Protein kinase C inhibitor chelerythrine attenuates the morphine-induced excitatory amino acid release and reduction of the antinociceptive effect of morphine in rats injected intrathecally with pertussis toxin

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Abstract

Neuropathic pain syndromes respond poorly to opioid treatment. In our previous studies, we found that intrathecal (i.t.) injection of pertussis toxin (PTX) produces thermal hyperalgesia, which is poorly responsive to morphine and is accompanied by an increase in cerebrospinal fluid (CSF) levels of excitatory amino acids (EAAs) and protein kinase C (PKC) activation. In the present study, rats were implanted with an i.t. catheter for drug injection and a microdialysis probe for CSF dialysate collection. On the fourth day after injection of PTX (2 μg, i.t.), there was a significant reduction in the antinociceptive effect of morphine (10 μg, i.t.) which was accompanied by an increase in levels of EAAs. Pretreatment with the PKC inhibitor, chelerythrine (25 μg, i.t.) one hour before morphine injection markedly inhibited both effects. These results suggest that, in PTX-treated rats, PKC plays an important role in inhibiting the morphine-induced spinal EAA release, which might be related to the reduced antinociceptive effect of morphine.

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Introduction

Pertussis toxin (PTX)-sensitive G protein-coupled receptors, including opioid, α₂-adrenergic, GABA, and A 1-adenosine receptors, are involved in antinociception, and PTX treatment results in ADP-ribosylation of the α–subunit of inhibitory guanine nucleotide binding regulatory proteins (Gi/Go), thus disrupting G-protein mediated signal transduction (Birnbaumer, 1990; Kurose et al., 1983). McCormack et al. (1998) suggested that intrathecal (i.t.) injection of PTX might be used as a model for studying the central mechanisms of neuropathic pain. In a previous study (Wen et al., 2003a), we demonstrated that i.t. injection of PTX induced an increase in levels of excitatory amino acids (EAAs) and a decrease in the levels of the inhibitory amino acid, glycine, in CSF dialysates, suggesting that it might affect the intrinsic balance between the inhibitory and excitatory nociceptive systems and result in excitation. Intrathecal injection of PTX not only attenuates the antinociceptive effect of opioid receptor agonists (Galeotti et al., 1996; Hoehn et al., 1988; Wong et al., 1992), but also produces hyperalgesia and allodynia (Womer et al., 1997; Womer and Shannon, 2000). Moreover, in our report (Wen et al., 2003a), we found increased levels of the EAAs, glutamate and aspartate, in the spinal CSF dialysate in PTX-treated rats.

Animal and human studies have shown that, in neuropathic pain, the analgesic effect of μ-opioid agonists is reduced (Arner and Meyerson, 1988; Courteix et al., 1993). In a previous study using i.t. injection of PTX to uncouple Gi-proteins to study the mechanisms of opioid tolerance, we found a reduction in the
antinociceptive effect of opioid peptide PL-017 (Wong et al., 1992). Several studies have demonstrated that activation of the EAA receptor system, and subsequently of PKC, plays an important role in neuropathic pain development (Mao et al., 1992, 1995a). In a recent study, we found that PTX-induced thermal hyperalgesia is accompanied by increased PKCγ expression in both the synaptosomal membrane and cytosolic fractions of the dorsal horn of the rat lumbar spinal cord, and that both effects are inhibited by the NMDA receptor antagonist, D-AP5 (Wen et al., 2003b). PKC activation is known to attenuate opioid receptor activity (Katada et al., 1985; Nestler, 1993). Acute single injection of PKC inhibitors (Granados-Soto et al., 2000; Smith et al., 2002), but not of the NMDA antagonist, MK-801 (Mayer et al., 1999), reverses the reduction in the antinociceptive effect of morphine in tolerant rats. Intrathecal perfusion of the PKC activator, phorbol ester, evokes mechanical allodynia and thermal hyperalgesia, accompanied by spinal EAA release (Palecek et al., 1999). Urban et al. (1994) found that PKC-mediated NMDA receptor phosphorylation enhances NMDA receptor activity, which then triggers EAA release, and thus produces hypersensitization of spinal nociceptive neurons. These reports suggest that inhibition of PKC activity prevents the reduction in the analgesic effect of opioids on neuropathic pain. The aim of the present study was to examine the EAAs-PKC interaction as a possible mechanism for the reduction in the effect of opioids in PTX-induced thermal hyperalgesia in rats.

Materials and methods

Intrathecal catheter and microdialysis probe implantation

Male Wistar rats (400–420 g) were used. An i.t. catheter and a microdialysis probe were inserted via the atlantooccipital membrane into the i.t. space to the level of the lumbar enlargement of the spinal cord, and externalized and fixed to the cranial aspect of the head (Yaksh and Rudy, 1976). The rats were then returned to their home cages for a 4-day recovery period; each rat was housed individually and maintained on a 12 h light/dark cycle with food and water freely available. Rats were excluded from the study if they showed evidence of gross neurological injury or the presence of fresh blood in the CSF. The use of animals in this study conformed to the Guiding Principles in the Care and Use of Animals of our Institute and was approved by the Care and Use Committee of our Institute.

Construction of the microdialysis probe

The microdialysis probe was constructed as described previously (Marsala et al., 1995), using two 7 cm PE5 tubes (0.008 inch inner diameter, 0.014 inch outer diameter) and a 4 cm cuprophan hollow fiber (300 μm outer diameter, 200 μm inner diameter, 50 kDa molecular weight cut-off; DM-22, Eicom Co., Kyoto, Japan). To make the probe firm enough for implantation, a Nichrome–Formavar wire (0.0026 inch; A–M system, Everett, Inc., WA, USA) was passed through a poly-carbonate tube (194 μm outer diameter, 102 μm inner diameter; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), and connected to a PE5 catheter with epoxy glue. The fiber was then bent in the middle section of the cuprophan hollow fiber, forming a “U”-shaped loop. The two ends of the dialysis fiber, consisting of silastic tubes, were sealed with silicon sealant. The dead space of the dialysis probe was 8 μl.

Intrathecal PTX injection and antinociceptive test

After the recovery period, PTX (2 μg) or saline was injected i.t. (day 0), then, on day 4, saline or chelerythrine (25 μg, i.t.) was injected, followed, 1 h later, by morphine (10 μg, i.t.). The dose of chelerythrine used did not alter the tail-flick latency or produce any other behavioral change in naïve rats. The rats were placed in plastic restrainers for drug injection and antinociception examination. The tail-flick latency was measured by the hot water immersion test (52 ± 0.5 °C). At this temperature, the mean tail-flick latency of naïve rats was approximately 2.2 ± 0.3 s. An automatic cut-off time was set at 10 s to prevent tissue injury. Antinociceptive effects were examined at 30, 60, 90, or 180 min after i.t. morphine injection. Each tail-flick latency value was the average of two measurements over a six-minute test period on each rat. The latency response was converted into the maximum percent effect (MPE) using the equation:

\[ \text{MPE}(\%) = \frac{\text{Test response time} - \text{Basal response time}}{\text{Cut-off time} - \text{Basal response time}} \times 100\% \]

Latencies less than the baseline or higher than the cut-off time were assigned MPE values of 0% or 100%, respectively.

CSF sampling and excitatory amino acid measurement

After the 4-day recovery period, the rats were transferred to a free-moving animal system and one end of the externalized microdialysis probe was connected to a syringe pump (CMA-100) for cerebrospinal fluid (CSF) sampling. The dialysis system was perfused with artificial CSF, consisting of 151.1 mM Na⁺, 2.6 mM K⁺, 122.7 mM Cl⁻, 21.0 mM HCO₃⁻, 0.9 mM Mg²⁺, 1.3 mM Ca²⁺, 2.5 mM HPO₄²⁻, and 3.5 mM dextrose, bubbled with 5% CO₂ in 95% O₂ to adjust the pH to 7.3. CSF samples were collected using a standard procedure of a 30 min washout period, followed by a 30 min sample collection period, at a flow rate of 5 μl/min. On the fourth day after PTX or saline injection, after collection of a basal CSF dialysate sample, the rats were injected as above and CSF dialysates collected in polypropylene tubes on ice every 30 min for 180 min after morphine injection and stored at −80 °C until assayed. Concentrations of EAAs were analyzed by high performance liquid chromatography (HP1100) using a fluorescence detector as described previously (Wen et al., 2004). In brief, amino acids were assayed by precolumn derivatization with o-phthalaldehyde/t-butylthiol (OPA) reagent and iodoacetic acid/methanol scavenger. Derivatization was performed by adding 4 μl of OPA reagent to 40 μl of sample, shaking the
mixture, then allowing it to react for 2 min. Four milliliters of reagent B (185 mg of iodoacetamide/ml of methanol) was added and the mixture allowed to react for another 2 min. The derivatized sample was then injected onto a C18 reverse phase column and eluted at a flow rate of 0.45 ml/min. A linear gradient from 100% eluent A [0.1 M sodium acetate buffer, pH 6.8/acetonitrile (80:20)] to 100% eluent B [acetonitrile/double-distilled water (80:20)] was used to separate the amino acids. All solvents were vacuum-filtered through a 0.22 \( \mu \)m membrane (Millipore) and degassed by sonication before use. External standard solutions containing 0, 10\(^{-8}\), 10\(^{-7}\), 10\(^{-6}\), 10\(^{-5}\) M standard amino acids were run before and after each sample group. The percentage change relative to the basal EAA release was calculated as the area under the curve (AUC) from time 0 to 180 min after morphine injection, which was derived from the original data by the trapezoidal method (Rowland and Tozer, 1995).

Data and statistical analysis

All data are presented as the mean±S.E.M. For statistical analysis, all data were analyzed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls post-hoc test for multiple comparisons. A significant difference was defined as a \( P \) value <0.05.

Result

Effect of the PKC inhibitor, chelerythrine, on the antinociceptive effect of morphine in PTX-treated rats

In sham-operated rats, injection of morphine (10 \( \mu \)g, i.t.) had a significant antinociceptive effect (MPE = 100%), whereas, on the fourth day after injection with PTX (2 \( \mu \)g, i.t.), 10 \( \mu \)g of morphine had little antinociceptive effect (MPE=15%) (Fig. 1).
1). Pretreatment of PTX-treated rats with the PKC inhibitor, chelerythrine (25 μg, i.t.), 1 h before morphine injection partially restored the antinociceptive effect of morphine (MPE = 70%) (Fig. 1). Chelerythrine (25 μg, i.t.) alone did not have any antinociceptive effect in either saline control or PTX-treated rats (data not shown).

**Effect of chelerythrine on morphine-induced EAA release in PTX-treated rats**

In the CSF microdialysis study on the fourth day after PTX injection, acute morphine injection (10 μg, i.t.) resulted in a significant increase in glutamate and aspartate concentrations in the CSF dialysates of PTX-treated rats (Figs. 2 and 3), whereas, in non-PTX-treated rats, it resulted in a slight, but non-significant reduction in the EAA concentrations. Pretreatment of PTX-treated rats with chelerythrine (25 μg, i.t.) 1 h prior to morphine challenge resulted in morphine-induced glutamate and aspartate release even lower than that seen in non-PTX-treated saline controls.

**Discussion**

As our previous report (Wong et al., 1992), the present study showed that, in PTX-treated rats, there was a reduction in the antinociceptive effect of morphine and an increase in the levels of EAAs in intrathecal CSF dialysates. Both effects were markedly inhibited by treating the PTX-treated rats with PKC

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**Fig. 2.** Effect of chelerythrine on morphine-induced aspartate release in PTX-treated rats. (A) On the fourth day after i.t. injection with either 2 μg of PTX or saline and basal CSF dialysate collection at time -60 min, rats were injected i.t. with chelerythrine (25 μg) or saline, then, 1 h later, with morphine (10 μg, i.t.) (time 0 min). Another four dialysate samples were collected at 30, 60, 90, and 180 min after morphine challenge. (B) Area under the curve (time 0 to 180 min) for aspartate release using the data from A and expressed as the AUC (% change from baseline x min). Data are presented as mean±SEM for the number of rats indicated in A. #, P<0.05 compared to the PTX+salmine+morphine group; *, P<0.05 compared to the saline+salmine+morphine group. Values are mean±SEM (n = 6 for each group).
inhibitor chelerythrine one hour prior to morphine administration, and chelerythrine alone did not produce any antinociceptive effect. The results suggest that EAA-PKC signaling is involved in the PTX-induced reduction of the antinociceptive effect of morphine and the associated morphine-induced spinal EAAs release.

It has been suggested that the ineffectiveness of morphine in treating neuropathic pain is due to the reduction in spinal μ-opioid receptors seen following peripheral nerve ligation (Mao et al., 1995b; Porreca et al., 1998). Possible mechanisms for this reduction might be (1) downregulation of high affinity μ-opioid receptors, (2) reduction in the number of total μ-opioid receptors, and (3) changes in intracellular nociception signal transduction, such as increased EAA release or PKC activation. Studies have shown that PTX treatment alone does not affect total μ-opioid receptor numbers or the Gs-, Gi-, and Go-protein content of neuronal plasma membrane from rat brains and spinal cords (Gomes et al., 2002; Wong et al., 1992). Chen et al. (2002) demonstrated that GTPγS binding by μ-opioid receptors was significantly reduced in the spinal dorsal horn of diabetic neuropathic pain rats when compared to normal rats, while Wong et al. (1992) found that injection of PTX (1 μg, i.t.) decreased the antinociceptive effect of μ-opioid peptide PL017 and it was associated with a reduction of the high-affinity μ-opioid receptor sites, but not total μ-opioid receptors. It suggests an impairment of μ-opioid receptor function, rather
than a reduction of μ-opioid receptor numbers, it is responsible for the reduced analgesic effect of μ-opioid agonists in neuropathic pain models.

In addition to the disruption of μ-opioid receptor signal transduction by PTX treatment, activation of the NMDA-PKC signal transduction cascade might also play an important role for the poor response of morphine in the management of neuropathic pains (Mayer et al., 1999) and in PTX-treated rats (Wen et al., 2003a,b). PTX-induced glutamate release via activation of the presynaptic L-type Ca\textsuperscript{2+} channel has been demonstrated in cerebellar granule neurons (Huston et al., 1993). Activation of NMDA-gated Ca\textsuperscript{2+} channels results in an increase in intracellular Ca\textsuperscript{2+} levels which, in turn, results in Ca\textsuperscript{2+}-dependent glutamate exocytosis (Sanchez-Prieto et al., 1996). Moreover, PTX-induced EAA release had also been seen in chromaffin cells or dorsal root ganglia (Dolphin and Scott, 1987, 1990; Sontag et al., 1991) and in the spinal CSF dialysate of PTX-treated rats (Wen et al., 2003a). Chen and Huang (1991) also demonstrated that activation of PKC downregulated opioid receptor function as a result of phosphorylation of μ-receptors, thereby decreasing the ability of opioids to inhibit adenyl cyclase, which results in the loss of the antinociceptive effect of morphine (Katada et al., 1985; Nestler, 1993). Taken together, PTX treatment of rats disrupts opioid signaling by activating NMDA receptors and PKC, and reduces the antinociceptive effectiveness of morphine (Mayer et al., 1999; Pasternak, 1993; Wong et al., 1992).

Previous studies have shown that the NMDA receptor antagonist, MK-801, can prevent morphine tolerance when co-infused with morphine at the beginning of tolerance induction, but cannot restore the antinociceptive effect of morphine once tolerance has developed (Mao et al., 1995a,b). In contrast, the PKC inhibitor chelerythrine not only prevented CCI-induced neuropathic pain, but also acutely restores the antinociceptive effect of morphine in tolerant rats (Granados-Soto et al., 2000; Hua et al., 1999; Smith et al., 2003). Our present study shows that a single injection of chelerythrine restores the antinociceptive effect of morphine in PTX-treated rats, but itself chelerythrine has no antinociceptive effect in naïve rats. Similarly, Smith et al. (2002) also found that PKC inhibitors Go-7874 and sangivamycin had no effect on the antinociceptive effect of morphine in naïve mice, but a single injection of morphine-tolerant rats with Go-7874 or sangivamycin restored morphine’s antinociceptive effect. This evidence suggests that PKC directly downregulates opioid receptor function, and results in the reduction of morphine’s antinociceptive effect. Moreover, in our present study PKC inhibitor chelerythrine also prevented acute morphine challenge-induced EAA release and this may explain the restoration of the antinociceptive effect of morphine in PTX-treated rats.

In conclusion, we believe that PTX treatment causes induced presynaptic EAA release, and activates postsynaptic NMDA receptors. It triggers PKC activation, and thus reduces the antinociceptive effect of opioids. This positive feedback regulation of the NMDA-PKC cascade serves to enhance opioid receptor phosphorylation which reduces opioid receptor function (Chen and Huang, 1991). Taken together, we find that the PTX-induced thermal hyperalgesia and the reduction of morphine’s antinociceptive effect share, at least in part, a common cellular mechanism — activation of the EAAs-PKC signaling cascade. EAAs, acting via NMDA receptors to regulate the intracellular PKC activity, are probably the link between thermal hyperalgesia and the reduction of morphine’s antinociceptive effect in PTX-treated rats.

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References


