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Activation of cGMP-dependent protein kinase Iα is required for *N*-methyl-D-aspartate- or nitric oxide-produced spinal thermal hyperalgesia

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Abstract

The effect of a selective cyclic guanocine 3′,5′-monophosphate (cGMP)-dependent protein kinase Iα inhibitor, Rp-8-[(4-chlorophenyl)thio]-cGMPS triethylamine (Rp-8-p-CPT-CGMPS), on either *N*-methyl-D-aspartate (NMDA)- or *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12, a nitric oxide (NO) donor)-produced thermal hyperalgesia was examined in the rat. Intrathecal administration of NMDA (15 pg/10 μl) or NOC-12 (10, 20 and 30 μg/10 μl) produced a marked curtailment of the tail-flick latency. Maximal NMDA- or NOC-12-produced facilitation of the tail-flick reflex was significantly and dose-dependently blocked by intrathecal pretreatment with Rp-8-p-CPT-CGMPS (7.5, 15 and 30 μg/10 μl). Rp-8-p-CPT-CGMPS given alone did not markedly alter baseline tail-flick latency. These results suggest that the activation of cGMP-dependent protein kinase Iα is required for NMDA- or NO-produced facilitation of thermal hyperalgesia at the spinal cord level.

Keywords: Protein kinase, cGMP-dependent; *N*-methyl-D-aspartate (NMDA); Nitric oxide (NO); Nociception; Spinal cord; Hyperalgesia; Pain

1. Introduction

Nitric oxide (NO), which serves as an intracellular messenger, is implicated in a number of processes in the central nervous system. In the spinal cord, considerable evidence has demonstrated that NO contributes to the development of hyperalgesia in models of acute and chronic pain (Meller and Gebhart, 1993). Noxious stimulation increased NO synthase (NOS) expression (Lam et al., 1996) and cyclic guanocine 3′,5′-monophosphate (cGMP) content (Garry et al., 1994b) in the spinal cord. Administration of inhibitors of NOS and soluble guanylate cyclase caused analgesic effects (Moore et al., 1990; Meller et al., 1992a,b; Malmberg and Yaksh, 1993). Moreover, NO donors and cGMP analogues applied intrathecally caused a reduction in tail flick or paw withdrawal latency (Garry et al., 1994a; Inoue et al., 1997). Recently, sodium nitroprusside (an NO donor) was shown to evoke the release of immunoreactive cGMP from dorsal horn slices, which was suppressed by the application of methylene blue (a soluble guanylate cyclase inhibitor) (Garry et al., 1994c). These data indicate that the NO/cGMP signaling pathway contributes to spinal hyperalgesia via a cGMP-dependent mechanism.

It has been demonstrated that the *N*-methyl-D-aspartate (NMDA) receptors play a key role in multisynaptic nociceptive transmission and plasticity within the spinal cord (Aanonsen et al., 1990; Dickenson and Aydar, 1991). The NMDA receptors may be involved in changes such as central sensitization, wind-up, facilitation, hyperalgesia and allodynia, all of which may be manifestations of the same mechanisms. It is found that many of the effects of NMDA receptor activation appear to be ultimately mediated through the production of NO and cGMP (Meller and Gebhart, 1993). In the cerebellum, NMDA receptor activation results in a Ca^{2+}-dependent increase in cGMP through the production of NO (Garthwaite et al., 1988). In the spinal cord, NMDA-produced facilitation of the tail flick reflex was completely abolished by pretreatment with either a NOS inhibitor (N^G-nitro-L-arginine methyl ester) or a soluble guanylate cyclase inhibitor (methylene blue)

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(Meller et al., 1992a). Moreover, NMDA has been demonstrated to directly produce the release of NO in vivo at the spinal cord level (Rivot et al., 1999). These results indicate that NMDA may produce thermal hyperalgesia through the activation of the NO/cGMP signaling system in the spinal cord.

The NO/cGMP signaling pathway modifies several intracellular processes including activation of protein kinases, ion channels and phosphodiesterases. cGMP-dependent protein kinases belong to the large family of serine/threonine protein kinases. cGMP-dependent protein kinases have been found to serve as major effectors for the NO/cGMP signaling pathway in the vascular and nervous system (Meller and Gebhart, 1993). Our previous results showed that cGMP-dependent protein kinase Iα but not Iβ was distributed primarily in the superficial laminae of the spinal cord (Tao et al., 2000). The purpose of the present study was to determine whether the thermal hyperalgesia produced by NMDA or NO is mediated through the activation of cGMP-dependent protein kinase Iα.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats weighing 250–300g were used. They were kept under a standard 12 h/12 h light–dark cycle, with water and food pellets available ad libitum. The experimental procedures were approved by the Animal Care Committee at the Johns Hopkins University and were consistent with the ethical guidelines of the National Institute of Health and the International Association for the Study of Pain.

The agents administered intrathecally in the present study were Rp-8-[(4-Chlorophenyl)thio]-cGMPS triethylamine (Rp-8-p-CPT-cGMPS, a selective and potent cGMP-dependent protein kinase Iα inhibitor) (RBI, MA, USA), *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine (NOC-12, a NO donor) (Alexis Biochemicals, CA, USA), NMDA (an NMDA receptor agonist) (RBI) and dizocilpine maleate (MK-801, a selective NMDA receptor antagonist) (RBI).

2.2. Surgery

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg). Chronic intrathecal catheters were inserted by passing a polyethylene-10 (PE-10) catheter through an incision in the atlanto-occipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar subarachinoid space. The animals were allowed to recover for a week before experiments were initiated. Rats showing neurologic deficits post-operatively were removed from the study.

2.3. Thermal nociceptive test

Nociception was evaluated by the radiant heat tail-flick test with no anesthesia. Each rat was placed in an Animal Holder (IITC Life Science, CA, USA), 690 cm^3 in capacity with rubber stoppers in both ends with a rostral inlet and a caudal outlet. The tail of the rat protruded through the caudal hole. The tail-flick apparatus (Model 33B Tail Flick Analgesy Meter, IITC Life Science) generated a beam of radiant heat which was focused on the underside of the tail, 5 cm from the tip. A cut-off time latency of 13.5 s was used to avoid tissue damage to the tail. Nociception was assessed by the prolongation of the time required to induce tail-flick after applying radiant heat to the skin of the tail. The latency of reflexive removal of the tail from the heat was measured automatically to the nearest 0.01 s. Tail-flick latency was measured six times and the basal latency was defined as the mean of the last five times. The tail-flick data are expressed as percentage change calculated by the formula: (trial latency−baseline latency)/(baseline latency) × 100%.

2.4. Drug treatment

The rats were randomly assigned into fifteen groups as follows: saline (control) ($n = 6$); 7.5 μg of Rp-8-p-CPT-cGMPS ($n = 6$); 15 μg of Rp-8-p-CPT-cGMPS ($n = 6$); 30 μg of Rp-8-p-CPT-cGMPS ($n = 6$); 10 μg of NOC-12 ($n = 6$); 20 μg of NOC-12 ($n = 6$); 30 μg of NOC-12 ($n = 6$); 7.5 μg of Rp-8-p-CPT-cGMPS and 30 μg of NOC-12 ($n = 6$); 15 μg of Rp-8-p-CPT-cGMPS and 30 μg of NOC-12 ($n = 6$); 30 μg of Rp-8-p-CPT-cGMPS and 30 μg of NOC-12 ($n = 6$); 15 pg of NMDA ($n = 5$); 34 pg of MK-801 and 15 pg of NMDA ($n = 5$); 7.5 μg of Rp-8-p-CPT-cGMPS and 15 pg of NMDA ($n = 5$); 15 μg of Rp-8-p-CPT-cGMPS and 15 pg of NMDA ($n = 5$); 30 μg of Rp-8-p-CPT-cGMPS and 15 pg of NMDA ($n = 5$). The dose and time point of maximal effect of NMDA used above was determined based on a previous study (Siegan and Sagen, 1995). The drug solution was injected intrathecally in a volume of 10 μl, followed by an injection of 10 μl of saline to flush the catheter. The tail-flick test was conducted before injection and 15, 30, 60, 90 and 120 min after injection.

2.5. Data analysis

Data were expressed as the mean ± S.E.M. The results were assessed by an analysis of variance followed by Newman–Keuls test. Significance was set up at $p < 0.05$.

3. Results

No significant change in the tail-flick latency was seen before and after the injection of saline (Fig. 1, $p > 0.05$).

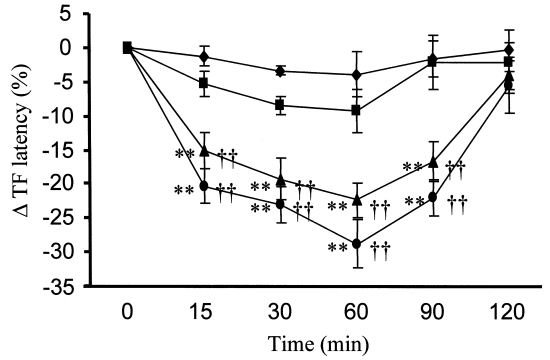

Fig. 1. Time course of the effects of intrathecally injected NOC-12 on the tail-flick latency in rats. Saline control (♦); 10 μg of NOC-12 (■); 20 μg of NOC-12 (▲); 30 μg of NOC-12 (●). All agents were dissolved in 0.9% saline before injection. Intrathecal NOC-12 produced curtailment of the tail-flick latency in a dose-dependent manner. Data represent the mean ± S.E.M. $^{**}p < 0.01$ vs. saline-treated groups. $^{††}p < 0.01$ compared with pre-examination (0 min) value in the same group.

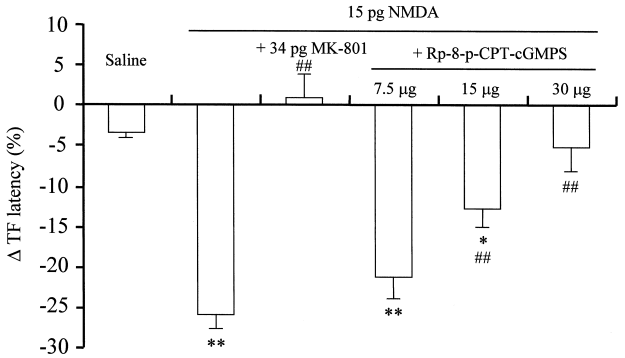

Fig. 3. Effects of MK-801 and Rp-8-p-CPT-cGMPS on NMDA-induced facilitation of the tail-flick latency when tested 30 min after the intrathecal administration of 15 pg NMDA. Data represent the mean ± S.E.M. $^{**}p < 0.01$ or $^{*}p < 0.05$ vs. saline-treated groups. $^{\#\#}p < 0.01$ vs. NMDA-treated groups.

The intrathecal administration of NOC-12 produced a dose-dependent decrease of the tail-flick latency during the period from 15 to 90 min with a maximum effect at 60 min (the baseline tail flick latency was maximally reduced from 6.53 ± 0.28 to 4.65 ± 0.32 s with the use of 30 μg NOC-12, Fig. 1, $p < 0.01$). The maximal decreases in the tail-flick latency (%) after administration of 10, 20 and 30 μg of NOC-12 were 5.4%, 18.5% (Fig. 1, $p < 0.01$) and 24.9% (Fig. 1, $p < 0.01$), respectively, compared to control (saline-treated group). The hyperalgesia evoked by NOC-12 was no longer observed 120 min after intrathecal injection.

Three doses of Rp-8-p-CPT-cGMPS (7.5, 15 and 30 μg) given alone had no significant effect on the baseline tail-flick latency between 15 and 120 min after administration ($p > 0.05$). However, pretreatment (10 min prior) with two high doses of Rp-8-p-CPT-cGMPS (15 and 30 μg) significantly blocked the NOC-12-induced decrease in the tail-flick latency when tested 30 and 60 min after the administration of 30 μg of NOC-12 (Fig. 2, $p < 0.01$), although a low dose of Rp-8-p-CPT-cGMPS (7.5 μg) did not influence the hyperalgesia induced by NOC-12 (Fig. 2, $p > 0.05$).

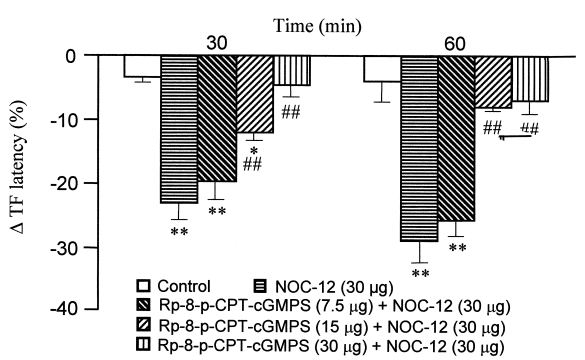

Fig. 2. Effect of intrathecally administered Rp-8-p-CPT-cGMPS on NOC-12-induced facilitation of the tail-flick latency when tested 30 and 60 min after the intrathecal administration of NOC-12. Data represent the mean ± S.E.M. $^{**}p < 0.01$ or $^{*}p < 0.05$ vs. saline control groups. $^{\#\#}p < 0.01$ vs. NOC-12-treated groups.

Consistent with the previous results (Meller et al., 1992a,b; Siegan and Sagen, 1995), intrathecal administration of NMDA induced a significant facilitation of the tail-flick reflex (the baseline tail-flick latency was reduced from 6.58 ± 0.57 to 4.88 ± 0.41 s, Fig. 3, $p < 0.01$). The NMDA-produced facilitation of the tail-flick reflex was not only completely abolished by prior treatment with NMDA receptor antagonist, MK-801 (Fig. 3, $p < 0.01$), but also dose-dependently blocked by prior administration with Rp-8-p-CPT-cGMPS. Rp-8-p-CPT-cGMPS given at 15 and 30 μg dramatically suppressed the NMDA-induced decrease of the tail-flick latency by 13.3% (Fig. 3, $p < 0.01$) and 20.7% (Fig. 3, $p < 0.01$), respectively. Rp-8-p-CPT-cGMPS at 7.5 μg failed to produce significant effect on the NMDA-evoked facilitation of the tail-flick reflex (Fig. 3, $p < 0.05$).

Subjective observation of rats injected with Rp-8-p-CPT-cGMPS, NOC-12, Rp-8-p-CPT-cGMPS + NOC-12, Rp-8-p-CPT-cGMPS + NMDA and MK-801 + NMDA revealed no obvious changes in animal behavior during a period of 2 h when compared with control animals, Although intrathecal administration of NMDA to some rats produced a caudally directed biting and scratching behaviors. No sedative or toxic effect was observed after intrathecal administration of any of the agents used in this study.

4. Discussion

Two isoenzymes of cGMP-dependent protein kinase have been recognized in mammals, cytosolic cGMP-dependent protein kinase I and membrane-bound cGMP-dependent protein kinase II. Furthermore, cGMP-dependent protein kinase I has been shown to exist in two isoforms,

designated Iα and Iβ (Lincoln and Cornwell, 1993). Our previous study showed that only abundant cGMP-dependent protein kinase Iα was detected in the superficial laminae at the different spinal levels, while cGMP-dependent protein kinase Iβ was absent or present at extremely lower levels in the spinal cord (Tao et al., 2000). The distribution of cGMP-dependent protein kinase Iα was similar to that of the substances related to the processing of nociceptive information in the spinal cord (Rustioni and Weinberg, 1989). This suggests that cGMP-dependent protein kinase Iα has important implications for the mechanisms of nociceptive processing at the spinal cord level. Sluka and Willis (1997) reported that the mechanical allodynia induced by capsaicin could be reversed by KT5823, a selective cGMP-dependent protein kinase but not selective cGMP-dependent protein kinase isoform inhibitor. Rp-8-p-CPT-cGMPS is a novel and selective cGMP-dependent protein kinase Iα isoform inhibitor without effects on cAMP-dependent protein kinase or cGMP-regulated phosphodiesterases (Butt et al., 1994). The present behavioral experiments indicated that intrahethecally administrated Rp-8-p-CPT-cGMS significantly and dose-dependently blocked NMDA- or NO-induced facilitation of the tail-flick reflex. To our knowledge, this is the first report to demonstrate the direct involvement of endogenous cGMP-dependent protein kinase Iα in spinal thermal hyperalgesia. Interestingly, the doses of Rp-8-p-CPT-cGMPS used in the present study, while completely blocking NMDA- or NO-induced facilitation of the tail-flick reflex, did not alter baseline tail-flick latency. This different effect on baseline and facilitation of the tail-flick reflex also occurred in NMDA receptor antagonists (DL-5-aminophosphonovaleric acid, AP5) and inhibitors of NOS (N^G-nitro-L-arginine methyl ester) and soluble guanylate cyclase (Methylene blue), all of which only blocked facilitation of thermal reflexes produced by NMDA (Meller et al., 1992a). These data above indicate that the neurotransmitters of primary afferents responsible for the reflex response to heat do not act on the activation of the NMDA receptors, neuronal NOS, soluble guanylate cyclase and cGMP-dependent protein kinase Iα in the spinal cord, but facilitation of the reflex response to heat (thermal hyperalgesia) induced by NMDA requires the production of endogenous NO and cGMP and the activation of cGMP-dependent protein kinase Iα. There is evidence that NMDA-induced activation of the NO/cGMP signaling pathway only occurred with the activation of the NMDA receptors but not other channels or receptors (Garthwaite et al., 1988; Bredt and Snyde, 1992; Kiedrowski et al., 1992). cGMP-dependent protein kinases have been found to serve as major effectors for the NO/cGMP signaling pathway in nervous system (Meller and Gebhart, 1993). The blockage of NMDA- or NO-induced thermal hyperalgesia by the cGMP-dependent protein kinase Iα inhibitor in the present study suggests that cGMP-dependent protein kinase Iα mediates the action of the NMDA receptors and NO/cGMP signaling pathway in spinal thermal hyperalgesia.

Several lines of previous evidence have shown the involvement of NMDA receptor and NO in multisynaptic nociceptive transmission and plasticity in the spinal cord. Intrathecal administration of NMDA or NO donors produced a marked hyperalgesia in thermal nociceptive tests (Aanonsen et al., 1990; Meller et al., 1992a,b; Inoue et al., 1997). Further, intrathecal injection of NMDA receptor antagonists or NOS inhibitors caused anti-nociception (Dickenson and Aydar, 1991; Meller et al., 1992a,b; Malmberg and Yaksh, 1993). Consistent with the previous studies above, the present study also showed that exogenous NMDA or NO could produce a facilitation of tail-flick reflex in the rats. More importantly, the previous data do strongly suggest that NMDA-produced facilitation of thermal reflexes is mediated through activation of NMDA receptors resulting in the production of NO and activation of soluble guanylate cyclase (Meller et al., 1992 a,b). Combined with the role of cGMP-dependent protein kinase Iα in the NMDA- and NO-induced thermal hyperalgesia in the present results, it is possible that facilitation of thermal nociceptive reflexes produced by NMDA in the spinal cord may be the result of a cascade of events as follows: NMDA receptor activation increases intracellular Ca^{2+} content which activates the calmodulin site on neuronal NOS to produce NO from the amino acid precursor, L-arginine. NO then activates soluble guanylate cyclase to increase intracellular content of cGMP, which results in the activation of cGMP-dependent protein kinase Iα within the target cells.

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