Biochemical Pharmacology, Vol. 58, pp. 493–501, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00107-0

Alteration of Spinal Protein Kinase C Expression and Kinetics in Morphine, but not Clonidine, Tolerance

*Yaohui Li and Sandra C. Roerig**

Department of Pharmacology and Therapeutics, Louisiana State University Medical Center, Shreveport, LA 71130, U.S.A.

ABSTRACT. Antinociceptive synergism between spinally administered morphine and clonidine decreases to an additive interaction in morphine- and clonidine-tolerant mice. Spinally administered protein kinase C (PKC) inhibitors also decrease the synergism to addition. To determine whether chronic morphine or clonidine treatment alters spinal PKC activity, the present studies measured PKC activity and expression of PKC isoform proteins in spinal cord cytosol and membrane fractions. Mice were treated for 4 days with either placebo pellets, morphine pellets, s.c. saline, or s.c. clonidine. Morphine pellet-implanted mice were tolerant to morphine-induced tail flick antinociception, but not cross-tolerant to clonidine. Clonidine-pretreated mice were tolerant to clonidine, but not cross-tolerant to morphine. Induction of morphine tolerance produced a 2-fold lower K_m value for PKC (8.24 ± 1.67 μM in placebo pellet vs 4.43 ± 1.24 μM in morphine pellet) in cytosol, but not membrane fractions from spinal cord. V_{max} values were not different. No difference in K_m or V_{max} values was found between proteins from saline- and clonidine-pretreated animals. Immunoreactive cPKCα, βI, and γ isoforms decreased 14, 26, and 17%, respectively, in cytosol from morphine-tolerant animals. No difference in PKC isoforms was found in the membranes or in fractions from clonidine-tolerant mice. Morphine tolerance, but not clonidine tolerance, enhanced PKC activity while decreasing protein expression. BIOCHEM PHARMACOL **58**;3:493–501, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. protein kinase C; morphine; clonidine; tolerance; antinociception; spinal cord

The effects of opioids on the cAMP†-regulated second messenger system have been characterized extensively [1–3] and likely are involved in antinociception produced by spinally administered opioids [4]. In contrast, relatively little is known about opioid-induced regulation of intracellular signals in the phospholipase C-stimulated second messenger pathway or how this pathway may contribute to antinociception. *In vitro* studies in cultured cells and *Xenopus* oocytes show that activated opioid receptors stimulate phospholipase C activity to increase the production of inositol trisphosphates and diacylglycerol, which leads to enhanced PKC activity [5–10]. However, there is also evidence for opioid-induced inhibition of inositol trisphosphate accumulation [11, 12]. Behavioral studies show that pretreatment with LiCl, to deplete inositol trisphosphates, attenuates antinociception produced by i.c.v. morphine [13]. Coadministration of i.c.v. inositol-1,4,5-trisphosphate with morphine restores the morphine antinociceptive potency [13]. Antinociception produced by i.t. or i.c.v. administered opioid agonists is attenuated by pretreatment with phorbol ester activators of PKC [14–16]. The phorbol ester-induced attenuation of the δ-opioid receptor agonist Delt II-induced antinociception is reversed by calphostin C, a selective PKC inhibitor [16]. Thus, opioid-induced antinociception also may involve phospholipase C activation and changes in PKC activity.

A role for PKC in the development of opioid antinociceptive tolerance has been suggested from a number of studies. The nonselective PKC inhibitors H7 and H8 block development of tolerance to i.c.v. morphine [17, 18]. Intrathecal pretreatment with the selective PKC inhibitor calphostin C blocks the development of acute antinociceptive tolerance to the i.t. μ-opioid agonist DAMGO and to Delt II [16, 19]. Inhibition of PKC activity by the selective PKC inhibitor chelerythrine or by H7 also reduces the severity of naloxone-precipitated withdrawal symptoms in morphine-tolerant animals [18, 20]. These results suggest that modulation of PKC function may be important in the development of opioid tolerance and physical dependence.

Additional evidence for a role of PKC in morphine tolerance is implied from studies showing a change in PKC activity in brain regions from morphine-tolerant rats. PKC activity in cytosol, but not membrane fractions, from pons/medulla but not midbrain or cortex, is increased after

* Corresponding author: Sandra C. Roerig, Ph.D., Department of Pharmacology and Therapeutics, LSU Medical Center, 1501 Kings Highway, Shreveport, LA 71130. Tel. (318) 675-7877; FAX (318) 675-7857; E-mail: sroeri@lsumc.edu

† *Abbreviations*: cAMP, cyclic AMP; PKC, protein kinase C; i.c.v., intracerebroventricular; i.t., intrathecal; Delt II, [D-Ala^2]-deltorphin II; DAMGO, [D-Ala^2], MePhe^4, Gly(ol)^5-enkephalin; IOD, integrated optical density; MP, morphine pellet(s); PP, placebo pellet(s); % MPE, percent maximum possible effect; and [^{3}H]PDBu, [^{3}H]phorbol-12,13-dibutyrate.

Received 20 July 1998; accepted 23 November 1998.

chronic systemic or i.c.v. morphine treatment [21, 22]. However, although the spinal cord expresses high concentrations of opioid receptors and is an important region for nociceptive transmission, spinal PKC activity has not yet been assessed in morphine-tolerant animals.

Expression of PKC isoform protein is also changed after chronic opioid treatment. The PKCαβ immunoreactivity in cytosol fractions from cerebral cortex decreases in rats treated chronically with systemic morphine and in human opioid addicts [23–25]. In the spinal cord, the immunoreactivity of PKCγ isoforms increases in the dorsal horn of rats tolerant to morphine [26]. Other PKC isoforms have not been evaluated in the spinal cord.

Recent studies from our laboratory [27] showed that chelerythrine or calphostin C decreases the analgesic synergism between spinal morphine and clonidine to an additive interaction. This finding suggests that activation of PKC in the spinal cord could be a factor regulating the synergistic interaction between spinal morphine and clonidine. The spinal morphine/clonidine synergism also changes to an additive interaction in morphine-tolerant [28] and clonidine-tolerant* mice. To facilitate understanding of the spinal morphine/clonidine interaction and the contribution of this interaction to development of morphine tolerance, the present experiments were designed to characterize PKC activity and expression of cPKC isoforms in the spinal cords of mice treated chronically with morphine or clonidine.

MATERIALS AND METHODS

Animals and Antinociceptive Testing

Male ICR mice (Harlan Sprague-Dawley) weighing 25–35 g were used in the experiments. The animals were housed five per cage and maintained in a room temperature of 22–24° and an alternating 12-hr light/dark cycle. Food and water were available *ad lib*. Animals were allowed at least 2 days of adaptation upon arrival and were used only once in each experiment.

Antinociception was determined by the radiant heat tail flick test [29]. The lamp intensity was adjusted to obtain a baseline latency at 2–4 sec. The cutoff time was set at 10 sec for the maximal antinociceptive response. The animals were tested before and 20 or 30 min after morphine sulfate or clonidine HCl administration, respectively. The % MPE was calculated using the formula:

$$\% \text{ MPE} = \frac{\text{post drug time} - \text{pre drug time}}{10 - \text{pre drug time}} \times 100$$

Drugs and Chemicals

Morphine sulfate, MP, and PP were obtained from the National Institutes on Drug Abuse. Clonidine HCl was purchased from the Sigma Chemical Co. Morphine sulfate and clonidine HCl were dissolved in saline and administered s.c. in a volume of 10 μL/g body weight. The PKC Biotrak enzyme assay system was purchased from Amersham. PKC antisera directed against the cPKCα isoform, chelerythrine, and calphostin C were purchased from Calbiochem (Calbiochem-Novabiochem Corp.). Antisera directed against the βI and γ cPKC isoforms were purchased from Santa Cruz Biotechnology, Inc. γ-[^{32}P]ATP was purchased from DuPont/NEN. SuperSignal^{TM} CL-HRP Substrate Working Solution was purchased from Pierce.

Chronic Drug Administration

MP were used for these studies because (a) previous investigators measuring PKC activity in morphine-tolerant mice used MP to induce tolerance [30], and (b) our previous studies measuring morphine/clonidine antinociceptive interactions in morphine-tolerant mice utilized the MP protocol [28]. Mice were implanted s.c. for 5 days with MP containing 75 mg of morphine base or PP containing only binding ingredients. On day 6 following pellet implantation, antinociceptive responses of all animals from MP and PP groups were assessed with different doses of s.c. morphine or clonidine by the tail-flick test.

Pellets containing clonidine were not available; thus, tolerance to clonidine was induced by once-daily s.c. clonidine injections. In preliminary studies, 0.3 mg/kg per day s.c. clonidine was administered for 4 days as described by Paul and Tran [31], who used CD-1 mice, but no tolerance developed to s.c. clonidine in the ICR mice (data not shown). After several other preliminary studies (data not shown), two clonidine dosing regimens were found in which different degrees of tolerance to s.c. clonidine could be measured. Thus, for chronic clonidine treatment, mice were injected s.c. with 1.25 mg/kg of clonidine HCl on days 1 and 2 and 2.5 mg/kg on days 3 and 4. This is a low-dose regimen. Mice were also treated with a high-dose regimen, 2.5 mg/kg of clonidine HCl for 2 days and 5 mg/kg for another 2 days. Vehicle-treated mice were injected once daily for 4 days with s.c. saline (0.9% NaCl). On day 5, antinociception produced by s.c. clonidine or morphine was assessed by the tail flick test.

Preparation of Spinal Cord Membrane and Cytosol Fractions

After chronic treatment as described above, animals were killed by decapitation, and spinal cords were excised quickly on an ice-cold petri dish. Each spinal cord was homogenized individually in 10 vol. of ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 48 mM β-mercaptoethanol, 0.32 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride (added fresh), and 10 mg/mL of leupeptin (added fresh). The homogenates were centrifuged at 4° for 10 min at 1000 *g*. The supernatants were again centrifuged at 4° for 30 min at 100,000 *g* to yield the supernatant and pellet fractions. The supernatant was

* Roerig SC, Busch T and Li Y, Manuscript submitted for publication.

the cytosolic fraction. The pellet was resuspended in the same buffer containing 1% Triton X-100 (v/v), stirred for 30 min at 4°, and centrifuged again at 100,000 *g* for 30 min at 4°. The supernatant contained the solubilized membrane fraction. Protein concentrations in these fractions were determined using a modification of the method of Lowry *et al*. [32].

PKC Activity Measurement

PKC activity was measured by tracing the transfer of the [^{32}P] phosphate group from γ-[^{32}P]ATP to a specific synthetic PKC substrate peptide. Spinal cord cytosol or membrane fractions were analyzed for PKC activity as described by the manufacturer. Briefly, samples containing membrane or cytosol fraction protein were incubated in 55 μL of a mixture containing calcium (1.3 mM), phosphatidylserine (3.4 μg/mL), dithiothreitol (3.4 mM), phorbol 12-myristate 13-acetate (2.7 μg/mL), the synthetic peptide substrate (102 μM), and 0.2 μCi γ-[^{32}P]ATP for 10 min at 37°. The reactions were stopped by the addition of 10 μL of 300 mM orthophosphoric acid, and the mixtures were applied to peptide-binding paper discs. The discs were washed twice with 75 mM orthophosphoric acid, and then were transferred to scintillation vials for liquid scintillation counting. In preliminary studies using protein amounts of 0.1 to 10 μg, there was a linear relationship between protein concentration and PKC activity ($r = 0.99$, data not shown). For the remainder of the studies, 1 μg protein was used in each experiment. Duplicate or triplicate samples were analyzed for each separate experiment.

For studies to determine kinetic parameters, PKC activity was measured in the same way as described above except that the final concentrations of the synthetic specific PKC substrate peptide were varied from 1 to 100 μM. Velocity of the reaction was expressed as picomoles of [^{32}P]ATP incorporated into substrate per minute per microgram of protein.

To assure that the activity measured was that of PKC and not another kinase, two different selective PKC inhibitors were tested. Chelerythrine inhibits enzyme activity by interaction of the catalytic domain [33], and calphostin C interacts at the PKC regulatory site [34]. These two compounds are potent PKC inhibitors in rat brain, with the K_i for chelerythrine being about 0.7 μM [33], and the IC_{50} for calphostin C being 0.05 μM [35]. In the present experiments, various concentrations of the two inhibitors were added to both membrane and cytosol fractions, and percent inhibition of kinase activity was measured.

Immunoblot Analysis

Spinal cord cytosol or membrane fractions (50 μg protein per sample) were electrophoretically separated on 10% SDS polyacrylamide gels and transferred to Immobilon P membrane. Immunoblotting was performed as previously described [36] with overnight incubation of the primary antisera in a 1:500 dilution for the βI and γ antisera and 1:100 dilution for the α isoform antiserum. Secondary antisera were conjugated to horseradish peroxidase, and immunopositive bands were developed using Super Signal. The blot was then exposed to X-ray film. The immunopositive bands of PKC isoforms were quantified using densitometric analysis with the NIH Image software program to obtain the IOD value for each band.

Data Analysis

Antinociceptive ED_{50} values were calculated from dose–response curves using the Graded Dose Response Method of Tallarida and Murray [37]. At least four drug doses and eight animals per dose were used to determine each ED_{50} value.

For PKC activity assays, results were calculated as picomoles of [^{32}P]incorporated into peptide per minute per microgram of protein. Student's *t* test was used for statistical analysis of the data. For the PKC kinetics study, V_{max} and K_m values were obtained by using the Kinetics Analysis Methods of Tallarida and Murray [37]. Values were compared using the unpaired Student's *t*-test. For the studies with PKC inhibitors, percent inhibition of control activity (DMSO vehicle) was determined. The IC_{50} values for the inhibitors were calculated using the Graded Dose Response Method of Tallarida and Murray [37].

For immunoreactivity data, the average IOD values calculated from band density of control mice in each gel were obtained, and then the percentage of each band compared with the average IOD values was calculated. The results thus were presented as mean ± SEM of percent of control. Student's unpaired *t*-test was used to compare the MP- or clonidine-treated groups with appropriate PP or saline controls.

RESULTS

To assess the effects of MP implantation on acute morphine antinociception, dose–response curves for s.c. morphine were generated and are shown in Fig. 1. The morphine ED_{50} values calculated from those data are shown in Table 1. MP implantation increased the morphine ED_{50} value 2.5-fold compared with PP implantation. Dose–response curves for clonidine also were generated (Fig. 1). There was no significant difference for clonidine antinociception between MP- and PP-treated mice (Table 1).

To determine the effect of chronic clonidine treatment on clonidine-induced antinociception, mice were treated with s.c. clonidine or saline for 4 days. Results from these experiments are shown in Fig. 2, and ED_{50} values are shown in Table 1. In initial studies, mice were treated with clonidine in a low-dose regimen of 1.25 mg/kg for 2 days and 2.5 mg/kg for another 2 days. When mice were tested with acute clonidine after this treatment, there was no change in the clonidine ED_{50} value (Table 1).

In subsequent experiments, the doses for chronic

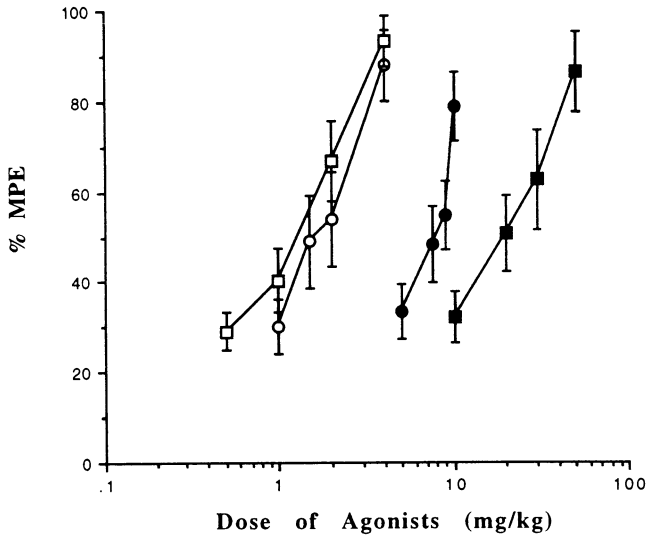

FIG. 1. Effect of MP implantation on morphine- and clonidine-induced antinociception in the tail flick test. Shown are dose–response curves for morphine (filled symbols) and clonidine (open symbols) after MP or PP implantation. The morphine (square symbols) or placebo (circle symbols) pellets were s.c. implanted for 5 days. Points represent the mean ± SEM values for 9–10 mice.

clonidine treatment were doubled, the high-dose treatment. Dose–response curves for acute s.c. clonidine and morphine then were obtained (Fig. 2, Table 1). The 4.5-fold increase in ED_{50} suggested that the mice were tolerant to s.c. clonidine. In contrast, ED_{50} values shown in Table 1 show that there was no significant difference for morphine antinociception between saline- and clonidine-treated groups.

To assure that the kinase activity measured in subsequent *in vitro* experiments was due to PKC activity, two different selective PKC inhibitors, chelerythrine and calphostin C,

TABLE 1. Effects of morphine pellet implantation or chronic clonidine treatment on morphine- and clonidine-induced antinociception

Treatment	ED_{50}* (mg/kg) (95% confidence interval)	
	Morphine sulfate	Clonidine HCl
Placebo pellet†	7.2 (6.3–8.2)	1.6 (1.3–2.0)
Morphine pellet†	18.4 (14.0–24.3)	1.1 (0.9–1.4)
Saline‡	3.9 (3.1–4.8)	1.3 (1.0–1.7)
Low-dose clonidine HCl‡		3.0 (1.4–5.9)
High-dose clonidine HCl‡	4.1 (3.6–4.7)	5.9 (3.6–9.6)

*The ED_{50} values were determined using at least four drug doses and at least eight mice per dose.

†Mice were implantede s.c. with morphine or placebo pellets for 5 days.

‡Mice were treated with s.c. saline or clonidine for 4 days.

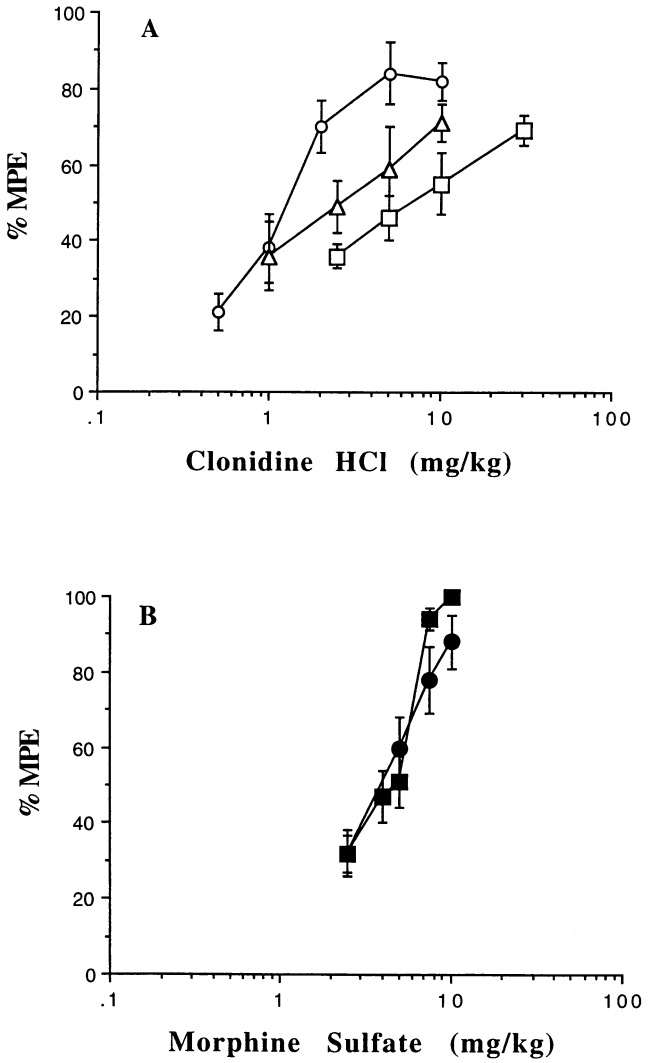

FIG. 2. Effect of chronic clonidine treatment on morphine and clonidine-induced antinociception in the tail flick test. Dose–response curves for clonidine (A) and morphine (B) after chronic saline or clonidine treatment. The saline (circles), low-dose clonidine (triangles; 1.25 mg/kg on days 1 and 2, 2.5 mg/kg on days 3 and 4), or high-dose clonidine (squares; 2.5 mg/kg on days 1 and 2, 5 mg/kg on days 3 and 4) was given s.c. once daily for 4 days. Points represent the mean ± SEM values for 9–10 mice.

were tested. PKC activity was measured in both membrane and cytosol fractions from spinal cords of untreated mice in the presence of 0.01, 0.1, and 1 μM calphostin C or 0.1, 1, 10, and 100 μM chelerythrine. Results of these experiments are shown in Fig. 3. Basal PKC activity (in the presence of the DMSO vehicle) in these experiments was 5.09 ± 0.75 and 2.18 ± 0.06 pmol [^{32}P]incorporated/min/μg protein in the cytosol and membrane fractions, respectively. The IC_{50} values also shown in Fig. 3 suggested that calphostin C was about 10-fold more potent than chelerythrine in inhibiting PKC activity. Chelerythrine showed similar inhibitory activity in membrane and cytosol fractions, and calphostin C was similarly active in membrane and cytosol fractions. These results showed that the enzyme activity measured in

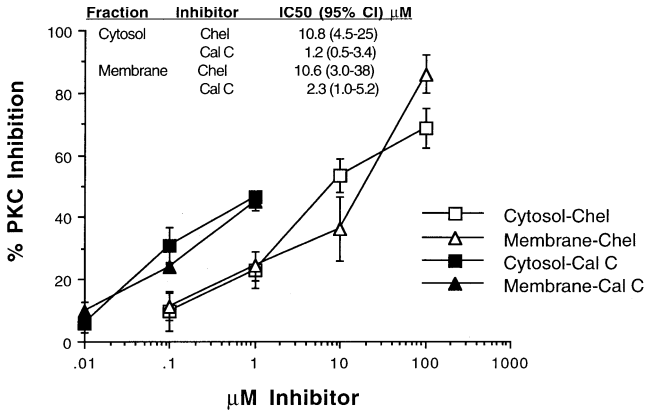

FIG. 3. Effects of chelerythrine and calphostin C on PKC activity in naive mouse spinal cord membrane and cytosol fractions. PKC activity was measured in the presence of the inhibitors or the DMSO vehicle. Percent inhibition of the DMSO (control) values is shown on the vertical axis. DMSO values were (in pmol [^{32}P]incorporated/min/μg protein): cytosol fractions = 5.09 ± 0.75; membrane fractions = 2.18 ± 0.06. Chel = chelerythrine, Cal C = calphostin C. Points represent the means ± SEM for fractions from three different spinal cords, with assays performed in triplicate. The IC_{50} values (95% confidence intervals) were calculated from the dose–response curves.

these experiments was blocked by selective PKC inhibitors with the expected potency difference, i.e. calphostin C was more potent than chelerythrine. The assay used measures the total enzyme activity of all PKC isoforms. Thus, although the present IC_{50} values are greater than those reported for rat brain [33, 35], this difference may be due to expression of a different mixture of PKC isoforms in mouse spinal cord.

The effect of MP implantation on total PKC activity in the cytosol and membrane fractions of spinal cord homogenates was determined. PKC activity in the cytosol fraction from the MP group was 6.43 ± 0.31 pmol [^{32}P]incorporated/min/μg protein, and from the PP group, it was 6.25 ± 0.51 pmol [^{32}P]incorporated/min/μg protein. In membrane fractions from the MP group, PKC activity was 2.49 ± 0.46 pmol [^{32}P]incorporated/min/μg protein, and activity from the PP group was 2.79 ± 0.44 pmol [^{32}P]incorporated/min/μg protein. Thus, chronic morphine treatment did not alter total PKC activity in cytosol or membrane fractions from spinal cord.

Because others have shown that chronic morphine treatment alters PKC activity in other tissues [21, 22], spinal cord fractions were analyzed further in a kinetic study. PKC activity in membrane and cytosol fractions was determined using increasing protein substrate concentrations, and K_m and V_{max} values were calculated. Results of these studies are shown in Table 2. In membrane fractions, neither V_{max} nor K_m was altered significantly in the MP group compared with the PP group. However, in cytosol fractions, the K_m value was significantly lower in the MP group than in the PP group.

PKC activity also was measured in the cytosol and membrane fractions of mouse spinal cord following high-dose chronic clonidine or saline treatment. In the cytosol fraction, the PKC activity was 8.77 ± 1.36 pmol [^{32}P]incorporated/min/μg protein in the chronic clonidine treatment group and 9.00 ± 0.79 pmol [^{32}P]incorporated/min/μg protein in the saline group. In the membrane fractions, PKC activity was 5.20 ± 0.55 pmol [^{32}P]incorporated/min/μg protein in the clonidine-pretreated group and 6.12 ± 0.61 pmol [^{32}P]incorporated/min/μg protein in the saline group. There were no significant differences between the saline and the clonidine-treated groups.

PKC kinetics were also determined in the cytosol and membrane fractions of spinal cord from mice treated chronically with clonidine. The results are shown in Table 2. Neither V_{max} nor K_m was altered after chronic clonidine treatment in cytosol or membrane fractions.

Immunoblots from the three antisera used showed single immunopositive bands that migrated at about 76 kDa. Representative immunoblots using the three antisera are shown in Fig. 4. For each antiserum, there was a decrease in band density in cytosolic fractions from MP animals compared with the PP mice. Results from densitometric analysis of these and other immunoblots are shown in Table 3. Chronic morphine treatment did not affect the expression of cPKCα, cPKCβI, or cPKCγ in the membrane fractions. However, in the cytosol fraction, expression of all three PKC isoforms decreased. The decreases were 14, 26, and 17% for cPKCα, cPKCβI, and cPKCγ, respectively.

Expression of PKC isoform proteins was also determined

TABLE 2. Effect of chronic morphine or clonidine on PKC kinetics in membrane and cytosol fractions of mouse spinal cord*

	Membrane fraction		Cytosol fraction	
Treatment	V_{max}	K_m (μM)	V_{max} (pmol/min)	K_m (μM)
Placebo pellet	1.93 ± 0.82	1.96 ± 0.97	7.90 ± 1.12	8.24 ± 1.67
Morphine pellet	2.02 ± 0.96	2.60 ± 1.05	7.03 ± 1.00	4.43 ± 1.24†
Saline	4.49 ± 0.72	6.19 ± 1.96	9.11 ± 2.06	5.31 ± 1.46
Clonidine	3.59 ± 0.65	4.53 ± 1.03	7.66 ± 1.11	5.29 ± 1.11

*Results are expressed as the mean ± SEM. N = 5 for membrane fraction and N = 9 for cytosol fraction of morphine- or placebo-pelleted groups. N = 4 for membrane fraction and N = 5 for cytosol fraction in the clonidine or saline groups.

†Significantly different from the PP group ($P < 0.05$).

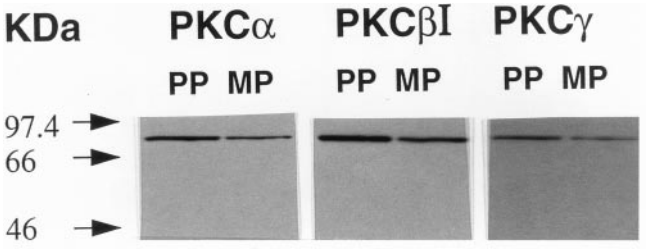

FIG. 4. Effect of morphine pellet implantation on expression of PKC isoform proteins in cytosolic fraction from mouse spinal cord. Mice were implanted with PP or MP for 5 days. Spinal cytosolic proteins (50 μg protein per lane) were separated electrophoretically, and membranes were immunoblotted with antisera selective for cPKCα (lanes 1 and 2), cPKCβI (lanes 3 and 4), or cPKCγ (lanes 5 and 6). Representative immunoblots from 4–5 separate experiments are shown. Arrows represent the electrophoretic mobility of the 97.4, 66, and 46 kDa protein standards.

in the spinal cord cytosol fractions from chronic saline- and clonidine-treated mice. The results from immunoblots of cPKCα, βI, and γ are shown in Table 3. The expression of cPKC isoforms did not differ in cytosol fractions between saline- and clonidine-treated mice.

DISCUSSION

Present studies show that the K_m value for PKC in the cytosol fraction of spinal cord from morphine-tolerant mice was decreased 2-fold compared with placebo treatment. There was no alteration in the PKC V_{max} value in cytosol or in the V_{max} and K_m values in the membrane fraction. The same cytosol fractions that showed enhanced PKC activity also showed a decrease in the expression of immunoreactive cPKCα, βI, and γ isoforms, while no change was found in the membranes. In spinal cord fractions from animals tolerant to clonidine, but not cross-tolerant to morphine, there were no changes in PKC activity, kinetics, or expression. Thus, induction of morphine tolerance, but not clonidine tolerance, increased the catalytic efficiency of PKC in the spinal cytosol, while protein expression was decreased.

TABLE 3. cPKC isoform proteins expression of in the spinal cords of mice tolerant to morphine or clonidine

Antibody	Fractions	Band density (%)	N
	Morphine*		
cPKCα	Membrane	100.4 ± 0.97	4
	Cytosol	86.0 ± 3.57†	8
cPKCβI	Membrane	97.1 ± 2.77	9
	Cytosol	74.1 ± 5.87‡	5
cPKCγ	Membrane	91.8 ± 3.2	4
	Cytosol	83.0 ± 4.36‡	5
	Clonidine§		
cPKCα	Cytosol	99.08 ± 8.40	5
cPKCβI	Cytosol	102.36 ± 7.15	5
cPKCγ	Cytosol	103.04 ± 10.3	5

*Values (means ± SEM) represent band density expressed as percent of placebo pellet.

†,‡Significantly different from the PP group: †$P < 0.05$, and ‡$P < 0.01$.

§Values (means ± SEM) represent band density expressed as percent of saline pretreatment.

Total PKC activity increases in the cytosol of certain brain regions after chronic morphine treatment [21, 22], while expression of PKCαβ decreases [23, 24]. Present results, measuring both enzyme activity and expression, suggest that similar changes occur in the spinal cord. Thus, similar adaptive changes in PKC activity to chronic opioid receptor stimulation may occur throughout the CNS. However, the results from spinal cord were different from brain regions in that total PKC activity was not changed in spinal cord cytosol. This difference in results prompted the kinetic studies, which then showed enhanced PKC catalytic activity after MP treatment. The enhanced kinetic activity may compensate for the decreased protein expression so that total activity is not changed. These alterations in enzyme function and expression may contribute to the development of opioid tolerance.

The consequences of altered PKC activity are not well understood; however, phosphorylation of receptors leading to desensitization may contribute to opioid tolerance expression. Phosphorylation of μ-opioid receptors is increased by morphine and by phorbol ester treatment, and the time course for receptor phosphorylation parallels that for desensitization in an *in vitro* system of expressed cloned receptors [38]. Also, the PKC inhibitor calphostin C blocks the development of acute analgesic tolerance *in vivo* [16, 19]. However, morphine-induced receptor phosphorylation is not blocked by PKC inhibition [38], and agonist-dependent phosphorylation of expressed cloned δ-opioid receptors appears to involve kinases other than PKC [39]. Recent provocative studies with a truncated δ-opioid receptor expressed in human embryonal kidney (HEK) 293 cells show that the nonphosphorylated receptor could be endocytosed in the presence of agonist [40]. Further studies are required to determine the role of phosphorylation, and of the various kinases, in the development of *in vivo* opioid tolerance.

Altered spinal PKC activity or expression was not observed in the mice that expressed tolerance to clonidine, but no cross-tolerance to morphine. The development of cross-tolerance between opioid and α_2 agonists appears to depend at least somewhat on method and route of chronic drug administration. Results in the present studies are consistent with other studies showing no cross-tolerance to s.c. or i.t. administered clonidine after continuous morphine treatment [28, 41, 42], and a similar result has been found for the α_2 agonist dexmedetomidine [43]. On the other hand, cross-tolerance to s.c. or i.t. clonidine (and dexmedetomidine) is observed when morphine is administered by repeated daily s.c. injection [31, 44, 45]. There may be differences between α_2-agonists because continuous morphine administration (i.t.) does produce cross-tolerance to norepinephrine [46] and the α_2-adrenergic agonists oxymetazoline [47] and ST-91 [48]. Chronic repeated

treatment with s.c. clonidine [31, 49] or continuous i.t. norepinephrine produces cross-tolerance to morphine, whereas continuous i.t. ST-91 does not [48]. Because in the present study clonidine was given by repeated administration, it is possible that a paradigm of continuous clonidine treatment would produce cross-tolerance to morphine and alteration of spinal PKC activity.

Both opioid [1] and α_2 [50] agonists inhibit cAMP production, and stimulation of both opioid [51] and α_2 [52] receptors increases phospholipase C activity to affect the actions of PKC. However, antinociception and antinociceptive tolerance may depend upon different mechanisms for the two types of agents. For example, while at least some of the antinociceptive actions of spinal morphine may be attributed to inhibition of adenylyl cyclase activity [4], Uhlen *et al.* [53] proposed that inhibition of cAMP production appears not to be directly linked to the antinociception produced by spinal α_2 agonists. These studies showed that stimulation of cAMP production by forskolin attenuates spinal morphine-induced antinociception [4], but has no effect on antinociception produced by the α_2 agonists UK14,304 or guanfacine [53]. In contrast, recent studies [27] showed that forskolin blocked antinociception produced by spinally administered clonidine as well as morphine. Further examination of the role of second messenger systems activated by opioid and α_2 receptors in spinal antinociception is required in order to fully understand the mechanisms involved in antinociception as well as development of antinociceptive tolerance.

Translocation of PKC from cytosol to membrane is associated with its activation (reviewed in Ref. 52). In a provocative series of studies, Mayer *et al.* [54] showed that GM1 ganglioside, a substance that interferes with PKC translocation, blocks development of tolerance to i.t. morphine. The increased membrane PKC (measured by [^{3}H]PDBu binding in spinal cord slices) found after chronic morphine treatment was also prevented by the GM1 ganglioside. However, the present studies, as well as others [22], failed to observe any change of spinal PKC in the membrane fraction after MP treatment. Using biochemical assays that measure transfer of radiolabeled phosphate to a peptide substrate, a change in PKC activity in homogenates from CNS regions has only been observed in cytosolic fractions [21, 22]. When [^{3}H]PDBu binding is used as an index of PKC activity, however, increased [^{3}H]PDBu binding affinity occurs in cortex and midbrain membrane fractions from rats treated chronically with morphine [55]. Multiple forms of PKC are expressed and differentially distributed in brain and spinal cord areas [56], and these may distribute differentially upon activation. Recent studies in cultured kidney MDCK-D1 cells show that activated PKCα has a different intracellular distribution from activated PKCβII [57]. Similar differential distribution may occur in neurons. To fully understand the complex regulation of PKC activity by opioids, measurement of the activities of the various individual PKC isoforms will be necessary.

In summary, chronic morphine treatment produced enhanced catalytic activity of PKC in the cytosol fraction of mouse spinal cord, while expression of cPKC isoforms decreased. These phenomena were not observed in mice after chronic clonidine treatment. Thus, enhanced catalytic activity of PKC may compensate for the loss of PKC protein during the development of morphine, but not clonidine, tolerance.

The authors wish to thank Mr. Timothy Busch and Dr. Matthew Chamberlain for their excellent technical assistance. These studies were supported by National Institutes of Health Grant DA07972. A preliminary report of this work was presented at the Pharmacology '97 meeting in San Diego, CA, March 7–11, 1997.

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