Inhibition of Calcium/Calmodulin-Dependent Protein Kinase II in Rat Hippocampus Attenuates Morphine Tolerance and Dependence

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ABSTRACT

Learning and memory have been suggested to be important in the development of opiate addiction. Based on the recent findings that calcium/calmodulin-dependent protein kinase II (CaMKII) is essential in learning and memory processes, and morphine treatment increases CaMKII activity in hippocampus, the present study was undertaken to examine whether inhibition of hippocampal CaMKII prevents morphine tolerance and dependence. Here, we report that inhibition of CaMKII by intrahippocampal dentate gyrus administration of the specific inhibitors KN-62 and KN-93 to rats significantly attenuated the tolerance to the analgesic effect of morphine and the abstinence syndrome precipitated by opiate antagonist naloxone. In contrast, both KN-04 and KN-92, the inactive structural analogs of KN-62 and KN-93, failed to attenuate morphine tolerance and dependence, indicating that the observed effects of KN-62 and KN-93 are mediated through inhibition of CaMKII. Furthermore, administration of CaMKII antisense oligonucleotide into rat hippocampal dentate gyrus, which decreased the expression of CaMKII specifically, also attenuated morphine tolerance and dependence, while the corresponding sense oligonucleotide of CaMKII did not exhibit such inhibitory effect. Moreover, the KN-62 treatment abolished the rewarding properties of morphine as measured by the conditioned place preference. These results suggest that hippocampal CaMKII is critically involved in the development of morphine tolerance and dependence, and inhibition of this kinase may have some therapeutic benefit in the treatment of opiate tolerance and dependence.

Opiate addiction is a phenomenon with complex physiological and social causes and consequences. It has been shown to result from neuronal adaptations produced by repeated drug exposure, which leads eventually to the complex behaviors, for example tolerance and dependence, that characterize an addictive state (Neatler and Aghajanian, 1997). The long-lived adaptations involve relatively stable changes in gene expression, which cause changes in neurotransmission and even in the structure of the target neurons (Neatler and Aghajanian, 1997). Opiate addiction is experience-dependent and in many respect is similar to learning and memory processes (Siegel, 1976; Wickelgren, 1998). In fact, learning and memory have been suggested to play an important role in opiate addiction (Wickelgren, 1998). A variety of different compounds that impair learning and memory (Li et al., 1997; Zou et al., 1998) prevent opiate tolerance and dependence (Trujillo and Akil, 1991; London et al., 1995). These compounds include N-methyl-D-aspartate (NMDA) receptor antagonists and nitric oxide synthase (NOS) inhibitors (Trujillo and Akil, 1991; London et al., 1995). Although there are many years of study, the mechanism underlying opiate addiction is still poorly understood, and little progress has been made in the treatment of the addictive disorders of opiate. Because learning and memory are suggested to be essentially involved in opiate addiction, it is intriguing to hypothesize that selective modulation of those genes that play key roles in learning and memory processes could affect the development of opiate tolerance and dependence. Calcium/calmodulin-dependent protein kinase II (CaMKII) belongs to this group of genes, because it is expressed predominantly in cerebral cortex and hippocampus (Erondu and Kennedy, 1985) and is essential in certain types of learning and mem-

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; NOS, nitric oxide synthase; CaMKII, calcium/calmodulin-dependent protein kinase II; KN-62, 1-[N.O-bis(5-isoquinolinsulfonfonyl)-N-methyl-L-trosyl]-4-phenylpiperazine; KN-94, [N-(1-1[P-(5-isoquinolinsulfonyl)benzyl]-2-(4-phenylpiperazinyl)ethyl]-5-isoquinolinsulfonamide; KN-93, 1-[N.O-bis(5-isoquinolinsulfonfonyl)-N-methyl-D-aspartate (NMDA) receptor antagonists and nitric oxide synthase (NOS) inhibitors (Trujillo and Akil, 1991; London et al., 1995). Although there are many years of study, the mechanism underlying opiate addiction is still poorly understood, and little progress has been made in the treatment of the addictive disorders of opiate. Because learning and memory are suggested to be essentially involved in opiate addiction, it is intriguing to hypothesize that selective modulation of those genes that play key roles in learning and memory processes could affect the development of opiate tolerance and dependence. Calcium/calmodulin-dependent protein kinase II (CaMKII) belongs to this group of genes, because it is expressed predominantly in cerebral cortex and hippocampus (Erondu and Kennedy, 1985) and is essential in certain types of learning and mem-

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ory (Silva et al., 1992; Lisman, 1994; Wolfman et al., 1994); inhibition or disruption of this kinase impairs spatial learning and memory tasks in rodents (Silva et al., 1992; Lisman, 1994; Wolfman et al., 1994). Interestingly, studies from our laboratory show that morphine treatment increases the expression of CaMKII in rat hippocampus but not in other brain regions (Lou et al., 1999). These data raise the possibility that inhibition or down-regulation of this kinase prevents the development of opiate tolerance and dependence. Here we report that intrahippocampal injection of specific CaMKII inhibitors or the antisense oligonucleotide strongly attenuated morphine tolerance and dependence. These results suggest that inhibition or down-regulation of CaMKII may have some therapeutic benefit in the treatment of opiate tolerance and dependence.

Materials and Methods

Animals. Male Sprague-Dawley rats (250–300 g) from Shanghai Center of Experimental Animals were used. They were individually housed in a temperature-controlled (22°C) colony room and maintained on a standard 12-h light/12-h dark cycle with food and water available.

Western Blot Analysis. Western blot analysis was carried out as described previously (Hashimoto and Soderling, 1987). Briefly, lysates from hippocampus were prepared in buffer containing 62.5 mM Tris-HCl (pH 7.8), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue. Samples containing 30 μg of proteins were separated by SDS-polyacrylamine gel electrophoresis before being transferred to nitrocellulose membrane. The membrane blots were blocked with 5% milk in Tris-buffered saline for 2 h and incubated for 2 h with either anti-CaMKII monoclonal antibody (1:2000) or anti-β-actin antibody (1:500). The blots were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h. Immunoreactive bands were detected with an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL).

Surgery and Microinjection Procedures. The cannulas were implanted into the hippocampi and striata of rats as described previously (Sommer et al. 1996; Valle et al., 1997). Briefly, rats were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg). The anesthetized rats were mounted on a stereotaxic apparatus. Two 26-gauge guide cannulas were implanted bilaterally into the hippocampal dentate gyrus before each morphine treatment. Each animal was confined there for 15 min. During the conditioning phase, rats were treated for 6 consecutive days with alternate injections of morphine-HCl (10 mg/kg s.c.) or saline, and placed into the environment as previously determined. Unless indicated, KN-62 was administered 15 min before each morphine treatment. Each animal was confined there for 50 min, after which it was returned to the home cage. During the test phase, the barrier was removed and the time spent at the drug side was recorded for 15 min.

Statistical Analysis. Individual comparisons within the group were made by the two-tailed Dunnett’s test, and between groups by the two-tail Student’s t test.

Results

Intrahippocampal Injection of CaMKII Specific Inhibitor KN-62 Attenuated Morphine Tolerance and Dependence. The present experiments were performed to determine whether down-regulation of CaMKII in hippocampus interferes with the analgesic effect of morphine and the development of morphine tolerance and dependence. The specific CaMKII inhibitor KN-62 was used by microinjection into the hippocampal dentate gyrus before each morphine treatment to inhibit the kinase activity, and KN-04, an inactive structural analog of KN-62, was used as a control. As shown in Fig. 1A, animals receiving morphine (10 mg/kg s.c.) displayed maximal analgesia on days 1 and 3 of treatment following microinjection into hippocampus of vehicle (0.1% DMSO in saline, 1 μl/site), KN-62 (10 nmol/site), or KN-04 (10 nmol/site). In vehicle- (n = 13) and KN-04-treated (n = 10) rats, the analgesic response to morphine displayed rapid development of tolerance, reducing latencies to the baseline (2–3 s) by day 9. In contrast, rats treated with KN-62 (n = 16) showed considerably less tolerance to morphine, and the analgesic response maintained throughout morphine treatment (~8 s by day 9).

CaMKII inhibitor KN62 did not affect the basal tail-flick latency, even after prolonged treatment, indicating that the effect of KN-62 on morphine tolerance is apparently not a
result of its own analgesic action (Fig. 1A). Acute administration of KN-62 (on day 10) could not restore potency of morphine in rats already tolerant to morphine (on days 1 through 9; Fig. 1B). Furthermore, animals treated with KN-62 and morphine on days 1 through 9 and given morphine alone on day 10 displayed considerable analgesia (Fig. 1B). It should be noted that after 9 days of treatment, KN-62 still remarkably inhibited CaMKII activity (data not shown). These data suggest that the CaMKII inhibitor KN-62 interferes with the development of morphine tolerance.

In addition to the attenuation of morphine tolerance, KN-62 pretreatment inhibited the naloxone-precipitated abstinence syndrome. Animals that had received repeated administration of vehicle (n = 13) or KN-04 (10 nmol/site; n = 10) together with morphine (10 mg/kg s.c.) for 9 days displayed numerous signs of an abstinence syndrome in response to an injection of the opiate antagonist naltrexone (Fig. 1C). In contrast, animals treated with KN-62 (10 nmol/site; n = 16) before each morphine administration showed very few signs of an abstinence syndrome after naloxone precipitation (Fig. 1C).

Dose-Dependent Effect of KN-62 on Development of Morphine Tolerance. The inhibitory effect of KN-62 on morphine tolerance was dose dependent (n = 10/group). Intrahippocampal administration of 3 nmol/site of KN-62 significantly attenuated the development of tolerance to the

![Fig. 1. Intrahippocampal administration of KN-62-attenuated morphine tolerance and dependence. A, animals received saline (Sal) or morphine (Mor; 10 mg/kg s.c.) twice per day for 9 days and KN-62 (10 nmol/site), KN-04 (10 nmol/site), or vehicle (Veh) was administered by intrahippocampal injection 15 min before each morphine treatment. Tail-flick latency was measured on odd days. Scores are expressed as mean ± S.E.M. *P < .05 versus the group receiving vehicle/morphine. B, analgesia scores on day 10 when treatments were reversed so that animals that had been treated with KN-62 or KN-04 followed by morphine on days 1 through 9 were challenged with vehicle followed by morphine; animals that were treated with KN-62 or KN-04 followed by saline on days 1 through 9 were challenged with KN-62 or KN-04 followed by morphine; and animals that were treated with vehicle followed by morphine on days 1 through 9 were challenged with KN-62 followed by morphine. The label under each bar indicates the treatment the animals received on days 1 through 9. *P < .05 versus the group receiving vehicle/morphine. C, effect of KN-62 on the withdrawal precipitated by naloxone. After 9 days of KN-62 (10 nmol/site) or KN-04 (10 nmol/site) treatment, animals were administered naloxone (1 mg/kg i.p.) and incidence of somatic signs of abstinence (mean ± S.E.M.) was measured within 15 min. Animal number in each group was the same as that described in Fig. 1. *P < .05, **P < .01 versus the group receiving vehicle/morphine treatment.](molpharm.aspetjournals.org)
analgesic effect of morphine. At the dose of 30 nmol/side, the development of morphine tolerance was completely inhibited (Fig. 2A). In contrast, administration of different doses of KN-04 (1–30 nmol/side) failed to affect morphine tolerance (data not shown). It is unlikely a result of the synergistic effect KN-62 and morphine, because treatment with different concentrations of KN-62 (1–30 nmol/site) failed to potentiate the analgesic effect of a mild dose (1 mg/kg s.c.) of morphine (Fig. 2B).

Intrastriatal Administration of KN-62 Failed to Attenuate Morphine Tolerance. In addition to the hippocampus, expression of CaMKII can be found in other brain regions including striatum (Erondu and Kennedy, 1985). To assess whether inhibition of CaMKII in other brain regions also attenuates morphine tolerance, KN-62 (10 nmol/site) was introduced into the striatum by microinjection before each morphine treatment ($n = 10/group$). As shown in Fig. 3, KN-62 treatment did not attenuate the development of tolerance to morphine’s analgesic effect.

Intrahippocampal Administration of KN-93 Attenuated Morphine Tolerance and Dependence. To confirm that the effects of KN-62 on morphine tolerance and dependence are due to its inhibition of CaMKII rather than its nonspecific effects, another CaMKII specific inhibitor, KN-93, and its inactive structural analog, KN-92, were used, and morphine tolerance and dependence were investigated. As shown in Fig. 4, animals ($n = 10/group$) receiving intrahippocampal injection of KN-93 (10 nmol/site) displayed considerably less of both tolerance to the analgesic effect of morphine and dependence precipitated by naloxone administration, while the same treatment of KN-92 (10 nmol/site) displayed no significant effect.

Intrahippocampal Injection of CaMKII Antisense Oligonucleotide Attenuated Development of Morphine Tolerance and Dependence. To further confirm that the effects of both KN-62 and KN-93 are due to their blocking CaMKII, phosphorothioated antisense oligonucleotide against the translation initiation site of CaMKII was used by intrahippocampal injection (Muthalif et al., 1996). Twenty-four hours after one injection of the antisense oligonucleotide (10 μg/site), there was a significant reduction in the protein level of CaMKII as detected by Western blot analysis using the monoclonal antibody against CaMKII (data not shown). In animals ($n = 6/group$) administered with CaMKII antisense oligonucleotide (10 μg/site) at an interval of 12 h for 9 days, hippocampal CaMKII remained at a significantly lower level as compared with that in the animals treated with vehicle or the sense oligonucleotide (Fig. 5A). In contrast, the same treatment of the sense oligonucleotide had no significant inhibitory effect on CaMKII expression. In a parallel experiment, it was shown that treatment with the CaMKII antisense oligonucleotide did not inhibit the expression level of P38 MAPK (Fig. 5A), indicating the sequence-specific and nontoxic effect of the CaMKII antisense oligonucleotide.

Animals ($n = 6/group$) were intrahippocampally administered with vehicle, the antisense, or the sense oligonucleotide before each morphine treatment. As shown in Fig. 5B, animals treated with the sense oligonucleotide showed tolerance to the analgesic effect of morphine (10 mg/kg s.c.) that was similar to that in vehicle-treated rats, whereas animals treated with the antisense oligonucleotide were analgesic.
throughout the testing period. It should be noted that the basal tail-flick latency was not altered in animals treated with the antisense oligonucleotide (Fig. 5B). Furthermore, animals that were treated with CaMKII antisense oligonucleotide during repeated morphine administration showed very few signs of an abstinence syndrome after naloxone administration compared with the sense oligonucleotide- and vehicle-treated animals (Fig. 5C). Taken together, these results illustrate that blocking of CaMKII by its antisense oligonucleotide considerably attenuate morphine tolerance and dependence.

Inhibition of Hippocampal CaMKII Prevented Morphin-Induced Conditioned Place Preference. To investigate whether the rewarding properties elicited by morphine are affected by inhibition of hippocampal CaMKII, the place-conditioning paradigm was used. During the preconditioning period, animals of each group spent the same time in each compartment (Fig. 6). Animals receiving vehicle and morphine (10 mg/kg s.c.; n = 14) or KN-04 (10 nmol/site) and morphine (10 mg/kg s.c.; n = 10) for 6 days displayed clear conditioned place preference indicated by a significant increase in the time spent in the drug-associated compartment during the test phase on day 7. This conditioned behavior was absent in rats (n = 12) receiving KN-62 (10 nmol/site) and morphine (10 mg/kg s.c.) for 6 days, which spent almost the same amount of time in the morphine-designed compart-

**Discussion**

The present study demonstrates that inhibition of CaMKII by microinjection of its specific inhibitors KN-62 and KN-93 into the hippocampal dentate gyrus strongly attenuated the development of tolerance and physical and psychological dependence in response to chronic morphine treatment. Admin-

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**Fig. 4.** Intrahippocampal injection of KN-93 attenuated morphine tolerance and dependence. A, animals (n = 10/group) were administered KN-93 (10 nmol/site) or KN-92 (10 nmol/site) followed by morphine (10 mg/kg s.c.) for 9 days. Tail-flick latency was measured on odd days. Scores are expressed as mean ± S.E.M. *P < .05 versus the group receiving vehicle/morphine. B, naloxone-precipitated withdrawal after 9 days of KN-93 (10 nmol/site) or KN-92 (10 nmol/site) and morphine (10 mg/kg s.c.) treatment. Data are mean ± S.E.M. *P < .05 versus the group receiving vehicle/morphine treatment.

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**Fig. 5.** Intrahippocampal administration of CaMKII antisense (AS) and sense (S) oligonucleotides attenuated morphine tolerance and dependence. A, protein immunoblot of CaMKII and P 38 MAPK after the antisense or sense oligonucleotide to CaMKII treatment. Animals (n = 6/group) receiving vehicle, antisense, or sense oligonucleotides (10 μg/site) twice per day for 9 days. Twelve hours after the last injection, a portion of hippocampus (30 μg) from each rat was separated by 10% SDS-polyacrylamide gel electrophoresis and examined by Western blot analysis using mouse monoclonal anti-CaMKII antibody or mouse monoclonal anti-P38 MAPK antibody. B, CaMKII antisense oligonucleotides attenuated morphine tolerance. Animals (n = 6/group) were treated with vehicle or the antisense (10 μg/site) or sense oligonucleotides (10 μg/site) to CaMKII by intrahippocampal injection 15 min before each morphine treatment (10 mg/kg s.c.) for 9 days. Tail-flick latency was measured on odd days. *P < .05 versus the group receiving vehicle/morphine treatment. C, naloxone-precipitated withdrawal on day 10. Animals in each group were the same as that described in B. Scores are expressed as mean ± S.E.M. *P < .05 versus the group receiving vehicle/morphine treatment.
istration of the kinase inhibitors in other regions of the hippocampus (CA1 and CA3) also produced similar results (data not shown). Furthermore, down-regulation of hippocampal CaMKII expression by its antisense oligonucleotide, which has been reported to reduce the kinase expression and activity (Muthalif et al., 1996), also remarkably attenuated the development of morphine tolerance and dependence. The effect of antisense oligonucleotide injection was selective to CaMKII because no attenuation of other proteins, for example P38 MAPK, was detectable. However, inhibition of striatal CaMKII did not attenuate morphine tolerance, indicating regional specificity. These results suggest that hippocampal CaMKII is vitally involved in the development of morphine addiction and down-regulation of this kinase may have some therapeutic benefit in the treatment of opiate tolerance and dependence.

As mentioned in the introduction, NMDA receptor antagonists and NO inhibitors, which impair learning and memory, prevent opiate tolerance and dependence (Trujillo and Akil, 1991; London et al., 1995). Interestingly, both NMDA and nitric oxide have been reported to regulate the expression of CaMKII in hippocampal dentate gyrus (Johnston and Morris, 1995), and a recent study shows that NMDA receptor is associated with CaMKII in the forebrain (Leonard et al., 1999). It is possible that NMDA antagonists and NO inhibitors exert their antiaddictive effects by indirectly regulating hippocampal CaMKII. The present study provides the further evidence for the possible involvement of learning and memory in opiate tolerance and dependence.

Although inhibition or down-regulation of hippocampal CaMKII attenuated morphine tolerance and dependence, the underlying molecular mechanism remains unclear. There are several possibilities for the consequences of inhibition of CaMKII. At the cellular level, tolerance manifests itself as a decreased response of opioid receptor to its agonist upon repeated exposure to the agonist, namely desensitization, and phosphorylation by protein kinases of receptors and/or many other essential signal molecules has been implicated as one of the important mechanisms in the opioid receptor desensitization (Strassheim and Malbon, 1994; Pei et al., 1995; Ueda et al., 1995; Zhang et al., 1996; Fan et al., 1997; Koch et al., 1997). CaMKII, a serine/threonine-dependent protein kinase, has been reported to phosphorylate opioid receptors and lead to the receptor desensitization in cellular models (Mestek et al., 1995; Koch et al., 1997). In addition, CaMKII has been shown to phosphorylate cyclic AMP response element-binding protein (Matthews et al., 1994; Sun et al., 1994), which is well known to be involved in morphine addiction (Maldonado et al., 1996; Lane-Ladd et al., 1997). Therefore, it is hypothesized from these data that inhibition or down-regulation of CaMKII prevents morphine-induced activation of the kinase and subsequent phosphorylation of opioid receptors and other signal proteins, and thus attenuates tolerance and dependence. On the other hand, opiate addiction may involve complex changes in neurocircuitry, appearing as augmented long-term potentiation (Mansouri et al., 1997). CaMKII is important in neuronal development and formation of long-term potentiation (Zou and Cline, 1996; Bortolotto and Collingridge, 1998), so inhibition of this kinase prevents the neuronal changes induced by chronic opiate treatment and the subsequent tolerance and dependence.

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References


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