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Modulation of Ca^{2+}/Calmodulin-Dependent Protein Kinase II Activity by Acute and Chronic Morphine Administration in Rat Hippocampus: Differential Regulation of α and β Isoforms

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ABSTRACT

Calcium/calmodulin-dependent protein kinase II (CaMK II) has been shown to be involved in the regulation of opioid receptor signaling. The present study showed that acute morphine treatment significantly increased both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent activities of CaMK II in the rat hippocampus, with little alteration in the protein level of either α or β isoform of CaMK II. However, chronic morphine treatment, by which rats were observed to develop apparent tolerance to morphine, significantly down-regulated both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent activities of CaMK II and differentially regulated the expression of α and β isoforms of CaMK II at protein and mRNA levels. Application of naloxone or discontinuation of morphine treatment after chronic morphine administration, which induced the withdrawal syndrome of morphine, resulted in the overshoot of CaMK II (at both protein and mRNA levels) and its kinase activity. The phenomena of overshoot were mainly observed in the β isoform of CaMK II but not in the α isoform. The effects of both acute and chronic morphine treatments on CaMK II could be completely abolished by the concomitant application of naloxone, indicating that the effects of morphine were achieved through activation of opioid receptors. Our data demonstrated that both acute and chronic morphine treatments could effectively modulate the activity and the expression of CaMK II in the hippocampus.

Calcium (Ca^{2+})/calmodulin-dependent protein kinase (CaMK II), a multifunctional protein kinase the activation of which depends on Ca^{2+}/calmodulin, is highly concentrated in brain tissues. At least five isoforms of CaMK II (α, β, β', γ, and δ) are found to express in rat brain and to function in the form of homomultimers or heteromultimers (Hanson and Schulman, 1992). Among them, the α and β isoforms are restricted in nervous tissues, especially in the hippocampus, where they constitute ~2% of the total protein (Schulman, 1993), whereas γ and δ are found in most tissues besides brain (Hanson and Schulman, 1992; Braun and Schulman, 1995). An important characteristic of CaMK II is its autophosphorylation, which is dependent on Ca^{2+}/calmodulin and essential for its activation (Kwiatkowski et al., 1988). Autophosphorylation enables the kinase to phosphorylate substrates in a Ca^{2+}/calmodulin-independent manner and thus prolongs the duration of its effect. Activation of CaMK II in hippocampus has been shown to play an important role in neuroplasticity, gene expression, learning, and memory (Hanson and Schulman, 1992; Braun and Schulman, 1995; Mayford et al., 1996; Cho et al., 1998; Giese et al., 1998; Koninck and Schulman, 1998).

The mechanisms underlying opiate dependence, tolerance, and addiction in response to repeated or chronic opiate administration are still poorly understood. Mounting evidence, however, shows that opioid receptor phosphorylation upon agonist stimulation plays a critical role in these processes. Protein kinases responsible for opioid receptor phosphorylation have been reported to include protein kinase C, cAMP-dependent protein kinase, G-protein-coupled receptor kinase (Yu, 1996; Nestler and Aghajanian, 1997). Recent evidence demonstrates that CaMK II is also involved in opioid receptor desensitization, a proposed cellular mechanism of animal opiate tolerance (Mestek et al., 1995; Koch et al., 1997). Mestek et al. report that the μ opioid receptor displays a stronger desensitization when active CaMK II, but not boiled CaMK II, has been injected into the μ opioid receptor expressed in *Xenopus* oocytes. It has been also reported that CaMK II significantly promotes the agonist-induced desensitization of μ opioid receptor expressed in human embryonic kidney 293 cells, and this effect is significantly attenuated by the mutation of CaMK II phosphorylation sites (Ser-261 and Ser-266) on the third intracellular loop of μ opioid receptors

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ABBREVIATIONS: CaMK II, Ca^{2+}/calmodulin-dependent protein kinase II; NS, normal saline; DTT, dithiothreitol.

(Koch et al.,1997). Furthermore, recent studies in our laboratory show that CaMK II is effectively involved in the regulation of opioid receptor-mediated cellular signaling (Fan et al., 1997). However, no information regarding the effect of opiates on CaMK II activity is available thus far. The present study, therefore, was undertaken to investigate the potential modulatory effect of morphine, a most frequently used opiate analgesic in clinic, on CaMK II activity and expression in rat hippocampus.

Experimental Procedures

Materials and Reagents. Hydrochloride morphine was generously provided by Professor Jing Wengqiao (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China). [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from DuPont-New England Nuclear (Boston, MA). Monoclonal antibody specific for the α or β isoform of CaMK II, respectively, and Sepharose-protein A were obtained from Life Technologies, Inc. (Grand Island, NY). ECL Western blot analysis system was purchased from Amersham International (Buckinghamshire, UK). Polypeptide substrate of CaMK II, autocamtide-2, was synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). P81 phosphocellulose paper was obtained from Whatman (Maidstone, England).

Animals and Treatment Protocol. Male Sprague-Dawley rats (200–250 g) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Rats were housed in groups and maintained on a 12-h light/dark cycle with food and water freely available. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For acute treatment, rats were s.c. injected with various doses of morphine or equal volume of normal saline (NS), then sacrificed at indicated times. For chronic treatment, rats were first s.c. injected with morphine for 9 consecutive days (20 mg/kg morphine or an equal volume of NS), and on the 10th day, rats were sacrificed 1 h after different treatments, according to the experimental protocol.

The hippocampus, brainstem, or spinal cord was homogenized in an ice-cold lysis buffer [50 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 1 mM EGTA, 10 mM sodium pyrophosphate, 0.4 mM sodium molybdate, 2 μg/ml leupeptin, 2 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT)]. The homogenate was centrifuged at 10,000*g* for 10 min, and the resultant supernatant was subjected to CaMK II activity assay and Western blot analysis as described below. Protein concentration was determined by Bradford method.

CaMK II Activity Assay. CaMK II activity was determined essentially according to the method described by Occur and Schulman (1991). Reactions were carried out in the mixture (final volume of 50 μl) containing 50 mM 1,4-piperazinediethanesulfonic acid, 1 mM DTT, 0.25 mM EGTA, 20 μM autocamtide-2, 100 μM ATP, 2 μCi of [γ-^{32}P]ATP (3000 Ci/mM), 20 μg/ml calmodulin, and 0.75 mM $CaCl_2$. To measure the Ca^{2+}/calmodulin-independent protein kinase activity of CaMK II, reactions were performed in the absence of Ca^{2+} and calmodulin and in the presence of 1 mM EGTA. All reactions were initiated by addition of 5 μg of homogenate and incubated at 30°C for 30 s. Phosphorylation was terminated by spotting 30 μl of sample onto P81 phosphocellulose paper and immediately immersing it into 75 mM H_3PO_4. The papers were washed four times in 75 mM H_3PO_4 and dried. Then, the radioactivity of samples was quantified by liquid scintillation counting.

Autophosphorylation of β Isoform of CaMK II. Autophosphorylation of CaMK II was performed according to the method of Popoli et al. (1995). Samples (20 μg of protein) were phosphorylated with [γ-^{32}P]ATP as described above. The reaction was stopped by the addition of SDS and heating for 3 min; then 4 volumes of buffer A (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) were added. To each sample, 2 μg of CaMK II β antibody were added, and the samples were incubated for 2 h at 4°C with shaking. Then, 15 μl of protein A-Sepharose beads were added, and incubation continued for 2 h. The beads were washed three times with 1 ml of buffer A containing 0.6 M NaCl and twice with buffer A. Bound kinase was solubilized by SDS-electrophoresis buffer and separated by SDS-polyacrylamide gel electrophoresis. The densities of the CaMK II β bands were quantified by scanning densitometry with a Bio-Rad model GS 700 imaging densitometer (Molecular Dynamics, Sunnyvale, CA) after autoradiography.

Immunoblot of CaMK II. Extract from both control and drug-treated rat hippocampus was boiled for 3 min in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 50 mM DTT) and then subjected to SDS-polyacrylamide gel electrophoresis with a 10% acrylamide gel. Protein bands were electrically transferred to nitrocellulose membranes. The blots were blocked for 2 h in TBST buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20 containing 5% nonfat milk) at room temperature and then incubated with monoclonal antibody against CaMK II α for 1 h. After being washed for three times with TBST, the blots were incubated with horseradish peroxidase-conjugated antimouse secondary antibody for 1 h at room temperature. The washings were repeated, and the blots were developed by enhanced chemiluminescence method. For detection of CaMK II β, the same immunoblot was stripped of previous antibody and reprobed with anti-CaMK II β antibody. The densities of the CaMK II bands were quantified by scanning densitometry with the Bio-Rad imaging densitometer.

Northern Blot Analysis. Total RNA was isolated from rat hippocampus by the RNA isolation kit TRIzol obtained from Life Technologies, Inc. An equal amount of total RNA (10 μg) was fractionated on 1.0% agarose gel, transferred onto a nylon membrane (Amersham Life Science, Arlington Heights, IL), and immobilized by UV cross-linking. Fragments containing an entire coding region of the α or β isoform of CaMK II, respectively, were purified and used as probes. Probes were prepared by random priming labeling with [α-^{32}P]dCTP to a specific activity of 5×10^8 dpm/g DNA using the Ready To Go DNA Labeling kit (Pharmacia Biotech, Beijing, China). The blots were prehybridized in 0.5 M phosphate buffer (pH 7.2) containing 7% SDS, 1 mM EDTA at 65°C for 4 to 6 h, and hybridized to ^{32}P-labeled probes at 65°C for 20 to 24 h. After hybridization, the membrane was washed twice in 2× SSC/0.1% SDS (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at room temperature each for 15 min and then twice in 0.5× SSC/0.1% SDS at 65°C each for 15 min. The membrane was exposed to X-ray film for 2 to 5 days at −80°C and CaMK II mRNA was quantified by scanning densitometry. To confirm equal loading and potential effect of morphine treatment on α or β isoform of CaMK II, the same blot was routinely probed with an α or β probe, respectively, after being stripped of previous radioactivity.

Statistical Analysis. Results were given as mean ± S.D., except where indicated, and were compared by Student's *t* test with $P < .05$ taken as statistically significant.

Results

Effect of Acute Morphine Treatment on CaMK II Activity. Autocamtide-2 has been shown to be a CaMK II-specific substrate by the early reports from other laboratories (Hanson et al., 1989; Wenham et al., 1994) and further confirmed by our pilot experiments in the present study. The incorporation of ^{32}Pi into autocamtide-2 was essentially dependent on the presence of Ca^{2+} and calmodulin (increased about 50-fold relative to control), whereas cAMP, a cAMP-dependent protein kinase activator, and phosphatidylserine and diolein, protein kinase C activators, had no significant effect on ^{32}Pi incorporation into this peptide under the identical assay conditions. In addition, phosphorylation of this

peptide was completely blocked by the addition of KN62, a calmodulin-competitive antagonist, but not by the addition of protein kinase C inhibitors Gö 6976 and chelerythrine, or the cAMP-dependent protein kinase inhibitor H-89, further demonstrating the autocamtide-2 is a specific substrate of CaMK II. The control activity of CaMK II was 0.9 ± 0.2 pmol/min/μg protein in the absence of Ca^{2+}/calmodulin and 35.2 ± 0.8 pmol/min/μg protein in the presence of Ca^{2+}/calmodulin, which were comparable with the results reported (Blitzer et al., 1998).

Both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activities were significantly induced in the hippocampus by acute morphine (20 mg/kg for 1 h) administration (Table 1). The Ca^{2+}/calmodulin-independent activity of CaMK II was increased by 50–60% relative to the control. A time-course study showed that maximal stimulation of the enzyme was achieved 1 h after morphine administration; thereafter, the CaMK II activity declined and returned to basal level at about 4 h (Table 2). Morphine-stimulated CaMK II activation was completely abolished by the concomitant administration of naloxone, an opioid receptor antagonist, indicating that this effect of morphine was exerted by the activation of opioid receptors.

To further verify the morphine stimulation of Ca^{2+}/calmodulin-dependent CaMK II activity in hippocampus, autophosphorylation of CaMK II was determined. The β isoform of CaMK II in hippocampal homogenate from rats treated with morphine or NS was immunoprecipitated by its monoclonal antibody, and its autophosphorylation was assessed in the presence of Ca^{2+}/calmodulin. As shown in Fig. 1, morphine treatment significantly enhanced the Ca^{2+}/calmodulin-dependent autophosphorylation of CaMK II β by about 50 to 60% above the control. The data demonstrated that the acute morphine administration indeed stimulated CaMK II activation in the hippocampus.

We also observed the acute effect of morphine on CaMK II activity in the rat brainstem and spinal cord, which have been established to be important sites in the analgesia of morphine. The activity in the brainstem or spinal cord had not been significantly altered by the acute morphine treatment (Fig. 2). The data suggest that the CaMK II activation and analgesia produced by morphine treatment may rely on different pathways.

Effect of Acute Morphine Treatment on the Level of CaMK II Protein. The potential effect of acute morphine treatment on the protein level of CaMK II in the hippocampus was detected by Western blot analysis. One h after morphine treatment, the protein level of the β isoform was slightly increased (121 ± 5%), but that of the α isoform of CaMK II was slightly decreased (92 ± 5%) (Fig. 3), implying differential regulation by morphine of α and β isoforms of CaMK II. The effect of the morphine on the protein level of both the α and β isoforms could be blocked by the concomitant application of naloxone, which by itself had no significant effect on CaMK II activity (Fig. 3), indicating that the effect of morphine was mediated by opioid receptors.

Effect of Chronic Morphine Treatment on CaMK II Activity. After chronic morphine treatment for 9 days, rats developed apparent tolerance, which manifested in both behavioral performance and reduced sensitivity to morphine (data not shown). In contrast to the acute single treatment, both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activities 1 h after morphine treatment on the 10th day were decreased to ~60% of kinase level of the control rats (receiving NS for 10 days) (Table 3). However, the cessation of the morphine injection on the 10th day (using NS instead) led to the marked increase in CaMK II activity as compared with those receiving morphine treatment on the

TABLE 1

Dose-dependent of activation of CaMK II in the hippocampus Rats (n = 7) were sacrificed at 1 h after morphine treatment at the indicated dose; then the hippocampi were removed for CaMK II activity assay as described in *Experimental Procedures*. Results are expressed as mean ± S.E.M.

Dose	CaMK II Activity	
	$-Ca^{2+}$/calmodulin	$+Ca^{2+}$/calmodulin
mg/kg	*pmol/min/μg protein*	*pmol/min/μg protein*
Control	0.9 ± 0.2	35.2 ± 0.8
10	1.0 ± 0.1	40.4 ± 4.9
20	1.4 ± 0.4[a]	65.5 ± 10.5[a]
40	1.5 ± 0.4[a]	67.4 ± 10.2[a]
40 + Nal	0.8 ± 0.2	35.5 ± 3.5

[a] $P < .01$ versus control.

TABLE 2

Time course of activation of CaMK II in the hippocampus

Time	CaMK II Activity	
	$-Ca^{2+}$/calmodulin	$+Ca^{2+}$/calmodulin
h	*pmol/min/μg protein*	*pmol/min/μg protein*
0	0.8 ± 0.1	34.1 ± 1.8
0.5	0.9 ± 0.2	36.0 ± 3.4
1	1.4 ± 0.4[a]	66.3 ± 3.4[a]
2	1.3 ± 0.4[a]	46.5 ± 5.2[a]
4	0.9 ± 0.2	39.5 ± 3.2

[a] $P < .01$ versus control.

Rats were sacrificed at the indicated times after morphine treatment at the dose of 20 mg/kg; then the hippocampi were removed for CaM II activity assayed as described in *Experimental Procedures*. Results are expressed as mean ± S.E.M.

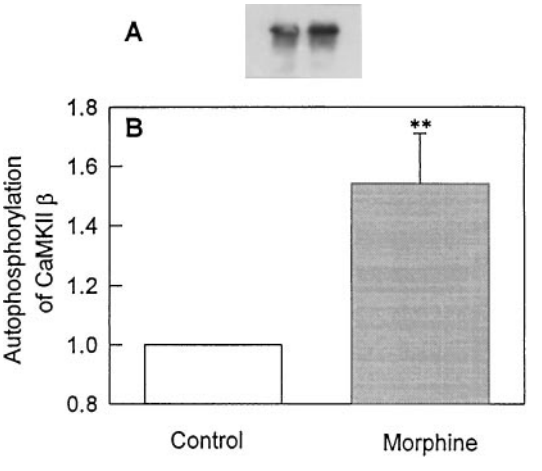

Fig. 1. Effect of acute morphine treatment on the autophosphorylation of CaMK II in hippocampus. After being treated with morphine (20 mg/kg for 1 h), rats were sacrificed, and the hippocampi were removed for autophosphorylation assay as described in *Experimental Procedures*. The β isoform of CaMK II was immunoprecipitated by specific antibody, and its autophosphorylation in the presence of Ca^{2+} and calmodulin was analyzed by autoradiography. A, representative of autoradiography; B, quantitative data estimated by scanning densitometry from three independent experiments. Results are expressed as mean ± S.D. **, $P < .01$ versus control.

10th day (Table 3). The increase in CaMK II activity resulted from discontinuation of the morphine treatment, which lasted at least 3 days (113 ± 6% and 120 ± 7% of control for Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activity, respectively). In addition, the effect of morphine was totally prevented by the simultaneous use of naloxone, indicating that the regulation of CaMK II activity by chronic morphine treatment was also through the activation of opioid receptors.

Effect of Chronic Morphine Treatment on the Protein Level of CaMK II. The effect of chronic morphine treatment on the protein level of α and β isoforms of CaMK II in hippocampus was examined further. As shown in Fig. 4, the continuous morphine treatment for 10 days reduced the protein level of both α (lane 4) and β (lane 4) isoforms of CaMK II, with a more pronounced decrease in the α isoform, as compared with the kinase level (lane 1) in the control rats receiving NS injection for 10 consecutive days. However, if the rats were injected with NS instead of morphine on the 10th day after continuous morphine treatment for 9 days, the protein level of the β isoform but not the α isoform was markedly increased (Fig. 4, lane 3), as compared with the samples from rats with the continuous morphine treatment on the 10th day (lane 4).

Effect of Chronic Morphine Treatment on the mRNA Level of CaMK II. To further examine the differential modulation by chronic morphine treatment of α and β isoforms of CaMK II, the mRNA level of the α and β isoforms was determined by Northern blot analysis using cDNA probes specific to each of them. The continuous morphine treatment for 10 days reduced the mRNA level of the β isoform but had no pronounced effect on that of α isoform (Fig. 5, lane 4), as compared with that (Fig. 5, lane 1) in the control rats receiving NS injection for 10 days continuously. Discontinuation of morphine treatment on the 10th day also markedly increased the mRNA level of the β isoform of CaMK II but not that of the α isoform (Fig. 5, lane 3), as compared with the samples from rats given the continuous morphine treatment on the 10th day (Fig. 5, lane 4). These results demonstrated that the expression of the α and β isoforms of CaMK II was indeed differentially regulated at both mRNA and protein levels by chronic morphine treatment.

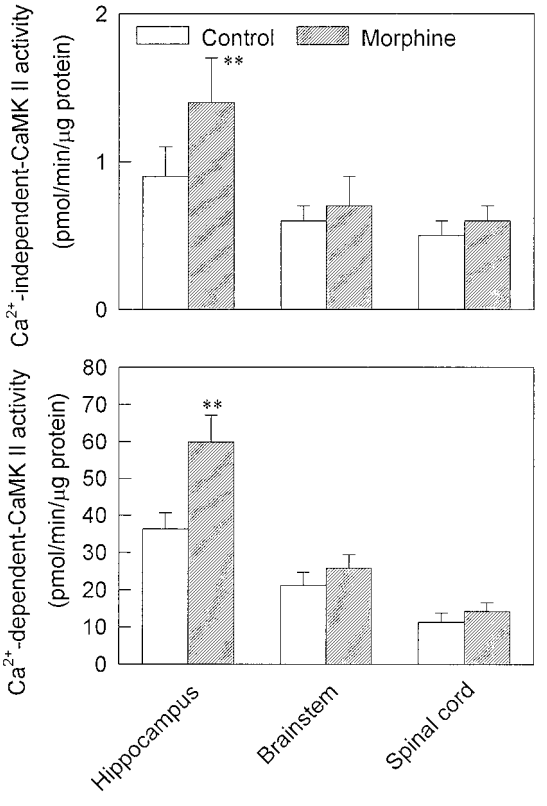

Fig. 2. Effect of acute morphine treatment on the activity of CaMK II in hippocampus, brainstem, and spinal cord. After morphine treatment (20 mg/kg for 1 h), rats (n = 5) were sacrificed, and the indicated tissues were removed for CaMK II activity assay as described in *Experimental Procedures*. Results are expressed as mean ± S.E.M. **, $P < .01$ versus control.

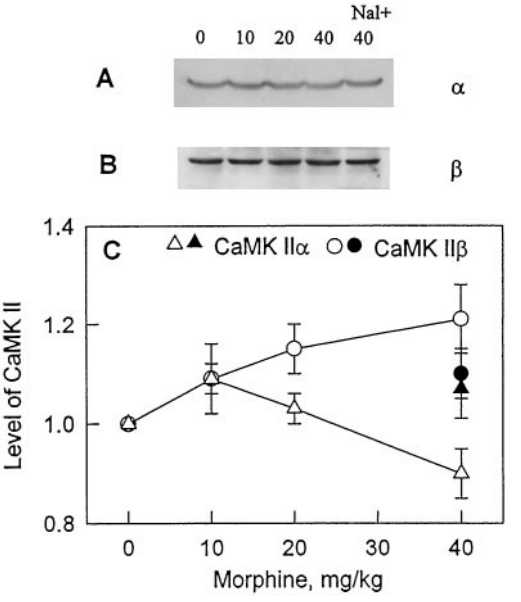

Fig. 3. Effect of acute morphine treatment on the protein level of the α and β isoforms of CaMK II in the hippocampus. After morphine treatment (20 mg/kg for 1 h), rats were sacrificed, and the hippocampi were removed for Western blot analysis as described in *Experimental Procedures*. A and B, representative immunoblot for the α and β isoforms of CaMK II, respectively; C, quantitative data estimated by scanning densitometry. Filled circle and triangle represent the treatment of 40 mg/kg morphine plus 10 mg/kg naloxone. Results are expressed as mean ± S.D. from four independent experiments.

TABLE 3

Effect of chronic morphine treatment on the activity of CaMK II in the hippocampus Rats (n = 7) received s.c. injections of morphine (20 mg/kg) for 9 consecutive days, and on the 10th day, they received different treatments as indicated in the table. After treatment, rats were sacrificed, and the activity of CaMK II was assayed as described in *Experimental Procedures*. Results are expressed as mean ± S.D.

No.	Treatment		CaMK II Activity	
	1–9 days	10th day	−Ca^{2+}/calmodulin	+Ca^{2+}/calmodulin
			pmol/min/μg protein	*pmol/min/μg protein*
1	NS	NS	0.8 ± 0.1	35.1 ± 3.9
2	NS	Mor	1.2 ± 0.2[a]	47.0 ± 5.2[a]
3	Mor	NS	0.8 ± 0.1[b]	33.4 ± 5.2[b]
4	Mor	Mor	0.5 ± 0.1[a]	23.1 ± 4.8[a]
5	Mor	Mor + NS	0.5 ± 0.1	22.5 ± 4.1
6	Mor	Mor + Nal	0.9 ± 0.2[c]	38.5 ± 4.5[c]

Nal, naloxone; Mor, morphine.
[a] $P < .05$ versus 1 (NS/NS).
[b] $P < .05$ versus 4 (Mor/Mor).
[c] $P < .05$ versus 5 (Mor/Mor + NS).

Effect of Naloxone-precipitated Opiate Withdrawal on the Activity and Expression of CaMK II. After 10 days of chronic morphine treatment, physical withdrawal was precipitated by administration of naloxone. All rats demonstrated behaviors characteristic of opiate withdrawal, including jumping, wet dog shakes, teeth chatter, ptosis, lacrimation, diarrhea, and irritability. Fifteen minutes after naloxone administration, the rats were sacrificed, and the hippocampus samples were prepared for CaMK II activity assay, Western blot, and Northern blot analysis. Naloxone-precipitated opiate withdrawal substantially increased both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activities (Table 3, row 6) by ~80% above the kinase activity in control rats receiving NS instead of naloxone (Table 3, row 5), indicating the overshoot of CaMK II activity. Overshoot of CaMK II was also observed at both the protein and mRNA levels of β isoform (2.1- and 1.6-fold of control, respectively, Fig. 4, lanes 5 and 6; Fig. 5, lanes 5 and 6), suggesting that the increase in CaMK II activity was mainly due to the increase of the β isoform. The expression of the α isoform at either protein or mRNA level was not significantly affected by this same treatment (Fig. 4, lanes 5 and 6; Fig. 5, lanes 5 and 6), indicating again the differential regulation of the α and β isoforms of CaMK II by morphine administration and withdrawal.

Discussion

The critical roles of opioid receptor phosphorylation in the opioid tolerance, dependence, and addiction have been well acknowledged (Nestler, 1997). Thus, investigation of the protein kinases potentially related to receptor phosphorylation will greatly promote the understanding of the mechanisms underlying these processes. Our results in the present study demonstrated that both acute and chronic morphine treatment could effectively modulate the CaMK II activity in the rat hippocampus in dose- and time-dependent manners. Both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activities were significantly enhanced by acute morphine treatment but attenuated by chronic morphine administration. The modulation of CaMK II activity by morphine treatment appeared to associate with the alteration of CaMK II expression at protein and mRNA levels, although α and β isoforms of CaMK II were differentially regulated. Moreover, this modulatory effect of both acute and chronic

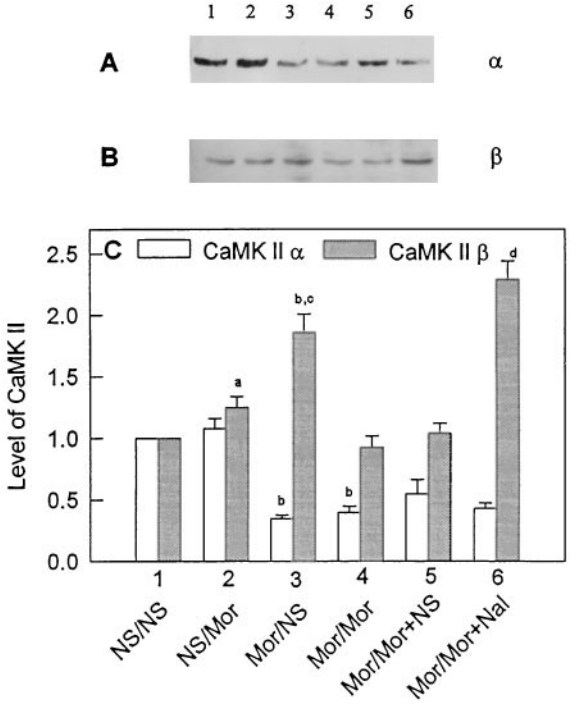

Fig. 4. Effect of chronic morphine treatment on the protein level of α and β isoforms of CaMK II in the hippocampus. Rats were s.c. injected with either NS or morphine for 9 days (lanes 1–4) or 10 days (lanes 5 and 6), and on the 10th day, they received different treatments as indicated in the plot. The protein level of the α and β isoforms of CaMK II was determined by Western blot analysis as described in *Experimental Procedures*. A and B, representative immunoblot for the α and β isoforms of CaMK II, respectively; C, quantitative data estimated by scanning densitometry. Nal, naloxone; Mor, morphine. Results are expressed as mean ± S.D. from three independent experiments. [a], $P < .05$; [b], $P < .01$ versus 1 (NS/NS); [c], $P < .01$ versus 4 (Mor/Mor); [d], $P < .01$ versus 5 (Mor/Mor+NS).

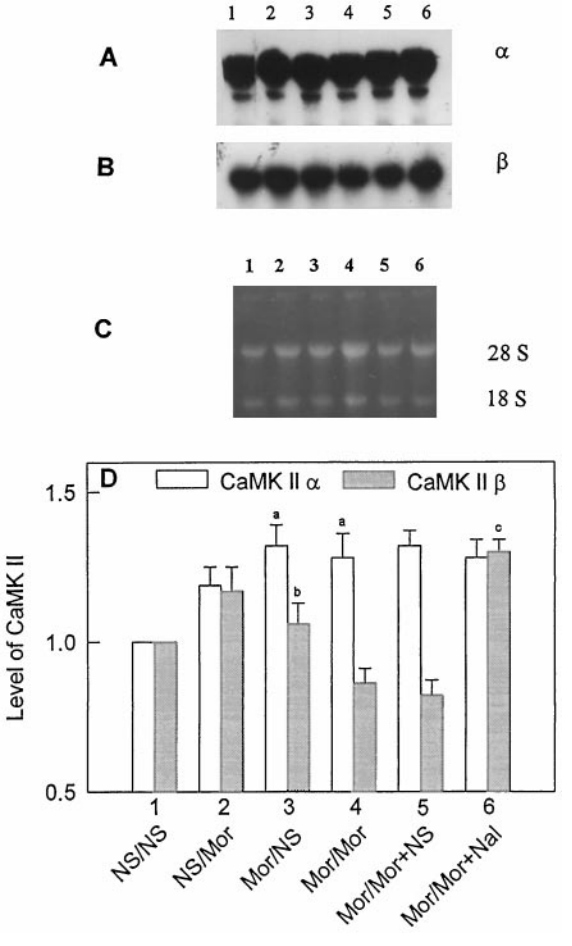

Fig. 5. Effect of chronic morphine treatment on the mRNA level of the α and β isoforms of CaMK II in the hippocampus. Rats were s.c. injected with either NS or morphine for 9 days (lanes 1–4) or 10 days (lanes 5 and 6), and on the 10th day, they received different treatments as indicated in the plot. The mRNA level of the α and β isoforms of CaMK II was determined by Northern blot analysis as described in *Experimental Procedures*. A and B, representatives of Northern blot analysis, respectively; C, photograph of the preparative electrophoretic gel after staining with ethidium bromide; D, quantitative data estimated by scanning densitometry. Nal. naloxone; Mor, morphine. Results are expressed as mean ± S.D. from two independent experiments. [a], $P < .05$ versus 1 (NS/NS); [b], $P < .05$ versus 4 (Mor/Mor); [c], $P < .01$ versus 5 (Mor/Mor+NS).

morphine treatments on CaMK II could be completely blocked by the concomitant application of opiate antagonist naloxone, indicating that these effects are mediated by opioid receptors.

In spite of the fact that several recent studies indicate that CaMK II is involved in the modulation of opioid receptor signaling (Mestek et al., 1995; Fan et al., 1997; Koch et al., 1997), no direct evidence for opiate regulation of the activity of CaMK II has been provided yet. To measure the activity of CaMK II, autocamtide-2, a widely used specific CaMK II substrate (Ocorr and Schulman, 1991; and present results), has been applied. The present study, using this method, showed clearly that acute morphine treatment significantly increased both Ca^{2+}/calmodulin-independent and Ca^{2+}/calmodulin-dependent CaMK II activities, which was further supported by the results from the autophosphorylation assay. Interestingly, modulation of CaMK II activity by morphine treatment was somehow different from that by high K^+-induced depolarization or brain-derived neurotrophic factor treatment (Gorelick et al., 1988; Ocorr and Schulman, 1991; Blanquet and Lamour, 1997). In later cases, Ca^{2+}/calmodulin-independent but not Ca^{2+}/calmodulin-dependent CaMK II activity was reported to increase in the hippocampus, suggesting that different stimulations via distinct receptor or ion channel may differentially modulate the kinase activity of CaMK II.

The acute morphine effect on CaMK II appeared to be site specific, because this treatment did not significantly affect the activity of CaMK II in the brainstem and spinal cord, which play important roles in the analgesia of morphine. This is somewhat difficult to explain, given that opioid receptors in the above two sites are more abundant than those in the hippocampus. However, it is likely that the abundance of CaMK II in the hippocampus enables it to be more sensitive to morphine treatment. Our data suggest that CaMK II activation and analgesia after morphine treatment may be produced through distinct pathways.

The mechanisms by which morphine regulates CaMK II activity are not yet clear. It has been shown that morphine can elevate the intracellular free Ca^{2+} by stimulating the opioid receptor, leading to the phosphatidylinositol hydrolysis, inositol formation, and subsequent release of Ca^{2+} from the intracellular store (Zimprich et al., 1995). The increased free Ca^{2+} concentration may result in the activation of CaMK II. Thus, it is likely that morphine regulation of CaMK II acts through the opioid receptor pathway. Another possible mechanism for morphine regulation of CaMK II activity and expression may be related to the up-regulation of the cAMP pathway after chronic morphine treatment (Nestler, 1997; Nestler and Aghajanian, 1997), as revealed by the very recent finding that the cAMP pathway can directly regulate the activity of CaMK II (Blitzer et al., 1998).

Overshoot or supersensitization of signaling components resulted from the withdrawal of opioid agonists after chronic treatment was shown to relate to opiate dependence and withdrawal (Collier, 1980; Nestler, 1992; Nestler et al., 1993). However, previous studies involving overshoot mainly focused on the changes in activity of related enzymes such as adenylyl cyclase (Avidor-Reiss et al., 1995; Avidor-Reiss et al., 1996, Ma et al., 1997), with little attention on the alterations of effector molecules at protein and mRNA levels. Our present results demonstrate that overshoot of CaMK II after withdrawal of morphine took place not only at the kinase activity level but also at the protein and mRNA levels. In addition, our data showed that overshoot of CaMK II could occur within a very short time and lasted for at least 3 days.

α and β CaMK II are the predominant isoforms specifically distributed in nervous system (Hanson and Schulman, 1992). The ratio of α to β is brain region specific and developmentally regulated (Hanson et al., 1989). Thus far, no significant functional difference between the two isoforms has been reported. Our results in the present study suggest that there exists a significant functional difference between them in response to morphine treatment: 1) acute morphine administration slightly led to an increase in protein level of β isoform of CaMK II and a decrease in that of α isoform; 2) discontinuation of morphine treatment after chronic morphine administration dramatically increased the protein and mRNA level of the β isoform but did not significantly affect that of the α isoform; and 3) naloxone-precipitated opiate withdrawal remarkably elevated the level of protein and mRNA of CaMK II β isoform but had no marked effect on that of α isoform. Our results indicate that the β isoform of CaMK II appears to be a major isoform in response to morphine treatment. The mechanisms underlying the differential modulation of isoforms of CaMK II remain to be further investigated.

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