding protein (CREB;

anti-pCREB antibody, 1/1000; Santa Cruz Biotechnology). The membrane was then washed and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1/1,000; Amersham Biosciences, Piscataway, NJ), washed, and developed using an enhanced chemiluminescence detection system (Amersham Biosciences). The membrane was then stripped and reprobed with the mouse anti- $\beta$ -actin antibody (1/10,000; Sigma-Aldrich, St. Louis, MO), followed by another incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (1/20,000; Amersham Biosciences), and developed as above. Enhanced chemiluminescence signals were captured by a ChemiDoc Imaging System and analyzed using Quantity One Program (Bio-Rad, Hercules, CA). Ratios of the optical densities of pCaMKII to that of  $\beta$ -actin were calculated for each sample.

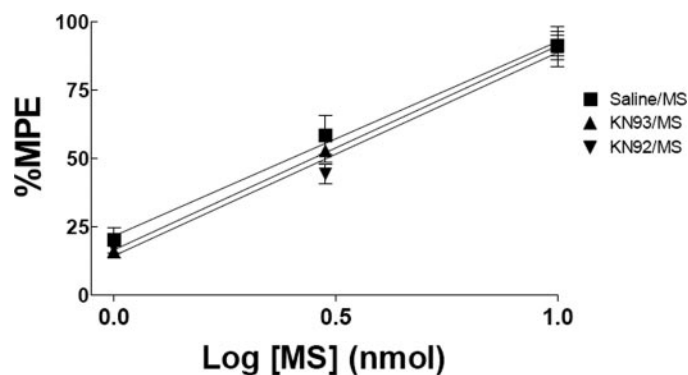
**Statistical Analysis.** Data are expressed as mean  $\pm$  S.E.M. Differences in responses between the treatment groups were determined using analysis of variance followed by Student's *t* (two groups) or Dunnett's *t* (multiple groups) tests. Statistical significance was established at 95%.

## Results

**Effect of the Acute CaMKII Inhibition on Acute Morphine Tolerance.** We first tested our hypothesis in an acute model of opioid tolerance. Tolerance to morphine was established in 2 to 6 h after the administration of morphine (100 mg/kg s.c.) (Bilsky et al., 1996), as evidenced by the significant reduction of i.c.v. morphine-induced antinociception (Fig. 1, A and B). Brain CaMKII activity increased over a time course of 1 to 4 h after s.c. morphine administration (Fig. 1C) correlated with the time course of the development of tolerance (Bilsky et al., 1996). KN93 (15–30 nmol i.c.v.), a selective CaMKII inhibitor, administered 35 min before the antinociceptive test (15 min before the i.c.v. test dose of morphine), reversed the already-established tolerance to morphine. The effect was KN93-dose-dependent, as KN93 at higher doses (15 and 30 nmol) was able to significantly reverse the established tolerance to morphine ( $p < 0.001$ ) while ineffective at a lower dose (5 nmol) (Fig. 1, A and B). In contrast, KN92, a kinase-inactive structural analog of KN93, had no effect on morphine-antinociceptive tolerance (Fig. 1B). The Western blotting experiments verified that acutely administered KN93 (i.c.v.) significantly reduced the supraspinal CaMKII activity in morphine-treated mice (Fig. 1D).

**Effect of KN93 and KN92 on Basal Nociception and Morphine Antinociception.** One potential problem in interpreting the above data was that KN93 might directly affect basal nociception or interfere with the antinociceptive effect of morphine. To account for this possibility, we tested the effects of KN93 and KN92 on basal nociception and morphine antinociception. KN93 or KN92 did not alter the basal tail-flick withdrawal latencies (data not shown). In all three of the morphine doses examined, morphine-induced antinociception was not affected by the administration of KN93 or KN92 (Fig. 2), indicating that KN93 or KN92 did not affect acute morphine antinociception.

**Effect of the Acute CaMKII Inhibition on Acute Morphine Dependence.** In morphine-treated (100 mg/kg s.c.) mice, dependence on opioids developed in 2 to 6 h (Bilsky et al., 1996). Challenging these mice with naloxone i.p. (5 h after morphine administration) precipitated withdrawal jumps in a naloxone dose-dependent manner, which was largely absent in saline-treated control mice (Fig. 3, A and B). KN93 (30 nmol i.c.v.), given 15 min before naloxone, was able

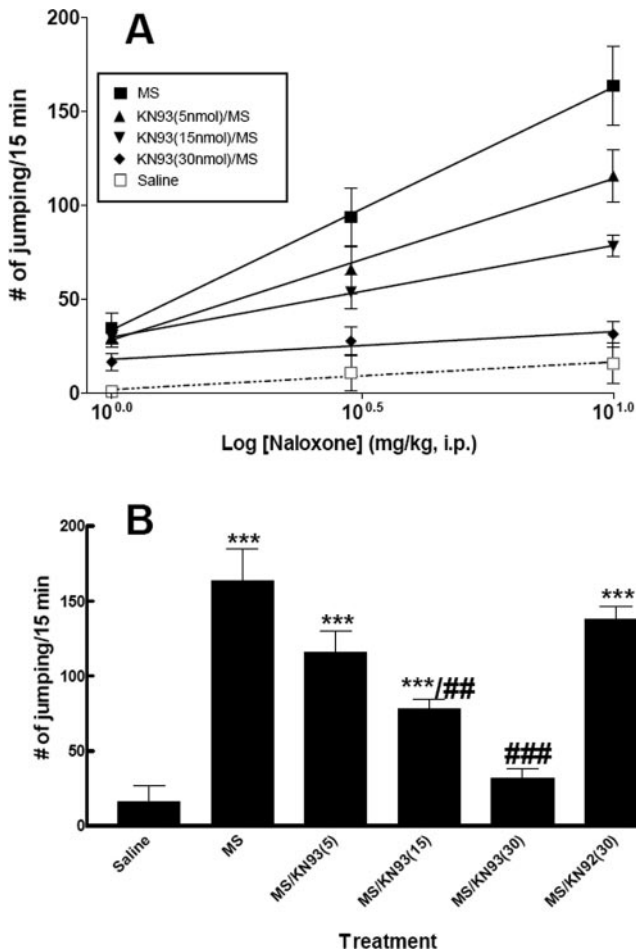


**Fig. 2.** Effects of KN93 and KN92 on MS antinociception. Groups of eight male ICR mice received i.c.v. injections of KN93 (30 nmol in 5  $\mu$ l of saline), KN92 (30 nmol in 5  $\mu$ l of saline), or saline (5  $\mu$ l). Fifteen minutes later, morphine (1, 3, or 10 nmol i.c.v.) was administered to mice to determine its antinociception by the tail-flick assay. Neither KN93 nor KN92 altered acute morphine antinociception ( $p > 0.05$ ). Data are expressed as %MPE (mean  $\pm$  S.E.M.).

to completely suppress the naloxone-induced withdrawal jumping (Fig. 3, A and B). Withdrawal jumping was significantly attenuated ( $p < 0.01$ ) at a lower dose (15 nmol), whereas KN93 at the lowest dose used (5 nmol) did not significantly affect naloxone-induced withdrawal jumping. Nor did the negative control compound, KN92 (30 nmol), show a significant effect (Fig. 3B).

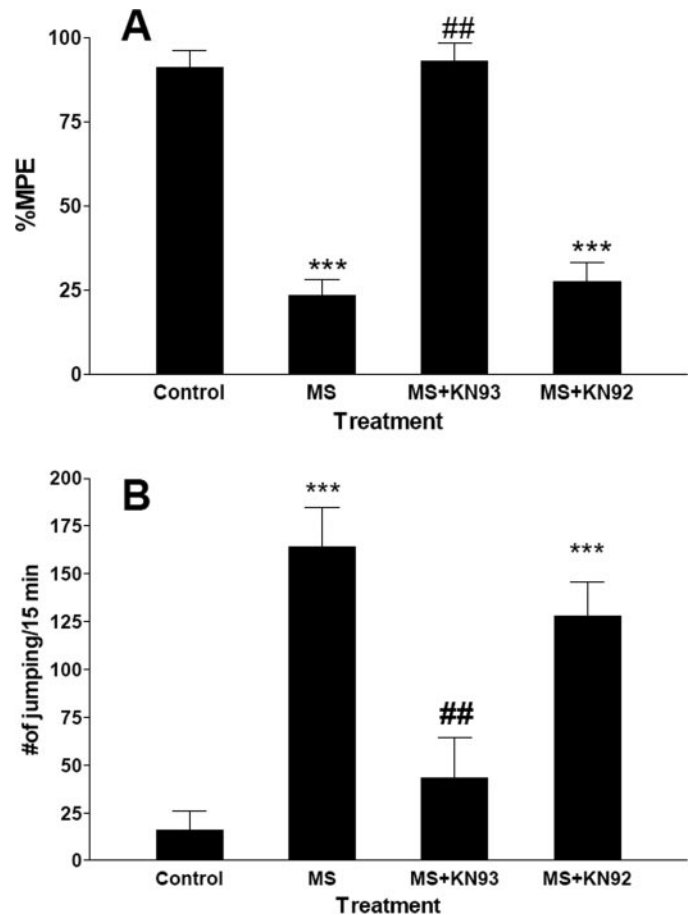
**Prevention of Opioid Tolerance and Dependence by the CaMKII Inhibition.** We next tested whether a broad supraspinal inhibition of CaMKII could prevent the development of tolerance to and dependence on opioids. In these studies, KN93 or KN92 (30 nmol i.c.v.) was administered immediately before the injection of morphine (100 mg/kg s.c.). Five hours later, mice received morphine or "morphine plus KN92" developed antinociceptive tolerance to morphine, whereas tolerance was absent in mice receiving "morphine plus KN93" [ $p < 0.01$  compared with morphine (MS) group; not significantly different from the control group] (Fig. 4A). Similar cotreatment with KN93, but not KN92 (not significantly different from the MS group), prevented the development of opioid dependence by significantly reducing the numbers of naloxone-induced withdrawal jumps ( $p < 0.01$  compared with MS group; not significantly different from the control group) (Fig. 4B). These data indicated that a broad supraspinal inhibition of CaMKII was effective in preventing the development of both opioid tolerance and dependence in mice.

**KN93 Reversed Opioid Tolerance in a Chronic Model of Opioid Tolerance.** To determine that the pharmacological effect of CaMKII inhibition was not limited to a particular animal model of opioid tolerance, we further tested our hypothesis in a chronic model of opioid tolerance. Mice developed tolerance to opioids over 2 to 6 days after receiving the s.c. implantation of morphine pellets (Ho et al., 1975; Patrick et al., 1975). The antinociception produced by morphine (10 nmol i.c.v.) before the implantation of pellets was  $91.0 \pm 5.0\%$  MPE. On day 6, the same test dose of morphine produced a significantly reduced antinociceptive response in morphine-pelleted mice ( $11.5 \pm 2.0\%$  MPE,  $p < 0.001$ ) while remaining fully active in mice implanted with placebo pellets ( $93.1 \pm 4.0\%$  MPE) (Fig. 5A), indicating the presence of antinociceptive tolerance in morphine-pelleted mice. Su-



**Fig. 3.** Effect of acute CaMKII inhibition by KN93 on morphine dependence. **A**, dose-response curve of KN93 in reversing the established opioid dependence. Groups of eight male ICR mice received s.c. injections of MS or an equal volume of saline (Saline). All mice received i.p. injections of naloxone (1, 3, or 10 mg/kg) 5 h later. Fifteen minutes before the injection of naloxone, KN93 (5, 15, or 30 nmol dissolved in 5  $\mu$ l of saline i.c.v.) was given to several groups of morphine-treated mice. The remaining groups of mice received i.c.v. injections of saline (5  $\mu$ l). The numbers of vertical jumps were recorded for each group of mice and expressed as mean  $\pm$  S.E.M. **B**, comparison of the effects of KN93 and KN92 on opioid dependence. In morphine-treated mice (100 mg/kg s.c.), dependence on morphine was revealed by 10 mg/kg i.p. naloxone-precipitated withdrawal jumping 5 h later. The withdrawal jumping was completely blocked by an acute treatment with KN93 at the highest dose (30 nmol, i.c.v.) and attenuated by KN93 at 15 nmol ( $p < 0.01$ ). KN93 at the lowest dose (5 nmol, i.c.v.) or KN92 (30 nmol i.c.v.) did not significantly alter morphine dependence ( $p > 0.05$ ). \*\*\*,  $p < 0.001$  compared with the Saline group; ##,  $p < 0.01$ ; ###,  $p < 0.001$  compared with the MS group.

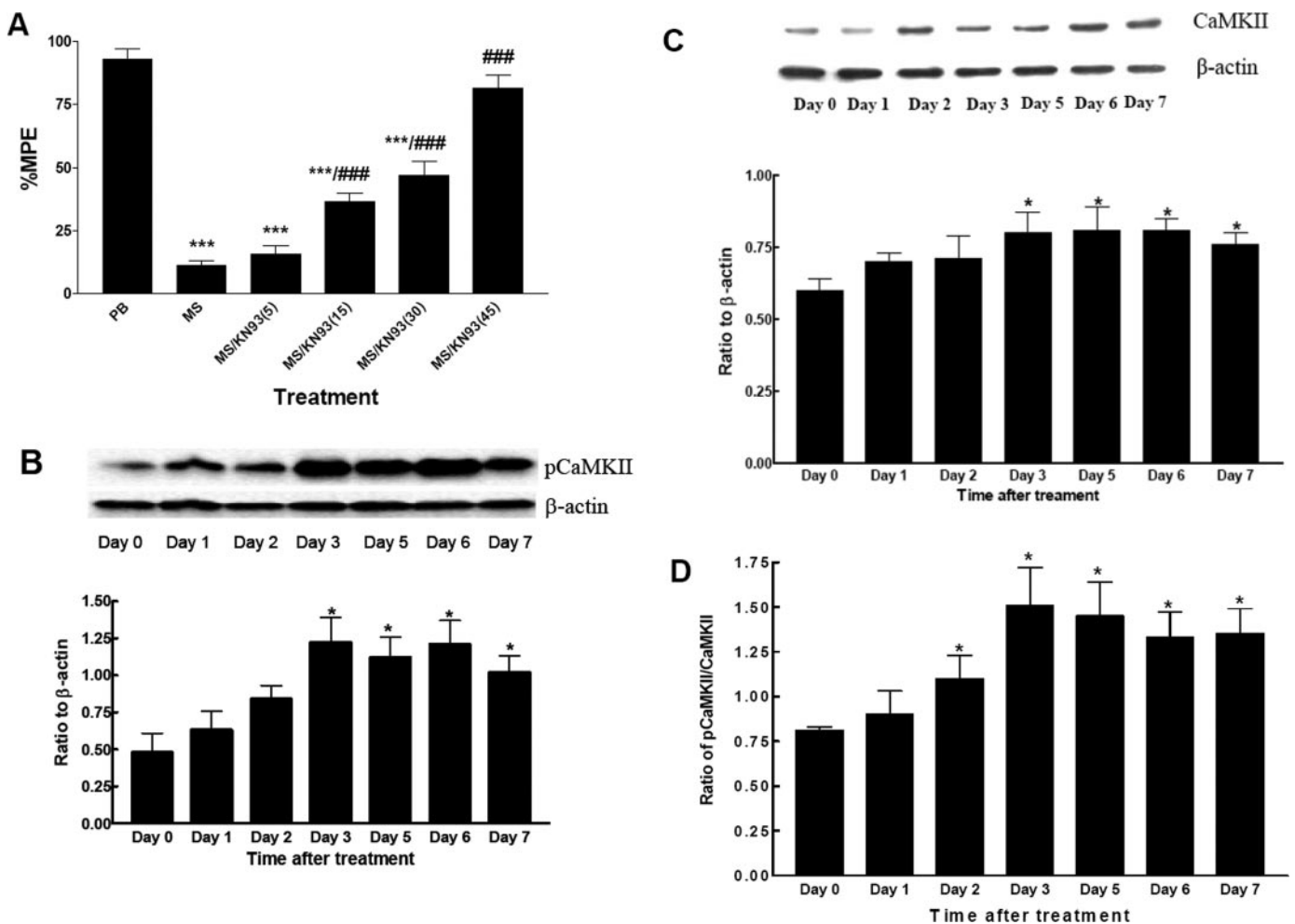
praspinal CaMKII expression and activity increased significantly over the time course of 6 days as the tolerance developed (Fig. 5, B–D). When KN93 (15–45 nmol i.c.v.) was given 15 min before morphine-antinoceptive tests, KN93 was able to significantly reverse the established tolerance in these mice ( $p < 0.001$  for all three doses) (Fig. 5A). KN93 at the lowest dose used (5 nmol) was not effective. Compared with the acute model, a higher dose of KN93 (45 nmol) was needed to achieve a complete reversal of tolerance (not significantly different from the “placebo-pelleted” group). These results were in agreement with those from the acute model, suggesting that the acute inhibition of supraspinal CaMKII effectively disrupted the established opioid-antinoceptive tolerance.



**Fig. 4.** Prevention of MS-antinoceptive tolerance and physical dependence by KN93. Groups of eight male ICR mice received KN93 (30 nmol in 5  $\mu$ l of saline i.c.v., designated as KN93+MS group), KN92 (30 nmol in 5  $\mu$ l of saline i.c.v., KN92+MS group), or saline (5  $\mu$ l i.c.v., MS group) immediately prior to the administration of morphine (100 mg/kg s.c.). Control mice received only s.c. injection of saline. **A**, development of tolerance to morphine was prevented by KN93 but not by KN92. **B**, development of morphine dependence, as revealed by 10 mg/kg i.p. naloxone-precipitated withdrawal jumping, was prevented by KN93 but not by KN92. Data are expressed as mean  $\pm$  S.E.M. \*\*\*,  $p < 0.001$  compared with the Control group; ##,  $p < 0.01$  compared with the MS group.

**KN93 Reversed Opioid Dependence in a Chronic Model of Opioid Dependence.** We next tested the effect of an acute CaMKII inhibition on opioid dependence in a chronic opioid dependence model. On day 6 after morphine-pelleting, naloxone precipitated a significant number of withdrawal jumps in morphine-implanted mice compared with placebo-pelleted mice, indicative of the presence of opioid dependence (Fig. 6). Acutely administered KN93 (i.c.v. 15 min before naloxone administration) dose-dependently decreased the number of naloxone-withdrawal jumping in morphine-pelleted mice. At the highest dose (45 nmol), KN93 completely suppressed the withdrawal jumping ( $p < 0.001$  compared with the morphine-pelleted mice; not significantly different from the placebo-pelleted mice). KN93 at lower doses (5–30 nmol) also significantly reduced the number of withdrawal jumping (Fig. 6), suggesting that acute inhibition of CaMKII was effective in reversing established opioid dependence in the chronic mouse model of opioid dependence.

**Downstream Effectors of CaMKII.** CaMKII affects a number of downstream effectors, including receptors and transcription factors. The transcriptional factor CREB, a



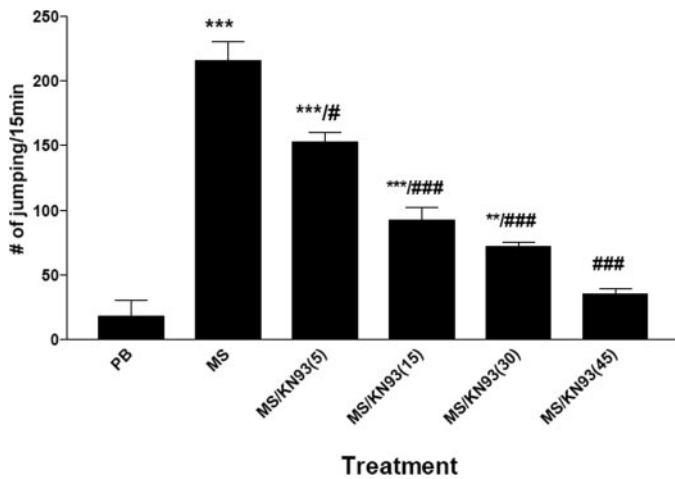
**Fig. 5.** A, reversal of morphine-antinoceptive tolerance by KN93 in a chronic model of opioid tolerance. Groups of six male ICR mice were implanted s.c. with morphine pellets ( $1 \times 75$  mg of morphine/pellet/mouse) or placebo pellets (PB group) on day 0. On day 6, KN93 (5–45 nmol, dissolved in  $5 \mu\text{l}$  of saline i.c.v.) was given to several groups of morphine-pelleted mice. The remaining groups of mice received an i.c.v. injection of saline ( $5 \mu\text{l}$ ). Fifteen minutes later, morphine antinoception (10 nmol i.c.v.) was determined. Data are expressed as %MPE (mean  $\pm$  S.E.M.). Antinoceptive tolerance to morphine was detected in mice that received only morphine pellets (MS). In contrast, in morphine-pelleted mice that also received KN93, tolerance was completely reversed (45 nmol,  $p > 0.01$  compared with PB group) or significantly attenuated (5–30 nmol). \*\*\*,  $p < 0.001$  compared with PB group; ###,  $p < 0.01$  compared with the MS group. B, activation of supraspinal CaMKII by morphine. Groups of three mice were implanted s.c. with morphine pellets (1 morphine pellet/mouse) or placebo pellets (day 0 group). Brain samples were taken at the indicated time points for the analyses of supraspinal CaMKII activity. The activated CaMKII was determined by the Western blotting method using an antibody specific for Thr286-pCaMKII. Data, expressed as mean  $\pm$  S.E.M., were from the representative figure shown and three other experiments. \*,  $p < 0.05$  compared with day 0. C, chronic treatment with morphine increased supraspinal CaMKII expression. Groups of three mice were implanted s.c. with morphine pellets (1 morphine pellet/mouse) or placebo pellets (day 0 group). Brain samples were taken at the indicated time points for the analyses of supraspinal CaMKII expression. Data, expressed as mean  $\pm$  S.E.M., were from the representative figure shown and three other experiments. \*,  $p < 0.05$  compared with day 0. D, activation of supraspinal CaMKII by morphine normalized by the expression of total CaMKII. Experiments were performed as described in B and C. Data from these panels were analyzed together to obtain the ratio of pCaMKII/total CaMKII. \*,  $p < 0.05$  compared with day 0.

key CaMKII downstream effector, has been previously proposed to be important in opioid tolerance and dependence (Nestler, 2001). We examined the activation of CREB (pCREB) in mice that have been treated with morphine. As expected, chronic treatment with morphine increased the levels of pCREB (Fig. 7).

## Discussion

The current study tested the hypothesis that CaMKII can directly regulate opioid tolerance and dependence. Previous studies using a rat model of opioid tolerance and dependence (10 mg/kg s.c. morphine every 12 h) demonstrated that opioid tolerance and dependence were prevented by chronically microinjecting into hippocampus

chemical CaMKII inhibitors or antisense oligodeoxynucleotides (Fan et al., 1999; Lu et al., 2000). The same chronic treatments in striatum (tolerance) and amygdale (dependence) were not effective. Neither was the acute CaMKII inhibition in hippocampus able to affect opioid tolerance (Fan et al., 1999). Based on the hippocampus-specific action of CaMKII inhibition and the requirement of chronic administration, it has been suggested that CaMKII modulated opioid tolerance and dependence through the learning and memory pathways. This was not entirely surprising since opioid tolerance and dependence have been hypothesized to involve learning and memory (Siegel, 1976). Several antagonists of NMDA receptor, another member of a group of genes essential for long-term poten-

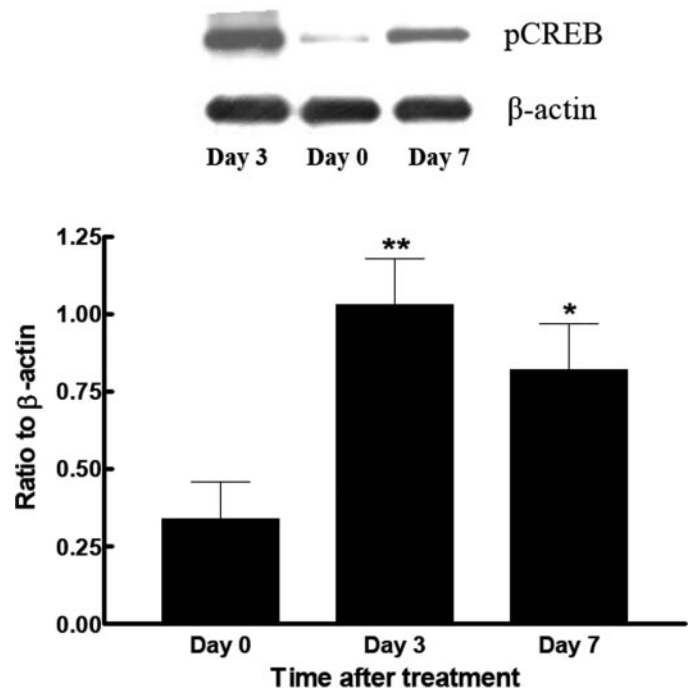


**Fig. 6.** Reversal of morphine dependence by KN93 in a chronic model of opioid dependence. Groups of six male ICR mice were implanted s.c. with morphine pellets (1 morphine pellet/mouse) or placebo pellets (PB group) on day 0. On day 6, KN93 (5–45 nmol, dissolved in 5  $\mu$ l saline i.c.v.) was given to several groups of morphine-pelleted mice. The remaining groups of mice received an i.c.v. injection of saline (5  $\mu$ l). Fifteen minutes later, opioid dependence was revealed by 10 mg/kg i.p. naloxone-induced withdrawal jumping in mice treated only with morphine pellets (MS group). In morphine-pelleted mice that also received KN93, naloxone-induced withdrawal jumping was completely blocked (45 nmol,  $p > 0.05$  compared with PB group) or significantly attenuated (5–30 nmol). \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with the PB group; #,  $p < 0.05$ ; ###,  $p < 0.001$  compared with the MS group).

tiation and learning and memory, have also been previously found to prevent the development of opioid tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991). Further studies found that the effect of MK801, an NMDA receptor antagonist, did not depend entirely on its ability to interfere with associative learning (Gutstein and Trujillo, 1993). Instead, MK801 prevented morphine-antinociceptive tolerance in spinalized rats (Gutstein and Trujillo, 1993), indicating that the NMDA receptor directly (i.e., independent of learning and memory) affected opioid tolerance and dependence.

In this study, we tested whether such a direct role existed for CaMKII. Because the acute inhibition of hippocampal CaMKII did not affect opioid tolerance (Fan et al., 1999), it would suggest that the acute inhibition of CaMKII had no or minimal impact on learning and memory. We took the study paradigm a step further to study the acute supraspinal inhibition of CaMKII in two models of opioid tolerance and dependence in mice. An effect by the acute supraspinal inhibition served as an indication for a direct effect by CaMKII.

Unlike the acute hippocampal CaMKII inhibition, the acute supraspinal CaMKII inhibition by KN93 i.c.v., a selective CaMKII inhibitor (Niki et al., 1993), effectively reversed the established opioid tolerance and dependence in an acute model of opioid tolerance/dependence. Whereas morphine-treated groups of mice showed antinociceptive tolerance, acutely administered (15 min before) KN93 was able to dose-dependently reverse the antinociceptive tolerance. The effect was not observed in mice acutely treated with KN92, a kinase-inactive structural analog of KN93 (Tombes et al., 1995). Moreover, the effect of KN93 was not due to any direct effect on nociception or antinociception, because KN93 did not produce antinociception by itself or interfere with acute



**Fig. 7.** Activation of supraspinal CREB by morphine. Groups of three mice were implanted s.c. with morphine pellets (1 morphine pellet/mouse) or placebo pellets (day 0 group). Brain samples were taken at the indicated time points for the analyses of supraspinal CREB activity. The activated CREB was determined by the Western blotting method using an antibody specific for phosphorylated CREB (pCREB). Data, expressed in mean  $\pm$  S.E.M., were from the representative figure shown and three other experiments. \*,  $p < 0.05$ , compared with day 0.

morphine antinociception. A previous study also did not find an interference by KN93 i.c.v. on subcutaneous morphine-produced antinociception or hyperlocomotion (Narita et al., 2004). The Western blotting analysis, on the other hand, confirmed the inhibition of CaMKII activity by the acute i.c.v. administration of KN93.

A direct action of CaMKII in opioid tolerance and dependence was further supported by studies in a chronic model (1  $\times$  75 mg of morphine pellet/mouse for 6 days) of opioid tolerance and dependence in mice. The acute CaMKII inhibition by KN93 reversed the already-established morphine tolerance and dependence, indicating that the effect of acute CaMKII inhibition was not limited to a particular model. We have previously found that acute spinal inhibition of CaMKII reversed the established morphine tolerance in rats (Wang et al., 2003), suggesting that both spinal and supraspinal CaMKII are essential for the maintenance of opioid tolerance and dependence.

The time course of CaMKII activation correlated with the development of opioid tolerance and dependence in both of the models that we tested (Ho et al., 1975; Patrick et al., 1975; Bilsky et al., 1996). These data were in agreement with previous findings that CaMKII can be activated in different CNS regions after the treatment with morphine (Fan et al., 1999; Wang et al., 2003; Liang et al., 2004).

The direct action of CaMKII in opioid tolerance and dependence was supported by data from cellular studies. In cellular models, desensitization of  $\mu$  opioid receptor was found to be affected by CaMKII. DAMGO ([D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin)-induced activation of G-protein-gated inwardly rectifying potassium channels was signifi-

cantly desensitized in the presence of a constitutively active CaMKII and the  $\mu$ OR in *Xenopus* oocytes (Mestek et al., 1995; Koch et al., 1997). Anatomically, a direct mechanism by CaMKII was also plausible because  $\mu$ OR and CaMKII were found to coexist in dorsal root ganglia and superficial layers of spinal dorsal horn, areas critical for pain transmission (Bruggemann et al., 2000). Moreover, upon the activation of  $\mu$ OR, these receptors were internalized to colocalize with CaMKII in the cytoplasm (Bruggemann et al., 2000).

Besides opioid receptors, CaMKII may also interact with the NMDA receptors, leading to opioid tolerance and dependence.  $\text{Ca}^{2+}$  influx via the activation of NMDA receptors results in the activation and autophosphorylation of CaMKII at position Thr286 (Fukunaga et al., 1992; Strack et al., 2000). Activated CaMKII, in turn, can phosphorylate and activate NMDA receptors, leading to more  $\text{Ca}^{2+}$  influx through the channel (Kitamura et al., 1993). Therefore, CaMKII and the NMDA receptor can interact with each other in a feed-forward fashion. As noted above, the NMDA receptor has been shown to directly affect opioid tolerance and dependence (Gutstein and Trujillo, 1993).

In addition to opioid receptors and NMDA receptors, numerous other downstream effectors can be affected by CaMKII, including transcription factors, such as CREB (Sheng et al., 1991), activating transcription factor 1 (Shimomura et al., 1996), serum response factor (Misra et al., 1994), and CAAT-enhancer-binding protein  $\beta$  (Wegner et al., 1992). We found that activation of CREB (pCREB) was closely related to the activation of CaMKII in mice that were tolerant to and dependent on morphine. These data are in agreement with previous findings that CREB is important for opioid tolerance and dependence (Nestler, 2001; Valverde et al., 2004).

In summary, our data provided the initial evidence for a critical role of CaMKII in directly promoting opioid tolerance and dependence. This mechanism is in addition to its impact on tolerance and dependence via learning and memory. Identifying such a direct mechanism will not only have a profound impact on our understanding but may also lead to pharmacological interventions targeting the CaMKII pathway for the attenuation of opioid tolerance and dependence. Recently, we found that trifluoperazine, an orally available antipsychotic drug, was capable of disrupting opioid tolerance by inhibiting CaMKII (Tang et al., 2006). It is conceivable that CaMKII inhibitors, such as phenothiazine antipsychotics, should be tested in humans for the prevention and/or treatment of opioid addiction and tolerance.

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