Expression of Spinal NMDA Receptor and PKCγ after Chronic Morphine Is Regulated by Spinal Glucocorticoid Receptor

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Spinal NMDA receptor (NMDAR), protein kinase C (PKC), and glucocorticoid receptor (GR) have all been implicated in the mechanisms of morphine tolerance; however, how these cellular elements interact after chronic morphine exposure remains unclear. Here we show that the expression of spinal NMDAR and PKCγ after chronic morphine is regulated by spinal GR through a cAMP response element-binding protein (CREB)-dependent pathway. Chronic morphine (10 μg, i.t.; twice daily for 6 d) induced a time-dependent upregulation of GR, the NR1 subunit of NMDAR, and PKCγ within the rat’s spinal cord dorsal horn. This NR1 and PKCγ upregulation was significantly diminished by intrathecal coadministration of morphine with the GR antagonist RU38486 or a GR antisense oligodeoxynucleotide. Intradiscal coadministration of morphine with an adenylyl cyclase inhibitor (2′,5′-dideoxyadenosine) or a protein kinase A inhibitor (H89) also significantly attenuated morphine-induced NR1 and PKCγ expression, whereas intrathecal treatment with an adenylyl cyclase activator (forskolin) alone mimicked morphine-induced expression of GR, NR1, and PKCγ. Moreover, the expression of phosphorylated CREB was upregulated within the spinal cord dorsal horn after chronic morphine, and a CREB antisense oligodeoxynucleotide coadministered intrathecally with morphine prevented the upregulation of GR, NR1, and PKCγ. These results indicate that spinal GR through the cAMP–CREB pathway played a significant role in NMDAR and PKCγ expression after chronic morphine exposure. The data suggest that genomic interaction among spinal GR, NMDAR, and PKCγ may be an important mechanism that contributes to the development of morphine tolerance.

Key words: glucocorticoid receptor; NMDA receptor; morphine tolerance; RU38486; protein kinase C; protein kinase A; adenylyl cyclase; cAMP response element-binding protein; CREB

Introduction

Opioid analgesic tolerance is a pharmacological phenomenon that hampers the clinical use of opioids. Recent research has shed light on the neurobiology of opioid tolerance, including studies on intracellular adenylyl cyclase (AC)–cAMP and cAMP-dependent protein kinase A (PKA) (Nestler and Aghajanian, 1997), G-protein signaling (Gintzler and Chakrabarti, 2001), β-arrestin (Bohn et al., 2000), μ-opioid receptor oligomerization (He et al., 2002; Bailey et al., 2003), NMDA receptor (NMDAR) and protein kinase C (PKC) (Trujillo and Akil, 1991; Mao et al., 1995; Zeitz et al., 2002; Xu et al., 2003), and glutamate transporter (Mao et al., 2002). Moreover, chronic morphine has been shown to upregulate and activate spinal glucocorticoid receptors (GRs) initiated by cAMP and PKA activity, which also contributed to the mechanisms of morphine tolerance in rats (Lim et al., 2005b,c). Although these studies have implicated multiple cellular elements in the mechanisms of morphine tolerance, how some of these cellular elements interact in this process remains unknown.

Activation of central GR has been shown to modulate morphine-induced antinociception (Pieretti et al., 1991; Capasso et al., 1992) and dopamine-dependent responses (Schoffelmeer et al., 1996; Marinelli et al., 1998). GR is expressed in spinal cord dorsal horn neurons, which contributes to spinal nociceptive processing (DeNicola et al., 1989; Cintra et al., 1993; Wang et al., 2004, 2005). Activation of central GR also has been shown to regulate neuronal plastic changes after neuronal injury (Cameron and Dutia, 1999) as well as the process of learning and memory (Quirarte et al., 1997; Oitzl et al., 1998; Roosendaal et al., 1999, 2003).

An increasing body of evidence indicates that activation of central GR modulates NMDAR through both genomic and non-genomic mechanisms. For example, the NMDAR-mediated response to excitatory amino acids of dopamine-sensitive neurons within the ventral tegmental area can be potentiated by GR activation (Cho and Little, 1999). Central GR also plays a role in NMDAR-mediated long-term depression (Coussens et al., 1997) and the regulation of NMDAR- and GR-mediated neuronal degeneration (Abraham et al., 2000; Lu et al., 2003). Indeed, perip-
eral nerve injury has been shown to upregulate spinal GR followed by a downstream upregulation and functional modulation of NMDAR (Wang et al., 2004, 2005). Moreover, the phosphorylated form of a cAMP response element-binding protein (pCREB) was increased after nerve damage (Ma and Quirion, 2001; Ma et al., 2003; Miletic et al., 2004; Miyabe and Miletic, 2005), spinal cord injury (Crown et al., 2005), and chronic morphine (Guitart et al., 1992; Li and Clark, 1999; Ma et al., 2001), and there are interactions between GR and pCREB (Imai et al., 1993; Shepard et al., 1998; Focking et al., 2003; Bachmann et al., 2005). Thus, it is possible that GR may play an important role in the regulation of the cellular elements implicated in the mechanisms of morphine tolerance.

With a rat model of chronic intrathecal morphine administration, we examined the hypothesis that the expression of spinal NMDAR and PKCγ would be upregulated after chronic morphine exposure, which would be regulated by spinal GR expression and activation initiated by the intracellular cAMP–PKA–CREB pathway.

### Materials and Methods

#### Experimental animals

Adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 300–350 g were used. Animals were housed in cages with water and food pellets available *ad libitum*. The animal room was artificially illuminated from 7 A.M. until 7 P.M.. The general experimental protocol was approved through our Institutional Animal Care and Use Committee.

#### Intrathecal catheter implantation and drug delivery

An intrathecal catheter (PE-10) was implanted in each rat under intraperitoneal pentobarbital anesthesia (50 mg/kg) according to a previously published method (Yaksh and Rudy, 1976). Animals that exhibited neurological deficits (e.g., paralysis) after the catheter implantation were excluded from the experiments (Mao et al., 2002). Drugs were delivered intrathecally in a total volume of 10 μl followed by a saline flush. The following drugs were purchased from Sigma (St. Louis, MO): RO38486, morphine, MK-801, spironolactone, 2′,3′-dideoxyadenosine (dDA), H89, and forskolin. Morphine and MK-801 were dissolved in normal saline; the other drugs were dissolved in 10% ethanol solution (vehicle).

For the experiment with GR oligodeoxynucleotides (ONDs), sequences for antisense, sense, and mixed-base ONDs were chosen on the basis of previous studies (Engelmann et al., 1998; Wang et al., 2004; Lim et al., 2005b,c). The sequences overlapping the respective initiation codon (GenBank accession number M14053) used to target the rat’s GR mRNA were as follows: antisense (TGGAGTCCATGTCGTT), sense (ATTTGGCCATGGCAGCA), and mixed base (TGAAGTCCAGTGCTAC). For the experiment with CREB ONDs, the sequences for antisense, sense, and mixed-base ONDs were chosen on the basis of a previous study (Ma et al., 2003). The sequences overlapping the respective initiation codon used to target the rat’s CREB mRNA (Ma et al., 2003) were as follows: antisense (TGTTGCTCATTGACCGTG), sense (CACCGTGACTAGATGACCA), and mixed base (GACCTCAGG-TAGTGCTGTT). Sequencing was performed by MWG-Biotech (High Point, NC), and product stability was ensured at the time of delivery. ONDs were dissolved in 0.9% saline and injected intrathecally twice daily (5 μg for each GR OND; 10 μg for each CREB OND) for 6 consecutive days.

#### Induction of morphine tolerance and statistical analysis of behavioral data

Tolerance to the antinociceptive effect of morphine was induced by an intrathecal treatment regimen: 10 μg of morphine was given twice daily for 6 consecutive days. This same morphine regimen has been shown to induce reliable antinociceptive tolerance in rats (Lim et al., 2005b,c). The tail-flick test was used with baseline latencies of 3–4 s and a cutoff time of 10 s. At least two trials were made for each rat, with an intertrial interval of 1 min and with a change of the tail position receiving radiant heat stimulation at each trial. We used a probe dose of morphine (10 μg, i.t.) at different time points, as well as the generation of dose–response curves at the end of the morphine regimen, to compare differences in nociceptive responses between the morphine-treated and control groups. The percentage maximal possible effect (% MPE) was determined by comparing the tail-flick latency before baseline (BL) and after a drug injection (TL) using the following equation: % MPE = [(TL – BL)/(10 – BL)] × 100% (the constant 10 refers to the cutoff time). The data were analyzed by using two-way ANOVA with post hoc Newman–Keuls tests.

#### Immunocytochemistry

Rats were anesthetized with pentobarbital and transcardially perfused with 200 ml of normal saline followed by 200–300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The lumbar spinal cords were dissected, postfixed for 1.5 h, transferred to 30% sucrose in 0.1 M phosphate buffer, and kept overnight. Tissues from both the experimental and control groups were then mounted together on the same block with the OCT compound and frozen on dry ice. The spinal cords were cut (20 μm sections) on a cryostat, mounted serially onto microscope slides, and stored at −80°C for later immunostaining. Immunocytochemical staining was used to detect GR (1:1000, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), NR1 (1:500, mouse monoclonal; Novus Biologicals, Littleton, CO), and PKCγ (1:500, mouse monoclonal; Santa Cruz Biotechnology). Lumbar spinal cord sections were blocked with 1% goat serum in 0.3% Triton for 1 h at room temperature and incubated overnight at 4°C with a primary antibody. Controls were made in the absence of a primary antibody, and antigen absorption (e.g., GR) was used whenever possible. The sections were then incubated for 1 h at room temperature with a corresponding fluororescence isothiocyanate- or cyanine 3-conjugated secondary antibody (1:300; Chemicon, Temecula, CA). For double staining, a second primary antibody was added after the incubation with the first primary antibody following the same procedure described above. Four to six nonadjacent spinal sections were randomly selected, analyzed with an Olympus fluorescence microscope, photographed with a digital camera, and processed with Adobe Photoshop.

#### Western blot

Rats were rapidly (<1 min) killed through decapitation after being anesthetized with pentobarbital. Lumbar spinal cord segments, divided into dorsal and ventral horns, were removed and homogenized in SDS sample buffer containing a mixture of proteinase inhibitors (Sigma). The lumbar segments were harvested because the drug delivery was aimed at this site. Protein samples were separated on SDS-PAGE gel (4–15% gradient gel; Bio-Rad, Hercules, CA) and transferred to polyvinylidine difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 3% milk and incubated overnight at 4°C with a primary antibody [GR, 1:1000, rabbit polyclonal (Santa Cruz Biotechnology); PKCγ, 1:400, mouse monoclonal (Zymed, San Francisco, CA); NRI, 1:2000, mouse monoclonal (Novus Biologicals); pCREB (Ser133), 1:1000, rabbit polyclonal (Cell Signaling Technology, Beverly, MA)] and for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:700; Amersham Biosciences, Arlington Heights, IL). The blots were visualized in enhanced chemiluminescence solution (DuPont NEN, Boston, MA) for 1 min and exposed to Hyperfilm (Amersham Biosciences) for 1–10 min. The blots were then incubated in a stripping buffer (67.5 μM Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol) for 30 min at 50°C and reprobed with a polyclonal...
thermocycler with the following programs: GR: forward primer (AAA TCT TTT TTT GGC TGC TTG CCC GCT CTT TCT GGG C), reverse primer (GAA ACG CAG ATA G), program (95°C, 1 min; 55°C, 2 min; 72°C, 1 min); PKCγ: forward primer (GCT GCC ATC ATA CCT GGG TCA G), reverse primer (TGA GTC AGT GGT AGT CCT TCA G), program (95°C, 1 min; 55°C, 2 min; 72°C, 1 min); PKCα: forward primer (ACC TCT AGT GGA ATG AGT CCT TCA G), reverse primer (AAA TCT TTG TTG GGC ACC TCA GGC TTC CTT GT), program (95°C, 1 min; 55°C, 1 min; 72°C, 1 min). Each program ended after 7 min at 68°C, and products were stored at 4°C.

Every PCR was accompanied by one negative control reaction without template RNA. PCR products were analyzed by gel electrophoresis on an ethidium bromide-stained 1% agarose gel (Sigma) in Tris borate–EDTA buffer. The amount of RNA per RT-PCR sample was normalized by using PCR with primers for β-actin (30 cycles each): forward primer (TAC AAC CAA GCC TGT TCA ATG G); program (95°C, 1 min; 55°C, 1 min; 72°C, 1 min). Each band was then measured with a computer-assisted imaging analysis program and normalized against the corresponding loading control. Differences were compared with a one-way ANOVA followed by post hoc Newman–Keuls tests.

**Results**

**Morphine-induced NR1 and PKCγ expression**

Chronic morphine exposure (10 μg, i.t.; twice daily for 6 d) in rats induced antinociceptive tolerance on days 5 and 7 of the experimental period compared with the saline control group (Fig. 1 A, B) (p < 0.05; n = 5–7). In association with the development of morphine tolerance, there was a time-dependent upregulation of the NR1 subunit of NMDAR and PKCγ within the spinal cord dorsal horn compared with the saline control group (Fig. 2) (p < 0.05; n = 5–7), as revealed by both RT-PCR and Western blot.

Spinal NR1 expression began to increase on day 3 and continued to rise on days 5 and 7 of the experimental period, whereas PKCγ expression showed a significant difference from that of the saline control on days 5 and 7 (Fig. 2), indicating that increased PKCγ expression was preceded by NR1 expression, which occurred before the onset of morphine tolerance.

There was no significant difference in the expression of the NR2 subunit of NMDAR and PKCα within the spinal cord dorsal horn between the morphine and saline treatment groups, nor was...
Effect of the GR antagonist RU38486 on NR1 and PKCy expression

To examine whether spinal GR regulated the expression of NR1 and PKCy after chronic morphine exposure, spinal cord dorsal horn samples were taken on day 7 from several groups of rats treated (intrathecally twice daily for 6 d) with 10 μg of morphine plus vehicle, 10 μg of morphine plus the GR antagonist RU38468 (0.25 or 2 μg), 10 μg of morphine plus the MR antagonist spironolactone (3 μg), 2 μg of RU38468 alone, or a vehicle. The spironolactone dose was chosen because it has been shown to be effective in blocking MR function (Marinelli et al., 1998), and RU38468 doses were chosen on the basis of the literature (Mao et al., 1992) and our previous dose–response experiments showing the preventive effect of 2 μg (but not 0.25 μg) of RU38468 on morphine tolerance (Lim et al., 2005b,c).

The results from this experiment showed that upregulation of NR1 and PKCy expression within the spinal cord dorsal horn after chronic morphine was blocked by the coadministration of morphine with RU38468 (2 μg) on day 7 by both RT-PCR (Fig. 3) \( (p < 0.05; n = 4–5) \) and Western blot (Fig. 4A,B) \( (p < 0.05; n = 4–5) \). RU38468 (2 μg) alone did not change baseline NR1 or PKCy expression (Figs. 3, 4) \( (p > 0.05; n = 4–5) \), nor did a low RU38468 dose (0.25 μg) attenuate the upregulation of NR1 and PKCy expression after chronic morphine (Fig. 4C,D) \( (p > 0.05; n = 5) \). In contrast, coadministration of morphine with the MR antagonist spironolactone did not prevent the morphine-induced upregulation of NR1 and PKCy (Fig. 4A,B) \( (p > 0.05; n = 4–5) \). RU38468 (2 μg) also did not change the expression of NR2, PKCa, and MR (Fig. 3) \( (p > 0.05; n = 4–5) \), indicating a selective effect of RU38468 on the expression of spinal NR1 and PKCy after chronic morphine exposure. In addition, there was colocalization between GR and NR1 as well as GR and PKCy within the superficial laminae of spinal cord dorsal horn as revealed by immunohistochemistry (Fig. 5).

Effect of GR antisense OND on NR1 and PKCy expression

The role of spinal GR in the expression of both NR1 and PKCy was examined further by downregulating spinal GR with a GR antisense OND. Although mouse models of genetically altered GR expression have been reported, these mouse models are either lethal at birth or unavailable (Tronche et al., 1999; Gartner et al., 2002; St-Hilaire et al., 2003). As an alternative, we used a GR antisense OND that was delivered regionally to the spinal site of GR action. In this set of experiments, each group of rats received 10 μg of morphine in combination with 5 μg each of GR antisense OND, sense OND, mixed-base OND, or vehicle given twice daily for 6 consecutive days, and the spinal expression of NR1, PKCy, and GR was examined on day 7. This GR antisense treatment regimen significantly attenuated spinal GR expression (Fig. 6B) \( (p < 0.05; n = 5) \), which is consistent with previous findings that the same GR antisense treatment downregulated spinal GR expression and attenuated the development of morphine tolerance and neuropathic pain behaviors in rats (Wang et al., 2004, 2005; Lim et al., 2005b,c).

On day 7, the GR antisense OND treatment significantly attenuated the upregulation of spinal NR1 and PKCy expression induced by chronic morphine exposure (Fig. 6A,C) \( (p < 0.05; n = 5–7) \). In contrast, the expression of NR1 and PKCy remained elevated in the morphine plus mixed-base OND or sense OND group compared with the vehicle group on day 7 (Fig. 6A,C) \( (p < 0.05; n = 5–7) \).
Because NMDAR expression was downstream to spinal GR upregulation and activation and NMDAR activation has been shown to prevent the development of morphine tolerance (Trujillo and Akil, 1991), we examined whether inhibition of NMDAR would have a feedback effect on the expression of GR after chronic morphine exposure. The results showed that coadministration of morphine (10 μg, i.t.; twice daily) with the noncompetitive NMDAR antagonist MK-801 (10 nmol) for 6 d significantly diminished morphine-induced GR upregulation on day 7 compared with the vehicle or MK-801-alone group (Fig. 8) \( (p < 0.05; n = 5) \), indicating a feedback effect of NMDAR activation on GR expression after chronic morphine exposure.

Role of AC and PKA activity in NR1 and PKCγ expression

Because the cAMP–PKA pathway regulated GR expression after chronic morphine exposure in our previous study (Lim et al., 2005b), we examined whether the cAMP–PKA pathway would mediate morphine-induced expression of NR1 and PKCγ. The results showed that the AC inhibitor ddA (1 μg, not 0.25 μg) or the PKA inhibitor H89 (10 μg, not 2.5 μg), given intrathecally twice daily for 6 d, nearly blocked the upregulation of spinal NR1 and PKCγ expression on day 7, compared with the morphine plus vehicle group (Fig. 9A–D) \( (p < 0.05; n = 4–5) \). In contrast, ddA (1 μg) or H89 (10 μg) treatment alone did not change baseline NR1 or PKCγ expression (Fig. 9A–D) \( (p > 0.05; n = 4–5) \). The selected dose range for ddA and H89 was based on previous studies that showed an effective inhibition of cAMP production and PKA activation, respectively (Aley and Levine, 1997; Jolas et al., 2000; Lim et al., 2005b).

To further examine the role of cAMP in the expression of NR1 and PKCγ, either forskolin (an AC activator) (Jolas et al., 2000) or vehicle alone was administered intrathecally twice daily for 6 consecutive days. Forskolin (10 μg, not 2.5 μg) alone induced the expression of both NR1 and PKCγ within the spinal cord dorsal horn compared with the vehicle control on day 7 (Fig. 9A–D) \( (p < 0.05; n = 4–5) \), which mimicked increased NR1 and PKCγ expression after chronic morphine exposure at this same time point. When forskolin (10 μg) was coadministered with morphine (10 μg, i.t.; twice daily for 6 d), the expression of spinal NR1 and PKCγ was further increased compared with either the morphine-alone or the forskolin-alone group (Fig. 9A–D) \( (p < 0.05; n = 4–5) \). Forskolin treatment (10 μg, not 2.5 μg or vehicle, i.t.; twice daily for 6 d) alone also induced the upregulation of spinal GR expression on day 7 (Fig. 9E,F) \( (p < 0.05; n = 4–5) \). Moreover, spinal GR expression was further enhanced, compared with morphine (10 μg) alone, after forskolin (10 μg) was coadministered intrathe-
Effect of CREB antisense OND on GR, NR1, and PKC expression

Chronic morphine exposure (10 μg, i.t.; twice daily for 6 d) induced the upregulation of p-CREB expression within the spinal cord dorsal horn on day 7 compared with the saline group (Fig. 10A, B) (*p < 0.05 compared with the vehicle group). D1, 1 μg of dexamethasone; D4, 4 μg of dexamethasone. Coadministration of dexamethasone (4 μg, i.t.; twice daily for 6 d) and RU38486 (2 μg) prevented the dexamethasone-induced upregulation of spinal NR1 and PKCγ in naive rats, whereas RU38486 (2 μg) alone did not change baseline NR1 and PKCγ expression. V, Vehicle; R, RU38486.

Discussion

The present study shows that (1) chronic morphine exposure that resulted in antinociceptive tolerance induced a time-dependent upregulation of the NR1 subunit of NMDAR and PKCγ within the spinal cord dorsal horn as shown in the merged (yellow) images (c, GR/PKCγ; f, GR/NR1). Spinal cord samples were taken from rats receiving morphine treatment on day 7. Scale bar, 100 μm.

Figure 6. A, A GR antisense OND (a/m), but not a sense (s/m) or mixed base (m/m) OND, significantly attenuated the upregulation of NR1 and PKCγ as assayed by RT-PCR, within the spinal cord dorsal horn when examined on day 7. Each OND (5 μg, i.t.) was given in combination with morphine (10 μg, i.t.) for 6 d. B, A GR antisense OND (A), but not a sense (S) or mixed base (M) OND, substantially downregulated GR expression within the spinal cord dorsal horn. C, Statistical analysis for the groups shown in A. *p < 0.05 compared with the vehicle (V) group.
the rat’s spinal cord dorsal horn; (2) this upregulation of NR1 and PKCγ expression was diminished by coadministration of morphine with either the GR antagonist RU38486 or a GR antisense OND; (3) GR was colocalized with NR1 as well as PKCγ within the spinal cord dorsal horn; (4) coadministration of morphine with the AC inhibitor dDA or the PKA inhibitor H89 also significantly attenuated the upregulation of NR1 and PKCγ expression, whereas forskolin (an AC activator) alone mimicked the expression of spinal NR1, PKCγ, and GR induced by chronic morphine exposure; and (5) chronic morphine exposure induced the upregulation of p-CREB expression within the spinal cord dorsal horn, and CREB antisense OND treatment attenuated the upregulation of spinal GR, NR1, and PKCγ expression induced by chronic morphine exposure. Given that chronic morphine induced the upregulation of spinal GR that was preventable by blocking AC and PKA activity (Lim et al., 2005b), these results indicate that spinal GR through the cAMP–CREB pathway played a significant role in NMDAR and PKCγ expression after chronic morphine exposure.

Several methodological issues should be considered regarding interpretation of the data. Although RU38486 has been widely used as a GR antagonist, it also acts as an antiprogestosterone (Mao et al., 1992). The antiprogestosterone effect of RU38486 is unlikely to be contributory in this experimental paradigm because only male rats were used in the study. In addition, the selective effect of RU38486 on spinal GR after chronic morphine exposure was supported by the following: (1) the GR knock-down experiment using a GR antisense OND produced a result similar to that after RU38486; (2) RU38486 at 2 μg blocked NR1 and PKCγ expression induced by an exogenous GR agonist (dexamethasone) in naïve rats; and (3) there was a dose–response effect of RU38486 on NR1 and PKCγ expression. Spinal MR also did not play a significant role in this process, because the baseline MR expression was not altered after chronic morphine exposure, and the MR antagonist spironolactone at a dose known to block MR function (Marinelli et al., 1998) did not prevent the morphine-induced upregulation of NR1 and PKCγ expression. It should be pointed out that the present experimental paradigm focused on the spinal cord dorsal horn because morphine was delivered through an intrathecal treatment regimen at this spinal site. This approach, however, does not rule out the possibility that a similar GR-mediated regulation of NR1 and PKCγ expression could be involved in brain regions known to be involved in the mechanisms of morphine tolerance.

For several reasons, we examined the relationship among the spinal cAMP–CREB pathway, GR expression and activation, and the expression of NMDAR and PKCγ after chronic morphine. First, a considerable number of previous studies have indicated that NMDAR and PKCγ play a significant role in the mechanisms of morphine tolerance (Trujillo and Akil, 1991; Mao et al., 1995; Zeitz et al., 2002). Second, our recent studies have shown that spinal GR contributed to the cellular mechanisms of morphine tolerance (Lim et al., 2005b,c) as well as neuropathic pain (Wang et al., 2004), and a number of studies have indicated that morphine tolerance and neuropathic pain may share a similar cellular mechanism involving both NMDAR and PKCγ (Mao et al., 1995). Third, chronic morphine treatment has been shown to increase the GR expression that was mediated through the cAMP and PKA pathway and prevented by the opioid receptor antagonist naloxone (Lim et al., 2005b,c), and the cAMP–PKA–CREB pathway can be activated after chronic morphine exposure (Guitart et al., 1992; Nestler and Aghajanian, 1997; Li and Clark, 1999; Ma et al., 2001). Thus, spinal GR is likely to play a critical role in the cellular mechanisms of morphine tolerance involving both NMDAR and PKCγ.

Our data indicate that at least part of the GR-mediated downstream response after chronic morphine exposure was an enhanced expression of NMDAR and PKCγ, two known contributors to the cellular mechanisms of neural plasticity related to learning, memory, and opioid tolerance (Olds et al., 1989; Collingridge and Singer, 1990; Madison et al., 1991; Trujillo and Akil, 1991; Mao et al., 1995, 2002; Mao, 2002; Zeitz et al., 2002). Moreover, the cAMP–PKA–CREB pathway played an active role in this process because inhibition of AC and PKA activity or a CREB antisense OND substantially diminished the upregulation of GR as well as NR1 and PKCγ expression after chronic morphine exposure. These findings are consistent with known GR actions within the CNS. For example, activation of central GR has been associated with (1) NMDAR-dependent long-term depression (Couszens et al., 1997) and an elevated intracellular Ca2+ concentration in hippocampal neurons (Takahashi et al., 2002), (2) modulation of the NMDAR function (Nair et al., 1998), (3) the potentiated response to NMDA of dopamine-sensitive neurons in the ventral tegmental area (Cho and Little, 1999), (4) neuronal apoptosis mediated through intracellular mitogen-activated protein kinases (Dien et al., 2003), and (5) inhibition of bradykinin-induced Ca2+ influx via PKC activation (Qiu et al., 2003). Thus,
the role of GR in spinal NR1 and PKCγ expression is indicative of a broad cellular mechanism of GR regulation under various conditions, including chronic morphine exposure.

The present data using both RT-PCR and Western blot assays indicate that GR-mediated NMDAR and PKCγ expression after chronic morphine is mediated through transcriptional and translational regulation. The actions of GR through a genomic mechanism begin with activation of cytosolic GR (Neeck et al., 2002), which can be activated by circulatory corticosteroids and/or locally produced neurosteroids (Compagnone and Mellon, 2000; Plassart-Schiess and Baulieu, 2001; Vallee et al., 2001). Activation of GR requires the formation of a GR-homodimer after its dissociation from the cytosolic complex consisting of heat shock proteins (Drouin et al., 1992). A GR-homodimer serves as a transcription factor and binds to nuclear-specific DNA responsive elements, regulating gene transcription and translation for a number of cellular elements (Drouin et al., 1992). For instance, GR activation has been shown to induce the expression of a Ca2+ channel subunit in neurons of the basolateral amygdala (Karst et al., 2002) and of spinal NMDAR after peripheral nerve injury (Wang et al., 2005). The expression of spinal NMDAR and PKCγ was attenuated by a GR antisense OND in this study, lending support to a genomic mechanism of GR action. Of note is that spinal GR is mostly associated with neuronal cells because there is an abundant colocalization between spinal GR and neuronal-specific nuclear protein (a neuronal marker), as shown in a previous study (Wang et al., 2004), and among GR, NMDAR, and PKCγ, as shown in the present study. Our data also indicate that inhibition of NMDAR by MK-801 significantly reduced GR upregulation after chronic morphine, which is consistent with a reciprocal role between NMDAR and GR in the regulation of neuronal degeneration (Abraham et al., 2000; Lu et al., 2003), suggesting that the downstream response to GR may have a feedback effect on the cellular process leading to morphine-induced GR upregulation.

The regulatory role of GR in the expression of NMDAR and PKCγ may be critical to the cellular mechanisms of morphine tolerance, because either disruption of GR upregulation with a GR antisense OND or inhibition of GR activation with RU38486 blocked the development of morphine tolerance (Lim et al., 2005b,c). Moreover, our previous studies have shown that the GR-mediated effect on morphine tolerance was abolished in adrenalectomized rats, indicating that endogenous corticosteroids played a significant role in GR function after chronic morphine exposure (Lim et al., 2005b). Together with previous data indicating (1) attenuation of the development of morphine tolerance in rats after intrathecal coadministration of ddA or H89 with morphine (Lim et al., 2005a), (2) an upstream regulatory role of cAMP and cAMP-dependent PKA in morphine-induced GR upregulation (Lim et al., 2005b), and (3) interactions between cAMP and glucocorticoid effects (Kuwahara et al., 2003), the present data suggest that a genomic interaction among spinal GR, NMDAR, and PKCγ mediated by the cAMP–PKA–CREB pathway plays a critical role in the cellular mechanism of morphine tolerance. The results also suggest that a GR inhibitor such as RU38486 or agents blocking the NMDAR and PKCγ upregulation may be useful, alone or in combination, in preventing the...
development of opioid tolerance, an issue of significant clinical relevance (Mao, 2002; Ballantyne and Mao, 2003).

References


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Figure 10. A. Chronic morphine (M) (10 μg, i.t.; twice daily for 6 d) induced the upregulation of p-CREB (43 kDa) expression within the spinal cord dorsal horn on day 7. Coadministration of morphine and a CREB antisense ODN (C/M) (10 μg, given intrathecally twice daily for 6 d), attenuated the morphine-induced upregulation of spinal GR, NRT1, and PKCγ expression on day 7. The CREB antisense (C) treatment alone did not affect baseline expression of GR, NRT1, and PKCγ. The CREB antisense ODN treatment also prevented the upregulation of spinal p-CREB expression. B. Statistical data for A. C. The CREB sense (S) or mixed (M) ODN (10 μg each) treatment given intrathecally twice daily for 6 d did not alter spinal p-CREB expression. *p < 0.05 compared with the vehicle (V) group.

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