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Intrathecal morphine-3-glucuronide-induced nociceptive behavior via *Delta-2* opioid receptors in the spinal cord

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ABSTRACT

Intrathecal (i.t.) injection of morphine-3-glucuronide (M3G), a major metabolite of morphine without analgesic actions, produces severe hindlimb scratching followed by biting and licking in mice. The M3G-induced behavioral response was inhibited dose-dependently by pretreatment with an antisera against dynorphin. However, the selective κ-opioid receptor antagonist, nor-BNI did not prevent the M3G-induced behavioral response. Dynorphin is rapidly degraded by a dynorphin-converting enzyme (cystein protease), to leucine-enkephalin (Leu-ENK). The M3G-induced behavioral response was inhibited dose-dependently by pretreatment with the antisera against Leu-ENK. We also showed that M3G co-administered with Leu-ENK-converting enzyme inhibitors, phosphoramidon and bestatin produced much stronger behavioral responses than M3G alone. Furthermore, the M3G-induced behavioral responses were inhibited dose-dependently by i.t. co-administration of the non-selective δ-opioid receptor antagonist, naltrindole or the selective δ_2-opioid receptor antagonist, naltriben, whereas the selective δ_1-opioid receptor antagonist, BNTX had no effect. An i.t. injection of M3G also produced a definite activation of ERK in the lumbar dorsal spinal cord. Western blotting analysis revealed that antisera against dynorphin, antisera against Leu-ENK, naltrindole or naltriben resulted in a significant blockade of ERK activation induced by M3G in the spinal cord. Taken together, these results suggest that M3G-induced nociceptive responses and ERK activation may be triggered via δ_2-opioid receptors activated by Leu-ENK, which is formed from dynorphin in the spinal cord.

1. Introduction

Morphine, with its potent analgesic property, has been widely used for the treatment of various types of acute pain and for the long-term treatment of severe chronic pain. However, the clinical use of morphine is complicated by unwanted side-effects, including a paradoxical increase in pain sensitivity (i.e., hyperalgesia and allodynia) (Arner et al., 1988; DeConno et al., 1991; Sakurada et al., 2005). These clinical observations have been confirmed in laboratory studies. At doses far higher than those required for antinociception, morphine injected intrathecally (i.t.) into the spinal subarachnoid space of mice produces a spontaneous vocalization/squeaking and agitation as well as hyperalgesia, allodynia and scratching, biting and licking compared with antinociception at low doses (Yaksh et al., 1986; Sakurada et al., 1996, 2005; Komatsu et al., 2007). Previous studies have also demonstrated that these pain-related behaviors evoked by i.t. high-dose morphine are not an μ opioid receptor-mediated event because behaviors evoked by i.t. high-dose morphine are not reversed by pretreatment with naloxone, an opioid receptor antagonist. Morphine is known to be metabolized by the conjugation of glucuronide to two major metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in humans (Boerner et al., 1975; Christrup, 1997). Most rodents do not form M6G but only form M3G (Handal et al., 2002; Lötsch, 2009). M6G has a high affinity for the μ-opioid receptor and appears to be a more potent opioid agonist than morphine. In contrast, M3G does not bind to μ-, δ-, or κ-opioid receptors, NMDA, $GABA_A$ or glycine receptors and appears to be devoid of analgesic activity. However, despite these apparent lacks of activity, i.t. and intracerebroventricular (i.c.v.) administrations of M3G have been reported to evoke a range of excitatory behaviors in rodents (Smith, 2000; Komatsu et al., 2009a,b, Hemstapat et al., 2009). Despite the increasing amount of evidence for the involvement of M3G in morphine-induced nociceptive responses, very few studies have addressed the underlying signaling mechanism. In the present set of

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studies, we employed behavioral and biochemical approaches to examine the mechanism of i.t. M3G in morphine-induced nociceptive responses using specific components affecting the signaling pathway.

Elevation in spinal dynorphin content has also been observed in the opioid-induced pain state (Vanderah et al., 2000). Although dynorphin was originally identified as an endogenous κ-opioid receptor agonist and may act as an endogenous antinociceptive peptide under specific conditions, considerable evidence indicates that enhanced expression of spinal dynorphin is pronociceptive. Furthermore, i.t. administration of 15 nmol of dynorphin A(1–17), dynorphin A(2–17), or dynorphin A(2–13) in rats produced evoked allodynia. Similarly, dynorphin A(2–17) (3 nmol, i.t.) in mice also induced allodynia (Laughlin et al., 1997; Vanderah et al., 1996a). Pain-related behavior associated with nerve injury is also blocked by an antiserum to dynorphin (Bian et al., 1999; Malan et al., 2000; Wagner and Deleo, 1996; Wang et al., 2001).

Dynorphin-converting enzymes, belonging to the cysteine protease family, cleave dynorphin A and dynorphin B between Leu^5–Arg^6 and Arg^6–Arg^7 bonds, thereby generating leucine-enkephalin (Leu-ENK) and Leu-ENK-Arg, which are primarily active in δ-opioid receptors (Silberring et al., 1992). The i.t. administration of dynorphin A(1–17) also produces an anti-analgesic activity against morphine via activation of the δ_2-opioid receptor by the increased release of Leu-ENK in the spinal cord (Rady et al., 1999, 2001; Tseng et al., 1994). Furthermore, i.t. Leu-ENK in combination with peptidase inhibitors produces nociceptive behavior via activation of the glutamate receptor, which results in the release of nitric oxide via the δ_2-opioid receptor in the spinal cord (Komatsu et al., 2014). Thus, nociceptive behavior induced by M3G in morphine may potentially occur via δ_2-opioid receptors activated by Leu-ENK, which is formed from dynorphin in the spinal cord.

Extracellular signaling-regulated kinase (ERK) is activated in the dorsal spinal cord by nociceptive stimuli, including formalin, capsaicin or carrageenan injection (Galan et al., 2002; Ji et al., 1999; Karim et al., 2001). Inhibition of ERK signaling reduces nociceptive behavior after nociceptive stimuli, suggesting that ERK activation contributes to acute nociceptive processing in the spinal cord (Ji et al., 1999; Karim et al., 2001).

The purpose of the present research study was to determine whether δ_2-opioid receptor activation by Leu-ENK, which is formed from dynorphin, is involved in M3G-induced nociceptive behavior and ERK activation in the spinal cord.

2. Materials and methods

2.1. Animals

Pathogen-free adult male ddY-strain mice weighing an average of 24 g (Shizuoka Laboratory Center, Japan) were used in all experiments. The mice were maintained in a controlled 12 h light–dark cycle with food and water ad libitum. Room temperature and humidity were controlled at 22–24 °C and 50–60%, respectively.

2.2. Intrathecal injections

The i.t. injections were administered by percutaneous lumbar puncture through an intervertebral space at the level of the 5th or 6th vertebrae using the Hylden and Wilcox technique (Hylden and Wilcox, 1980). The drugs were administered i.t. in a volume of 5 μl with a 50-μl Hamilton microsyringe. A tail flick was used as an indication that the needle had penetrated the dura.

2.3. Behavioral experiments

Mice were acclimatized initially for 1 h in an individual plastic cage (22.0 × 15.0 × 12.5 cm) which also served as the observation chamber. The animals were challenged i.t. with M3G observed for 5 min. The observation of items of the induced behaviors was the total response time (s) of the following behaviors: hindlimb scratching, biting or licking of the hindpaw.

2.4. Drugs

The following drugs were used: M3G, bestatin, naltrindole, naltriben, *nor*-binaltorphimine dihydrochloride (nor-BNI), 7-benzylidenenaltrexone (BNTX), 4-(hydroxymercuri) benzoic acid sodium salt (PHMB) (Sigma Chemical Co., St. Louis, MO, USA), phosphoramidon (Nakalai tesq, Kyoto, Japan), dynorphin A antibody (Phenix Pharmaceutical, Inc., USA), Leucine-enkephalin polyclonal antibody (Millipore Corporation, USA), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126) (Calbiochem, Darmstadt, Germany). Monoclonal anti-phospho-p44/42 MAP kinase antibody and anti-p44/42 MAP kinase antibody were obtained from Cell Signaling Technology, Inc. U0126 was initially dissolved in 100% DMSO as stock solution, further diluted by artificial CSF and adjusted to 6.71% DMSO as the final concentration. The other drugs were dissolved in 50% dimethylsulfoxide (DMSO) to prepare the concentrated stock solution and working solutions were then diluted in artificial cerebrospinal fluid (CSF), containing NaCl 7.4 g, KCl 0.19 g, $MgCl_2$ 0.19 g and $CaCl_2$ 0.14 g/1000 ml of distilled and sterilized water, in a stepwise fashion. The highest concentrations of drugs used contained 0.9% and 1.4% DMSO, respectively. Low concentrations of DMSO resulted in no substantial effect on M3G-induced behavioral changes.

All antagonists were co-administered i.t. with M3G in a volume of 5 μl. Antiserum against dynorphin or leucine-enkephalin were injected i.t. 5 min prior to i.t. M3G.

2.5. Sample preparation

At 3 min after i.t. injection, the mice were decapitated and the entire spinal cord was obtained by pressure expulsion with physiological saline. The dorsal part of lumbar spinal cord was dissected quickly on an ice-cooled glass dish for Western blotting analysis.

2.6. Western blotting analysis

Tissue samples were homogenized in 0.1 ml of lysis buffer reagent (150 mM NaCl, 1.0% NP-40, 50 mM Tris–HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mM sodium vanadate and 1 mM EDTA pH 8.0) and centrifuged at 15,000 × *g* for 30 min at 4 °C. Supernatants were collected and total protein amounts were measured using the Protein Assay (BIO-RAD, Hercules, CA). An equal volume of 2× sample buffer (100 mM Tris–HCl pH 6.8, 2.5% SDS, 20% glycerol, 0.006% bromophenol blue and 10% β-mercaptoethanol) was added to 30 μg of total protein. The samples were boiled, electrophoresed in a 10% SDS-polyacrylamide gel (BIO-RAD, Hercules, CA) and then transferred to a Hybond-P membrane (Amersham Biosciences).

The blotted membrane was then incubated overnight with 5% skim milk (Wako Pure Chemical Industries, LTD, Osaka, Japan) in T-PBS (PBS containing 0.1% v/v Tween 20). All antibody applications were performed in T-PBS. After the membranes were washed, primary antibody incubations were performed for 2 h at room temperature using the appropriate dilutions (anti-phospho-p44/42 MAP kinase 1:1000 and anti-p44/42 MAPK antibody 1:1000). The membranes were extensively washed with T-PBS and incubated for 2 h with the secondary antibody (anti-rabbit or anti-mouse IgG peroxidase-conjugated antibody 1:5000) (Amersham Biosciences). After washing, the proteins were detected using the ECL-Plus Western blotting detection system (Amersham Biosciences) and visualized using the Dolphine-Chemi Image System (Wealtec). MagicMark western protein standard (Invitrogen) was simultaneously resolved on the gel, and the molecular weight of the proteins was estimated.