



Spinal PKC activity and expression: role in tolerance produced by continuous spinal morphine infusion

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Abstract

It has been hypothesized that spinal morphine tolerance results from protein kinase C (PKC) mediated phosphorylation. Chronic lumbar intrathecal (i.t.) infusion of morphine (20 nmol/ μ l/h) was shown to produce antinociception on day 1 (d1) that disappeared by d5 (tolerance). On d6, a bolus i.t. probe dose of morphine (60 nmol) produced a more profound antinociception in saline-infused rats than in morphine-infused rats. Coinfusion of morphine with a PKC inhibitor, chelerythrine, prevented tolerance to the probe morphine dose. Bolus i.t. chelerythrine or GF109203X (GF), another PKC inhibitor, on d5, but not the inactive homologue of GF Bisindolymaleimide V, also blocked development of tolerance after 24 h. I.t. morphine infusion, but not saline, produced a 2-fold increase in dorsal horn PKC phosphorylating activity and in the expression of PKC α/γ . Bolus chelerythrine on d5 after spinal morphine infusion blocked upon an increase in PKC activity, confirming that at the behaviorally active dose the drug had the intended biochemical effect upon spinal PKC activity. PKC activity and protein expression did not change when assessed 1 h after bolus i.t. morphine in naive rats. Thus, tolerance produced by morphine infusion is dependent upon an increase in local phosphorylating activity by PKC. Blocking the PKC activity prevents expression of the morphine tolerance. © 2000 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Continuous intrathecal (i.t.) infusion of μ opioids in humans yields a potent dose-dependent analgesia in chronic pain patients. Although controversial, it is clear that over time, the magnitude of the effect produced by a given dose will diminish, suggesting the development of tolerance (Yaksh and Onofrio, 1987). Systematic studies have shown similar phenomena in rat models. Thus, in rats with chronic intrathecal catheters, continuous infusion of morphine over a period of days will display a dose-dependent antinociception that declines over the infusion interval. At the end of this infusion, bolus dose-response curves for the μ agonist display a right shift and a reduced maximum effect (Dunbar and Yaksh, 1996). These characteristics define the phenomenon of opiate tolerance. The mechanism

of this tolerance is uncertain. Concurrent blockade of the NMDA receptor diminishes the loss of effect with repeated (Mao et al., 1994) or continuous (Dunbar and Yaksh, 1996) exposure of the rat to spinal morphine. Our observation that there is only a modest increase in spinal glutamate release during chronic spinal morphine infusion (Jhamandas et al., 1996) suggests that the ongoing role of spinal NMDA receptors may reflect an enhanced sensitivity of the existing receptors. Other work has shown that opiates can lead to a facilitation of NMDA ionophore function through activation of protein kinase C (Mayer et al., 1995). Using [³H]phorbol-12,13-dibutyrate (PdBu) binding, Mayer et al. (1995) showed that daily spinal injection of morphine leads to an increase in membrane-bound PKC, particularly in spinal laminae I and II, indirectly suggesting an increase in phosphorylating activity in dorsal horn neurons during the development of tolerance.

In the present work we sought to characterize the role of spinal PKC in continuous spinal opiate infusion. This model has several virtues. (i) It shows a direct effect upon spinal receptors and obviates concerns of changes in spinal and supraspinal linkages (Roerig and Fujimoto, 1989). (ii)

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Continuous infusion minimizes the stress typically associated with handling and injecting, a factor that may be implicated in associative learning and tolerance (Collingridge and Bliss, 1995). (iii) This model has been widely used and has shown that the reduction in drug effects is not the result of either altered drug levels or multiple testing paradigms (Yaksh, 1993). (iv) Finally, continuous infusion provides a constant drug level and therefore a stable receptor occupancy. This last variable is critical, as we believe that time variant exposure to the toleragen (e.g., the peaks and valleys in drug levels that occur with bolus injection or pellets) represents a crucial variable in tolerance (Ibuki et al., 1997; Dunbar and Pulai, 1998).

Given the above, we undertook to investigate (i) if chronic spinal morphine alters spinal phosphorylating activity, and (ii) whether the blockade of spinal PKC activity attenuates the morphine tolerance.

2. Materials and methods

2.1. Animals

Approval for this study was obtained from the University of California San Diego Animal Care Committee. Male Holtzman Sprague–Dawley rats (350–400 g) were implanted with catheters (details see below), and thereafter housed in individual standard cages at room temperature on a 12:12 h light/dark cycle (lights on at 07:00 h). Testing was performed during the light cycle at 12:00 h. Animals had free access to food and water. Each rat was implanted as described below with a subarachnoid catheter attached to a subcutaneous osmotic pump filled with saline or drug(s) and randomly assigned to treatment groups.

The preparation of the intrathecal infusion catheter has been described (Sosnowski and Yaksh, 1990). In brief, a 16-cm length of PE-10 tubing was connected by heat fusing with a hot air jet to a 2-cm length of PE-60 tubing. A 0.5-cm piece of Silastic tubing (Tuzik, Norwell, MA), previously soaked in chloroform, was then passed over both ends of the PE-10 tubing to form a loop of 3 cm from the end of the PE-10 tubing fused to the PE-60. The long end of the catheter was stretched to reduce the diameter, soaked in alcohol (70%) overnight, and cut to a length of 9 cm. Alzet osmotic mini-pumps (model 2001, 1 μ l/h; Alzet, Palo Alto, CA) were filled with drug(s) or saline and attached to the saline-flushed catheter. This pump delivered an infusion for 5 days after an initial activation period in the animal of 4 h. The catheter (Yaksh and Rudy, 1976) and pump were implanted between the hours of 09:00 and 12:00 h, with the additional modification of the subcutaneous osmotic pump. In brief, rats were anesthetized with halothane (2%), placed in a stereotaxic head holder, and the atlantooccipital membrane exposed. The membrane was pierced and the PE-10 end of the catheter was passed intrathecally to the level of the thoracolumbar junction (8.5 cm). The pump was

then attached to the end of the PE-60 catheter and implanted subcutaneously in a pouch to lie just behind one of the shoulders. A 19-gauge needle was used to make a small hole in the forehead. The loop end of the catheter is passed through this hole. This PE-10 loop can then be cut and used to administer external doses of drugs at the end of the 5 day infusion period. The wound was sutured, including a loose ligature at the base of the loop to prevent it from moving. Animals displayed full motor recovery within 15–30 min after implantation. Control rats typically displayed normal thermal escape thresholds within 1–2 h post-operatively. Rats that showed any signs of motor impairment were euthanized with an overdose of barbiturate.

2.2. Drugs and injection

The following drugs were used for continuous spinal infusion: morphine sulfate (Merck, Sharp and Dohme, West Point, PA), and chelerythrine chloride, bisindolylmaleimide I (GF-109203X), and bisindolylmaleimide V (BM-V, the inactive homologue of GF-109203X) (all from Calbiochem, San Diego, CA). The drugs were dissolved in DMSO (10%), except chelerythrine, which was dissolved in saline. Drug doses, calculated as the free base, were expressed as nanomoles per μ l per hour (nmol/ μ l/h) for the infusion concentrations, or nmol per rat for the bolus injection. Morphine infusion was 20 nmol/ μ l/h in all animals.

2.3. Experimental paradigms

The study was divided into three phases. Phase 1 was directed at defining the change in response over time during chronic morphine infusion and its modification by the coadministration of chelerythrine, a PKC inhibitor. Phase 2 was designed to assess the effect of a single bolus injection of the PKC inhibitor on morphine tolerance. Phase 3 was designed to evaluate possible changes in PKC phosphorylating activity, and the expression of PKC α and PKC γ and their role on tolerance. Each rat was used only once.

2.3.1. Phase 1

In Phase 1, animals were prepared with intrathecal catheters and preloaded osmotic infusion pumps. The subsequent measurements of the thermal escape latencies were carried out on day 0, 1, 3 and 5 between the hours of 10:00 and 12:00 h, from day 0, the day of implantation, to day 5, the end of the 5-day infusion. Rats were randomly assigned to receive morphine infusion alone (20 nmol/ μ l/h) or morphine infusion with one of the doses of chelerythrine (0.3 or 3 nmol/ μ l/h). In a preliminary dose ranging study, we observed that chelerythrine doses exceeding 10 nmol/ μ l/h resulted in weight loss and motor weakness. A dose of 3 nmol/ μ l/h of chelerythrine was found to be without conflicting behavioral effects. On day 6, 24 h after termination of the infusion, a single probe dose of intrathecal morphine (60 nmol/rat) was administered to all animals. This dose had

been previously shown to be maximally effective in morphine naive rats. In the probe dose testing, thermal latencies were measured at 0, 30, 60, and 120 min.

2.3.2. Phase 2

This phase was directed to determine whether acute intrathecal PKC inhibitors would restore morphine sensitivity in morphine-tolerant rats. Groups infused with either saline or morphine sulfate alone were entered into the following study. On day 5, the external loop was cut, and the intrathecal part of the catheter was flushed with 10 μ l sterile saline. After 3 h, the rats received an intrathecal bolus dose of vehicle or PKC inhibitor, and the thermal latency was then assessed at 0, 15, 30, 60, and 120 min. On day 6, 24 h after termination of the infusion, a single probe dose of intrathecal morphine (60 nmol/rat) was administered to all animals. Thermal latencies were then measured at 0, 30, 60, and 120 min.

2.3.3. Phase 3

To determine if chronic morphine infusion led to changes in spinal PKC isozyme expression and PKC phosphorylating activity, rats were assigned to one of the following intrathecal drug infusion groups: (i) naive (no surgery or treatment); (ii) i.t. saline (1 μ l/h, 5 days); (iii) i.t. morphine (20 nmol/ μ l/h, 5 days); (iv) i.t. morphine (20 nmol/ μ l/h, 5 days) + i.t. chelerythrine (3 nmol) delivered as a bolus on day 5; (v) i.t. chelerythrine (3 nmol) delivered as a bolus; and (vi) i.t. morphine (60 nmol) delivered as a bolus. In the continuous infusion groups, the rats were sacrificed on day 6 and the spinal cord harvested. Lumbar dorsal and lumbar ventral horns were separated and assayed for PKC α and PKC γ using Western blots as well as for PKC phosphorylating activity (see below).

2.4. Antinociceptive testing

To assess thermal nociceptive responses, a paw thermal stimulator similar to that conceived by Hargreaves (Hargreaves et al., 1988) was employed (Dirig et al., 1997). The device consisted of a glass surface upon which the rats were placed individually in Plexiglas cubicles (9 \times 22 \times 25 cm). The glass surface temperature was maintained at 30 \pm 0.1°C by a feedback-controlled, under-glass, forced-air heating system. The heating system was driven by a thermocouple attached to the bottom surface of the glass plate. The thermal nociceptive stimulus originated from a focused projection bulb mounted in a stimulus tower that was manually manipulated in a two-dimensional axis on ball bearing slides to permit the stimulus to be delivered separately to both hind paws of each test subject. This stimulus was positioned under each footpad with the aid of an angled mirror mounted on the stimulus source that permitted an exact visual targeting of the stimulation site prior to stimulus initiation. A timer was automatically actuated with the light source, and the response latency was

defined as the time required for the paw to show an abrupt withdrawal. Paw withdrawal was detected by an array of photodiode motion sensors mounted on the stimulus tower that stops the timer and terminates the stimulus. Stimulus current from a regulated source was monitored continuously to determine the amperage delivered to the light source and the magnitude of the radiant stimulus to which the paw is subjected. In all cases, a cut-off of 20 s was employed to avoid tissue injury. Rats were acclimated to the test chamber for 20–30 min prior to testing. At each test exposure, the left and right paws were tested 1 min apart in random order. The response latency of a rat for a given test was represented by the of the latency of paw withdrawal for the left and right paw.

Thermal escape latency data were expressed either as mean latencies (in seconds) for each group or as a maximum percent effect (%MPE). Maximum percent effect was calculated as follows: % MPE = (Post-drug latency – baseline) \times 100/(Cutoff – baseline), where post-drug latency was the response measured at the particular time after initiation of infusion or after intrathecal dose of probe drug. Baseline was the pre-infusion or pre-probe latency, and the cut-off time was 20 s. Area under the %MPE against time curve was calculated by the trapezoidal method (Rowland and Tozer, 1995).

2.5. Preparation of PKC extracts

All steps were carried out at 4°C. Centrifugation was performed in a Beckman Optima TLX ultracentrifuge. Dissected frozen tissue samples were homogenized 1:8 (w/v) in ice-cold buffer A: 250 mM sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 40 μ g/ml leupeptin, 2 mM benzamidine, 0.1 mM microcystin, 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100 000 \times g for 30 min, the supernatant containing a cytosolic fraction of PKC was mixed with an equal volume of glycerol and stored at –20°C. The protein concentration was determined by the method of Bradford (1976).

2.6. PKC activity assay

PKC activity assays were performed as described by Kikkawa et al. (1982). All reactions were run in triplicate. The standard reaction mixture (80 μ l) contained: 20 mM HEPES (pH 7.4), 1 mM DTT, 5 mM MgCl₂, 100 μ M ATP, 1 μ Ci of γ -[³²P]ATP, 140 μ M phosphatidylserine/3.8 μ M diacylglycerol membranes (PS/DAG), 0.5 mM CaCl₂, 100 μ g/ml peptide substrate Ac-FKKSFKL-NH₂, corresponding to the PKC-specific phosphorylation domain in the MARCKS protein (Graff et al., 1989) and 0.25–0.5 μ g of protein extracts from the spinal cords. Protein amounts used in the assays were within the linear range of the standard curve for ³²P-incorporation with respect to time of incubation and the amount of protein assayed.

Control reactions containing 0.5 mM EGTA instead of CaCl₂ and 20 mM HEPES instead of PS/DAG were

performed as well in order to determine the non-PKC activity present in the extracts. After incubation at 30°C for 7 min, the reactions were terminated by adding 25 μ l of 0.1 M ATP/0.1 M EDTA (pH 8). Then, 85 μ l of each sample were transferred onto P81 Whatman filter paper (6 cm²). The papers were washed four times in 0.4% phosphoric acid for 10 min each, followed by one wash with 95% C₂H₅OH for 5 min. The radioactivity retained on the filters was determined by liquid scintillation counting. PKC activity was defined as pmol of incorporated phosphate per μ g protein per minute after subtracting the radioactivity due to non-PKC activity in the corresponding sample.

2.7. Detection of PKC α and PKC γ by immunoblotting

Proteins from the tissue extracts of the lumbar spinal cord were electrophoretically separated on 10% SDS–polyacrylamide gels and transferred to PVDF membranes (Immobilon-P, Millipore, 0.45 μ m pore size). After the transfer was accomplished, the membranes were incubated overnight at 4°C in 10% non-fat dry milk in phosphate-buffered saline containing 0.1% (v/v) Tween-20 (PBS-T) to block the nonspecific binding of the primary and secondary antibodies. Blots were washed 2 \times 10 min each in PBS-T and incubated for 1 h at room temperature with anti-PKC antibody in 2% (w/v) BSA in PBS-T. Anti-PKC α antibody (1:1000 dilution, Transduction Laboratories, San Diego, CA) and anti-PKC γ antibody (1:5000 dilution, Transduction Laboratories) were consecutively used for each membrane. The membranes were washed extensively in PBS-T buffer and the PKC antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibody and ECL detection system (SuperSignal Substrate, Pierce). After exposing the membranes to X-ray film, the bands were quantified by densitometry using the NIH 1.61 software package. The densitometry readings were normalized against the amount of protein loaded. To ensure consistency, two gels with different amounts of protein loaded on each were run for every experimental group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.8. Data analysis and statistics

Data analysis and statistics were done with computer software programs (SigmaStat, Jandel Scientific). Where applicable, data from testing (i.e. absolute latencies, calculated %MPE or AUC) were analyzed using one-way analysis of variance (ANOVA) to detect differences between groups. When differences were found, these findings were subjected to the Bonferroni test (significant at 95%). Unless stated, single points of comparison were made using a standard paired or unpaired *t*-test. Differences yielding critical values corresponding to $P < 0.05$ were considered statistically significant.

3. Results

3.1. Phase 1. Continuous intrathecal infusion of morphine and PKC inhibitor

There was no significant difference between thermal latencies on days 1 to 5 in saline- infused rats ($P > 0.05$) (Fig. 1, top left, clear symbols). All rats that received morphine infusion (20 nmol/ μ l/h) showed a significant increase ($P < 0.05$) of thermal latency on days 1 and 3 compared to the baseline on day 0, returning to near baseline on day 5 (Fig. 1, top left). There was no significant difference between latencies of saline- and morphine-infused rats on day 5 ($P > 0.1$).

On day 6, 24 h after stopping the infusion, spinal administration of the probe dose of 60 nmol of morphine to morphine- and saline-infused rats produced a significant increase in the thermal latency in both groups. The increase in thermal latency was significantly higher ($P < 0.05$) in saline-infused rats compared to that observed in morphine-infused rats (Fig. 1, bottom), indicating tolerance.

The coinfusion of saline and the PKC inhibitor chelerythrine (3 nmol/ μ l/h) did not modify the thermal latencies observed with saline alone. However, the thermal latency observed in the morphine-chelerythrine coinfusion group (Fig. 1, top right) was significantly higher ($P < 0.05$) than the group that received the infusion of morphine only (Fig. 1, top left). Thus, coinfusion of chelerythrine at 3 nmol/ μ l/h with morphine resulted in maintenance of elevated latencies during infusion, which returned to baseline after cessation of infusion. On day 6, 24 h after stopping the coinfusion, the thermal latencies observed in the morphine-chelerythrine group were no different from those observed in the saline-chelerythrine group ($P < 0.05$), indicating that chelerythrine is able to reduce the development of morphine tolerance (Fig. 1, bottom right).

Fig. 2 shows the antinociceptive effects produced by the intrathecal administration on day 6 of a probe dose of morphine (60 nmol) in rats infused chronically with saline, chelerythrine (3 nmol/ μ l/h), morphine (20 nmol/ μ l/h) or the combination of morphine (20 nmol/ μ l/h) and different doses of chelerythrine (0.3 and 3 nmol/ μ l/h). The effect observed in the morphine group was significantly lower than that observed in the saline or chelerythrine groups (ANOVA, $P < 0.001$). The effect observed in the morphine–chelerythrine groups was dose-dependent and significantly higher than that observed with the morphine alone group (ANOVA, $P < 0.05$).

3.2. Phase 2. Continuous intrathecal infusion of morphine and bolus injection of PKC inhibitor

Intrathecal administration of a probe dose of morphine (60 nmol) on day 6 in rats infused chronically for 5 days with morphine (20 nmol/ μ l/h) produced a significant increase in the antinociceptive effect ($P < 0.05$) in the

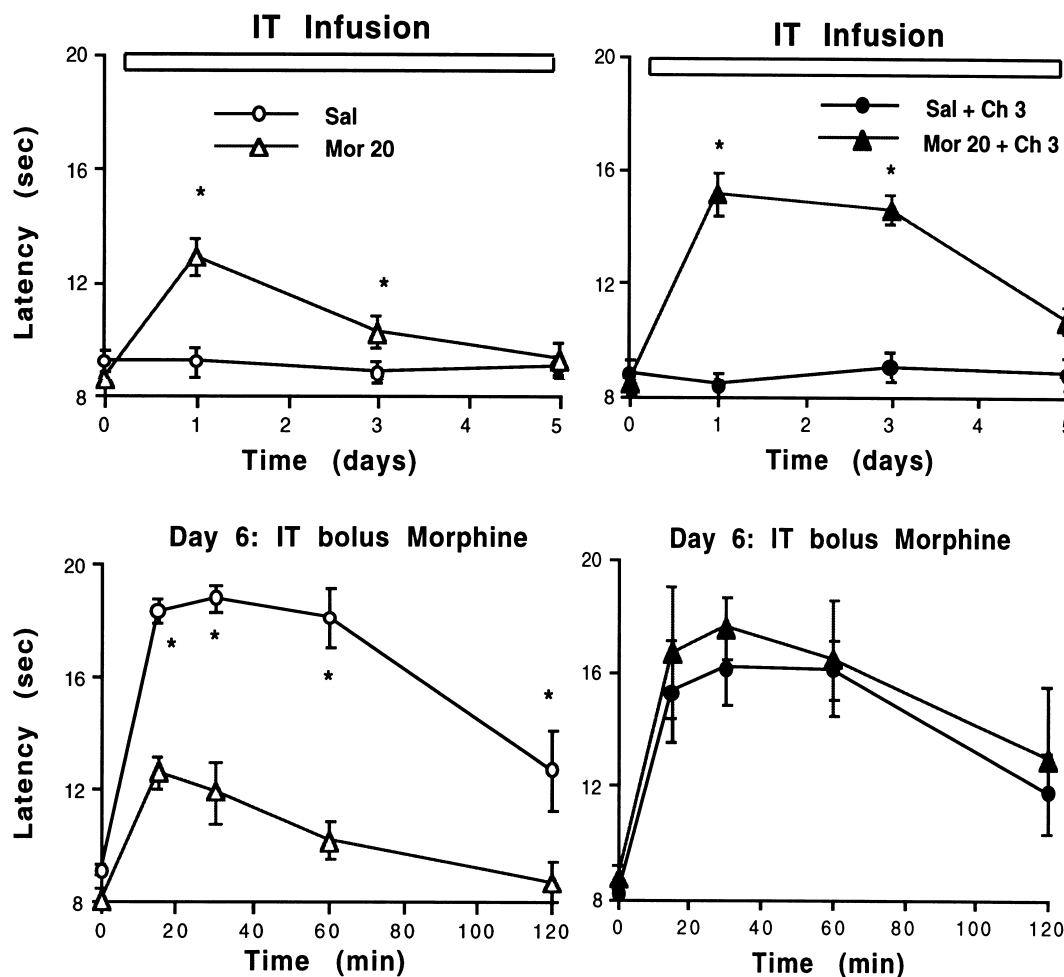


Fig. 1. (Top) time course of the antinociceptive effect expressed as latency observed with chronic infusion for 5 days of intrathecal saline, morphine alone or coinfused with chelerythrine. (Bottom) time course observed in rats that received chronic infusion for 5 days of saline, morphine or the combination with chelerythrine and that received an intrathecal probe dose of morphine (60 nmol) on day 6. As indicated, PKC inhibition diminished the onset of morphine tolerance and prevented the loss of response to the effects of bolus i.t. morphine given on day 6. Each line represents the mean \pm SEM of more than 5 rats tested at 0, 1, 3, and 5 days and at 0, 15, 30, 60, and 120 min on day 6. Doses used in chronic infusion are expressed in nmol/ μ h. Doses used as bolus injection on day 5 and day 6 are expressed as nmol/10 μ l. Open symbols: * $P < 0.05$, as compared with the saline group (top); * $P < 0.05$, as compared with the morphine group (bottom). Closed symbols: *Significantly different ($P < 0.05$) from the group morphine + chelerythrine (unpaired *t*-test).

group of rats that received an intrathecal bolus injection of chelerythrine on day 5 compared to the group that received saline on day 5 (Fig. 3, top). On the other hand, the intrathecal administration of the morphine probe dose on day 6 in rats infused chronically for 5 days with saline produced a similar antinociceptive effect in the groups that either received an intrathecal bolus injection of chelerythrine or saline on day 5 (Fig. 3, bottom). The antinociceptive effect observed in the morphine-infused rats that received chelerythrine (3 nmol) on day 5 was similar to that observed in the saline-infused rats regardless of the treatment received on day 5.

The PKC inhibitors chelerythrine (assured bars, 0.03, 0.3 and 3 nmol), GF-109203X (0.24, 2.4 and 12 nmol) or Bisindolymaleimide V (BM-V: an inactive homologue of GF-109203X, 12 nmol), injected i.t. after 5 days of intrathecal infusion of morphine (20 nmol/ μ l/h), failed to show a

significant increase in the antinociceptive effect, thus demonstrating a lack of acute effect of the intrathecal PKC inhibitors in restoring sensitivity to morphine (Fig. 4). However, 24 h after the PKC inhibitors were given to tolerant rats, both served to reverse the morphine tolerance in a dose-dependent manner (Figs. 5 and 6). The effect of PKC inhibitors was time-dependent, as the effect produced by the morphine probe dose on day 6 was significantly higher (ANOVA, $P < 0.05$) in rats that received chelerythrine (3 nmol) 24 h before, as compared to the group that received the PKC inhibitor 16 h before (Fig. 7).

3.3. Phase 3. Spinal PKC activity and PKC α/γ expression

3.3.1. PKC activity

In vitro phosphorylation assays revealed that the PKC activity in the lumbar dorsal horn was approximately 2-

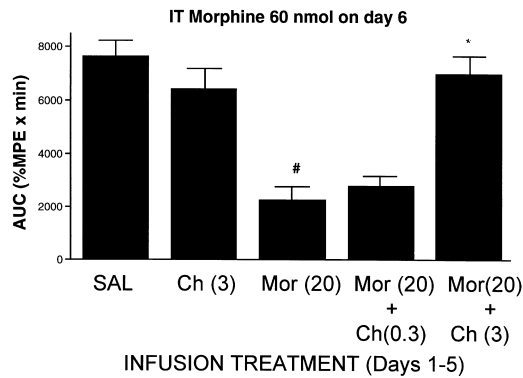


Fig. 2. Summary of antinociceptive effect, expressed as area under the curve, produced by the intrathecal administration on day 6 of a probe dose of morphine (60 nmol) in rats infused chronically with saline, chelerythrine, morphine or the combination of morphine with different doses of chelerythrine. Each bar represents the mean \pm SEM of 7–9 rats. Doses used in chronic infusion (days 1–5) are expressed in nmol/ μ l/h. [#]Significantly different ($P < 0.001$) from the saline group by ANOVA followed by the Bonferroni test. ^{*}Significantly different ($P < 0.05$) from the morphine group by ANOVA followed by the Bonferroni test.

fold higher than that in the ventral horn in the group of naive rats (Fig. 8). The PKC activity in both the dorsal and ventral horn of the saline group (i.e. 5-day infusion) did not differ significantly from that of the naive animals. Morphine infusion for 5 days (20 nmol/ μ l/h) profoundly increased PKC phosphorylating activity in the dorsal ($P < 0.01$), but not the ventral horn. In contrast, acute morphine (i.e. a bolus i.t. injection, 60 nmol) given in naive rats did not show any

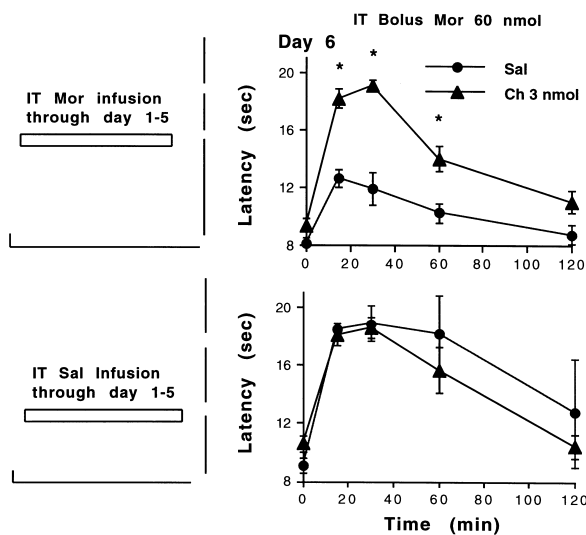


Fig. 3. Time course of effects of single bolus i.t. chelerythrine on day 5 on spinal morphine tolerance. The antinociceptive effect, expressed as latency (second), produced by the intrathecal administration of a probe dose of morphine (60 nmol) on day 6 in rats i.t. infused for 5 days with morphine (20 nmol/ μ l/h, days 1–5) (top) or saline (bottom) and treated with a single bolus i.t. injection of either saline or chelerythrine on day 5. Each line represents the mean \pm SEM of 7–9 rats. Doses used in chronic infusion (days 1–5) are expressed in nmol/ μ l/h. Doses used as bolus injection on days 5 and 6 are expressed as nmol/10 μ l. ^{*}Significantly different ($P < 0.05$) from the saline group by the unpaired *t*-test.

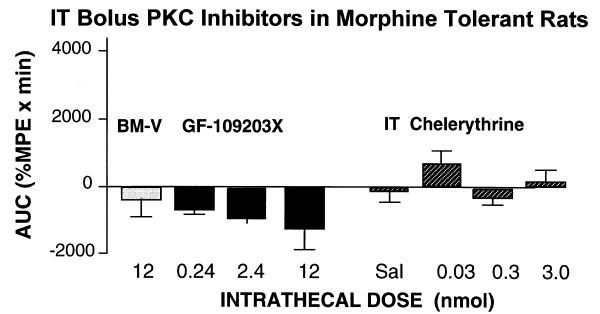


Fig. 4. Thermal paw withdrawal response 1 h after the bolus i.t. injection of GF-109203X and chelerythrine. Thermal withdrawal latency, expressed as area under the curve (%MPE \times min), produced by a single bolus i.t. of saline, BMV (bisindolylmaleimide V), GF-109203X (0.24, 2.4, and 12 nmol), or chelerythrine (0.03, 0.3, and 3 nmol), injected after 5 days of intrathecal infusion of morphine (20 nmol/ μ l/h). Thermal withdrawal latency was measured 1 h after the bolus i.t. injection on day 5. Each bar represents the mean \pm SEM of 5–9 rats.

effect on dorsal or ventral horn PKC activity. Chelerythrine (3 nmol) given i.t. on day 5 reversed the elevation of PKC activity in the dorsal horn induced by chronic morphine ($P < 0.01$). A bolus i.t. injection of chelerythrine (3 nmol) alone did not alter basal PKC activity in the dorsal horn.

3.3.2. PKC α and PKC γ expression

Western immunoblotting carried on the same tissue extracts as that for the PKC activity assays showed that both PKC α and PKC γ immunoreactivity in the dorsal horn of the chronic morphine infused rat (Fig. 9, top, lane 3) were higher than that in the naive rat or the rat receiving chronic saline infusion (Fig. 9, top, lanes 1 and 2, respectively). Single i.t. bolus chelerythrine on day 5 after spinal morphine infusion apparently reduced the expression of both PKC α and PKC γ (Fig. 9, top, lane 4). Single i.t. injection of morphine or chelerythrine in naive rats had no effect

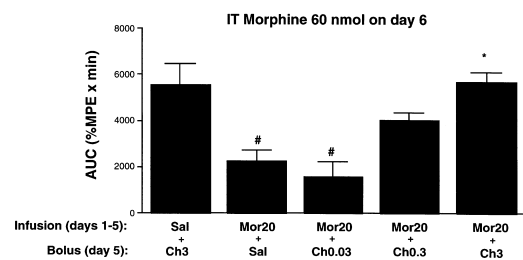


Fig. 5. Dose–response effects of single bolus i.t. chelerythrine on day 5 on spinal morphine tolerance. Antinociceptive effect, expressed as area under the curve, produced by the intrathecal administration on day 6 of a probe dose of morphine (60 nmol) in rats infused chronically with saline, morphine and treated with a single bolus i.t. injection of either saline or different doses of chelerythrine on day 5. Each bar represents the mean \pm SEM of 5–9 rats. Doses used in chronic infusion (days 1–5) are expressed in nmol/ μ l/h. Doses used in bolus injection (day 5) are expressed in nmol/10 μ l. [#]Significantly different ($P < 0.05$) from the saline + chelerythrine 3 μ l group (ANOVA followed by the Bonferroni test). ^{*}Significantly different ($P < 0.05$) from the morphine + saline group (ANOVA followed by the Bonferroni test).

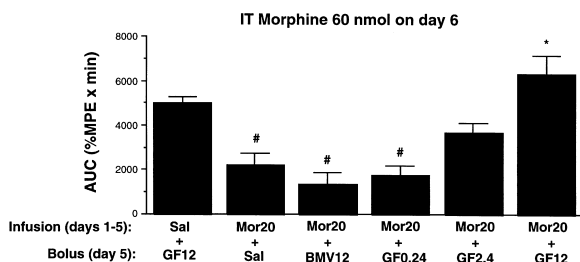


Fig. 6. Dose–response effects of single bolus i.t. GF-109203X on day 5 on spinal morphine tolerance. Antinociceptive effect, expressed as area under the curve, produced by the intrathecal administration on day 6 of a probe dose of morphine (60 nmol) in rats infused chronically with saline (Sal) or morphine (Mor), and treated with a single bolus injection of either saline (Sal), bisindolylmaleimide V (BMV) or different doses of GF-109203X (GF) on day 5. Each bar represents the mean \pm SEM of 5–9 rats. Doses used in chronic infusion (days 1–5) are expressed in nmol/ μ l/h. Doses used in bolus injection (day 5) are expressed in nmol/10 μ l. [#]Significantly different ($P < 0.05$) from the group of Sal + GF12 (ANOVA followed by the Bonferroni test). ^{*}Significantly different ($P < 0.05$) from the group of Mor20 + Sal (ANOVA followed by the Bonferroni test).

on PKC α nor PKC γ protein expression in the dorsal horn (Fig. 9, top, lanes 5 and 6, respectively). We observed similar results in three sample sets examined (Fig. 9, bottom). There was no change in the expression of PKC α or PKC γ in the ventral horn (data not shown).

4. Discussion

4.1. Intrathecal tolerance model

Continuous exposure of the spinal cord to a fixed concentration of morphine will result in a progressive loss of spinal opiate receptor-mediated antinociception and a right shift in the dose–effect curves generated with bolus doses of the

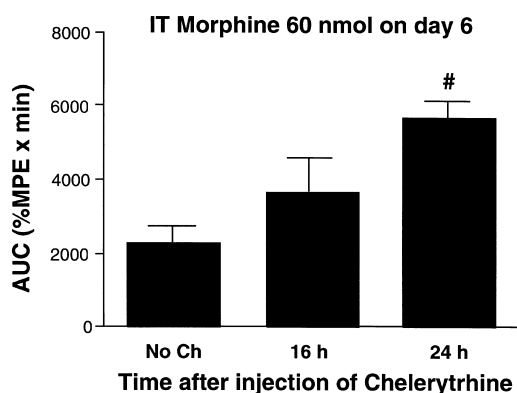


Fig. 7. Minimal hours required for offset of morphine tolerance by a bolus i.t. treatment with chelerythrine. The antinociceptive effect, expressed as area under the curve, observed after the intrathecal administration on day 6 of a probe dose of morphine 60 nmol in rats infused chronically for 5 days with morphine 20 nmol/ μ l/h and treated with a single bolus i.t. injection of either saline (No Ch) or chelerythrine 3 nmol/10 μ l. Morphine probe dose was given either at 16 or 24 h after i.t. chelerythrine treatment. Each bar represents the mean \pm SEM of 3–9 rats. [#]Significantly different ($P < 0.05$) from the no chelerythrine group (ANOVA followed by the Bonferroni test).

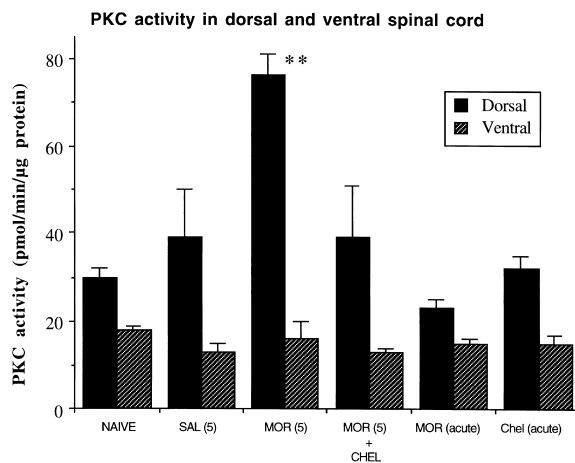
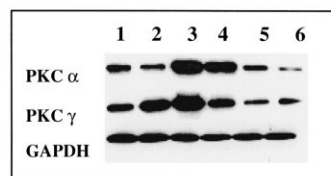


Fig. 8. Levels of PKC phosphorylating activity (pmol/min/ μ g protein) in the dorsal and ventral horns in the groups with different treatments ($n = 3$ each). Naive: no surgery and no drug treatment; SAL (5): chronic saline infusion for 5 days and with a bolus i.t. saline (10 μ l) on day 5; MOR (5): chronic morphine (20 nmol/ μ l/h) for 5 days and with a bolus i.t. saline (10 μ l) on day 5; MOR (5) + CHEL: chronic morphine (20 nmol/ μ l/h) for 5 days and with a bolus i.t. chelerythrine (3 nmol) on day 5; MOR (acute): 1 h after i.t. morphine (60 nmol) delivered as a bolus; Chel (acute): 1 h after i.t. chelerythrine (3 nmol) delivered as a bolus. In dorsal horn (Dorsal), the PKC activity level of the MOR (5) group is significantly higher than that in all the other groups ($P < 0.01$, ANOVA). In ventral horn (Ventral), the activities in all the groups are significantly lower than the corresponding group in the dorsal horn ($P < 0.05$, ANOVA). There is no statistically significant difference between the groups of the ventral horn.

Western blotting for PKC α and PKC γ in the rat dorsal horns



PKC α expression in dorsal horn

	1	2	3	4	5	6
Set 1	247	224	474	390	212	142
Set 2	322	546	739	211	436	506
Set 3	446	529	639	428	260	295

PKC γ expression in dorsal horn

	1	2	3	4	5	6
Set 1	288	272	325	331	239	232
Set 2	383	471	740	482	259	269
Set 3	336	465	652	490	296	392

Fig. 9. (Top) Western blot. 1. Naive: no surgery and no drug treatment. 2. Chronic saline infusion for 5 days and with a bolus i.t. saline (10 μ l) on day 5. 3. Chronic morphine 20 nmol/ μ l/h for 5 days and with a bolus i.t. saline (10 μ l) on day 5. 4. Chronic morphine 20 nmol/ μ l/h for 5 days and with a bolus i.t. chelerythrine (3 nmol) on day 5. 5. One hour after i.t. morphine (60 nmol) delivered as a bolus. 6. One hour after i.t. chelerythrine (3 nmol) delivered as a bolus. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) is the internal control. (Bottom) Summary of results of three independent repetitions of six treatment groups indicated in the Western blot at the top. The data from the densitometric analysis are expressed as AUC/mg protein loaded on the gels.

toleragen given 24 h after the termination of infusion (Dunbar and Yaksh, 1996). Such changes are consistent with a reduction in the number of receptors or in an uncoupling of the receptors from the intracellular second messenger that mediates the respective actions of these agonists. Binding studies often have shown a modest reduction in binding sites after exposure, but these results have not been consistent (Nishino et al., 1990; Wong et al., 1992; Gouarderes et al., 1993; Bhargava, 1995).

4.2. Intrathecal inhibitors and spinal PKC inhibition

Chronic spinal infusion of chelerythrine, a PKC inhibitor, did not produce a change in thermal latency when given to a rat that had been receiving saline infusion. However, when delivered concurrently with morphine, or injected as an intrathecal bolus on day 5 in tolerant rats, it preserved the antinociception produced by morphine. These results suggest that during continued opiate exposure, the change in spinal responsiveness may be mediated by the activation of PKC. The bolus injection of PKC inhibitors at the end of toleragen infusion unexpectedly served to reverse the loss of effects typically observed 24 h later. We believe these results indicate that during the course of opiate exposure, activation of PKC is in part responsible for the daily loss of effects that occurs with continuous morphine exposure. These results are consistent with the findings of other investigators who reported that PKC inhibitors attenuate tolerance development (Narita et al., 1994a,b, 1995, 1996; Mao et al., 1995; Mayer et al., 1995). In the present study we observed that acute PKC inhibition at the time morphine infusion was terminated reverses the tolerance after 24 h, but not acutely and only partially after 17 h. This suggests that with opiate exposure there is an ongoing activation of PKC and phosphorylation of specific cellular substrates. The validity of this interpretation requires consideration of several issues.

First, the implication of this reasoning is that there must be an increase in phosphorylation in the spinal dorsal horn after chronic morphine infusion. In these studies we demonstrated that after chronic morphine infusion, there was a significant increase in the PKC-dependent phosphorylating activity in the dorsal horn but not the ventral horn of rats receiving chronic infusion of morphine. These data parallel the increase in PKC α and PKC γ protein in the dorsal horn but not in the ventral horn of morphine infused rats. Using [³H]phorbol-12,13-dibutyrate (PdBu) binding, Mayer et al. (1995) showed that daily spinal injections of morphine leads to an increase in membrane-bound PKC, particularly in spinal laminae I and II. This lends support to our hypothesis that chronic exposure leads to an increase in PKC phosphorylating activity in the spinal dorsal horn.

Second, an important issue is whether the effects of the PKC antagonists in fact reflect their actions as PKC inhibitors. In this regard, we note the following. (i) The antinociceptive effect observed in morphine + PKC inhibitor-

infused rats returned to baseline after stopping infusion. This argues against any permanent neurologic effects that might have accounted for the observed persistence of the antinociceptive effect. (ii) The same results were obtained using two structurally different PKC inhibitors, chelerythrine and GF-109203X. Both display a high selectivity to PKC, i.e., chelerythrine: IC₅₀ for PKC 0.66 μ M and for PKA 170 μ M; and GF-109203X: K_i for PKC 0.01 μ M and for PKA 2 μ M (Herbert et al., 1990; Toullec et al., 1991). (iii) These effects were observed at doses that had no effect upon resting nociceptive thresholds or motor function. (iv) The effects were not observed with the highest equivalent dose of 12 nmol of bisindolylmaleimide V, the inactive homologue of GF-109203X. (v) At the dose of chelerythrine that was behaviorally effective, we confirmed that this agent served to block the elevated levels of PKC activity otherwise observed in the spinal cord of the morphine tolerant rat. It is interesting to note that the presence of antagonists also resulted in a reduction in the apparent levels of PKC α and PKC γ isozymes in the spinal cord. It is not clear whether PKC activity regulates its own expression levels or whether the inhibitor promotes degradation. These observations jointly suggest, however, that these agents at the doses employed in the *in vivo* model reflect an inhibition of spinal PKC in general and PKC α and PKC γ isozymes in particular.

4.3. Phosphorylating enzymes relevant to tolerance

There are a variety of kinases that may serve to phosphorylate spinal proteins and may be of functional importance to nociceptive processing. As discussed above, evidence supports the hypothesis of the relevance of PKC. Several PKC isozymes have been described (Newton, 1995). PKC γ is of particular interest, as it has been found in specific populations of dorsal horn neurons (Polgár et al., 1999). Mao et al. (1995) showed that daily spinal boluses of morphine in rats were associated with an increase in membrane-bound PKC γ isoforms, as in the present studies. It is, however, likely that other isozymes are relevant. Polgár and colleagues (Polgár et al., 1999) have shown that there is only a 5% overlap between cells expressing μ opiate receptors and PKC γ . Thus, even if the upregulation of PKC activity and PKC γ protein can be accounted for by this small population of neurons, one would have to hypothesize that all of the spinal morphine tolerance was the result of an action in this small population of μ opiate receptor/PKC γ -expressing neurons. In this regard, we also noted in the present studies that there was an increase in dorsal horn PKC α after chronic opiate exposure. We do not know whether PKC α is in fact expressed in dorsal horn neurons that contain opiate receptors. Until isozyme-specific interventions become available, it is not possible to determine if the net effect of PKC inhibition on tolerance development is mediated by one or several identified isozymes. Nonetheless, the present studies provide strong support for (i) the

importance of phosphorylation in the evolution of the change in responsiveness to chronically administered opiates, and (ii) the role of one or more PKC isozymes.

4.4. Role of spinal PKC in tolerance

The present studies emphasize the importance of spinal PKC in morphine tolerance. The mechanism whereby phosphorylation alters spinal function is likely complicated. Two possible components will be noted below.

4.4.1. Changes in opiate receptor–effector coupling

We believe that the preservation of the spinal morphine effects in the presence of PKC inhibitors may reflect several changes in receptor–effector coupling that occurs secondary to chronic agonist occupancy. Morphine acting at the μ opioid receptor inhibits adenylyl cyclase activity and thus decreases the cAMP formation (Bachrach et al., 1979). Adenylyl cyclase is inactivated or activated by G_i and G_s , glutamyl transpeptidase membrane-bound proteins, respectively. Decreased cAMP levels activate PKC. The activation of PKC phosphorylates the μ receptor-coupled G protein, thereby suppressing its ability to mediate receptor-evoked inhibition of adenylyl cyclase (Katada et al., 1985; Nestler, 1993). It has been suggested that G protein-coupled receptor kinase specifically phosphorylates and uncouples the agonist-activated receptors and facilitates their interaction with β -arrestins (Lefkowitz, 1993; Ferguson et al., 1996; Zhang et al., 1996), which serve to further uncouple the receptors from their G proteins (Lefkowitz, 1993). Thus, the development of tolerance has been proposed to result from receptor phosphorylation, resulting in a loss of agonist affinity and activity. There is evidence that after chronic systemic morphine administration, the phosphorylation state of many proteins is increased (Nestler, 1993; Zhang et al., 1996, 1998; Chakrabarti et al., 1998). This increase in phosphorylation is also accompanied by an upregulation of PKC and a concurrent tolerance to the antinociceptive effects of morphine. As reported by Narita et al (Narita et al., 1996), attenuation of the μ -opioid receptor agonist-induced antinociception by PDBu appears to be specifically mediated by the activation of a DAG binding site of PKC. This assertion is supported by the finding that attenuation of morphine-induced antinociception was prevented by concomitant pretreatment with the PKC inhibitor calphostin C. In our case, the blockade of morphine tolerance by either coinfusion or acute treatment with the PKC selective inhibitors chelerythrine or GF-109203X, but not the inactive homologue of GF-109203X (bisindolylmaleimide V) (Herbert et al., 1990; Toullec et al., 1991), is in accord with that observation.

4.4.2. Changes in non-opiate receptor function

Previous work has emphasized the importance of the NMDA receptor in spinal opiate tolerance. Concurrent delivery of NMDA antagonists and morphine will diminish

the loss of opiate effect, stressing the importance of the NMDA receptor in the development of spinal tolerance (Dunbar and Yaksh, 1996). The mechanism of this interaction is not certain. However, phosphorylation of the NMDA receptor itself increases the functionality of the NMDA ionophore, which accordingly serves to increase calcium influx (Chen and Huang, 1991). This positive feedback would thus serve to additionally enhance opiate receptor phosphorylation with an attendant decrease in μ receptor function (Chen and Huang, 1991).

In conclusion, these studies demonstrate that: (i) concurrent with the onset of tolerance, there is a clear increase in dorsal, but not ventral horn, PKC protein and PKC-mediated phosphorylating activity; and (ii) that blocking PKC phosphorylation will reverse the pharmacological and behavioral indices in morphine tolerance. We believe the potential role of spinal PKC specifically and phosphorylation in general has broad relevance to issues of tolerance. Previous work has shown that other spinal G protein-coupled receptors, such as for δ opioid and α_2 adrenergic agonists, also display tolerance with chronic agonist exposure (Stevens and Yaksh, 1989; Takano and Yaksh, 1993). This tolerance can be prevented by chronic NMDA receptor inhibition (Dunbar and Yaksh, 1997). Accordingly, we hypothesize that such tolerance is also reversed by spinal inhibition of PKC.

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