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Spinal ERK activation via NO–cGMP pathway contributes to nociceptive behavior induced by morphine-3-glucuronide

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ABSTRACT

Intrathecal (i.t.) injection of morphine-3-glucuronide (M3G), a major metabolite of morphine without analgesic actions, produces a severe hindlimb scratching followed by biting and licking in mice. The pain-related behavior evoked by M3G was inhibited dose-dependently by i.t. co-administration of tachykinin NK_1 receptor antagonists, sendide, [D-Phe^7, D-His^9] substance P(6-11), CP-99994 or RP-67580 and i.t. pretreatment with antiserum against substance P. The competitive NMDA receptor antagonists, D-APV and CPP, the NMDA ion-channel blocker, MK-801 or the competitive antagonist of the polyamine recognition site of NMDA receptor ion-channel complex, ifenprodil, produced inhibitory effects on i.t. M3G-evoked nociceptive response. The NO–cGMP–PKG pathway, which involves the extracellular signal-regulated kinase (ERK), has been implicated as mediators of plasticity in several pain models. Here, we investigated whether M3G could influence the ERK activation in the NO–cGMP–PKG pathway. The i.t. injection of M3G evoked a definite activation of ERK in the lumbar dorsal spinal cord, which was prevented dose-dependently by U0126, a MAP kinase-ERK inhibitor. The selective nNOS inhibitor N^{ω}-propyl-L-arginine, the selective iNOS inhibitor W1400, the soluble guanylate cyclase inhibitor ODQ and the PKG inhibitor KT-5823 inhibited dose-dependently the nociceptive response to i.t. M3G. In western blotting analysis, inhibiting M3G-induced nociceptive response using these inhibitors resulted in a significant blockade of ERK activation induced by M3G in the spinal cord. Taken together, these results suggest that activation of the spinal ERK signaling in the NO–cGMP–PKG pathway contributes to i.t. M3G-evoked nociceptive response.

1. Introduction

The intrathecal (i.t.) administration of morphine at doses far higher than those required for antinociception exhibited a nociceptive-related behavior consisting of scratching, biting and licking, hyperalgesia, allodynia, spontaneous vigorous agitation or vocalization in mice and rats [1–4]. Morphine is known to be metabolized by conjugation with glucuronide to two major metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide [5]. Morphine-6-glucuronide is an analgesic active metabolite, which has a relatively high affinity for the μ-opioid receptor [6,7]. The metabolite, M3G has been detected in concentrations more than twice that of the morphine administered systemically to rats and humans [8]. This metabolite is devoid of analgesic activity, which is consistent with its lack of binding to opioid receptors [8]. The i.t. and intracerebroventricular administration of M3G to rats, is known to have potent neuroexcitatory side effects, inducing myoclonus, allodynia, wild-running and seizures, which are independent of opioid mechanisms [2,3,8,9]. Despite the increasing amount of evidence for the involvement of M3G in morphine-induced nociceptive response, very few studies have addressed the molecular signaling mechanisms. In the present set of studies, we employed behavioral and molecular approaches to examine the mechanism of i.t. M3G-induced nociceptive response by using specific components affecting signaling pathway. Our previous research has demonstrated that high-dose morphine may stimulate a simultaneous release of substance P and glutamate from primary afferent terminals, which occurs secondary to nitric oxide (NO) production induced by nitric oxide synthase (NOS) [10]. The major action of NO is to activate the soluble form of the enzyme guanylate cyclase (sGC), which

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converts guanosine-5′-triphosphate (GTP) to cyclic guanosine-3′, 5′-monophosphate (cGMP) [11]. The function of NO is also expressed through the production of cGMP and activation of cGMP-dependent protein kinase (PKG) [12]. The NO–cGMP pathway and PKG activation in the spinal cord may play an important role in the modulation of nociceptive transmission [12–18].

Extracellular signaling-regulated kinase (ERK) is believed to be a member of the mitogen-activated protein kinase family, which is activated in the dorsal spinal cord by nociceptive stimuli like formalin, capsaicin or carageenan injection [19–21]. Inhibitor of ERK signaling reduces a short-lived nociceptive behavior after formalin or capsaicin administration, suggesting that ERK activation contributes to acute nociceptive processing in the spinal cord [19,20].

In the present study, we investigated whether activation of the spinal ERK pathway contributes to M3G-induced behavioral response. In addition, we sought to determine whether the NO–cGMP–PKG–ERK pathway in the spinal cord is involved in elicitation of M3G-induced nociceptive behavior.

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 (light 8:00 a.m. to 8:00 p.m.) with food and water ad libitum. All experiments were performed from 9:00 a.m. to 6:00 p.m. Room temperature and humidity were controlled at 22–24 °C and 50–60%, respectively.

2.2. Intrathecal injections

The i.t. injections were given by percutaneous lumbar puncture through an intervertebral space at the level of the 5th or 6th vertebrae by the Hylden and Wilcox technique [22]. The drugs were administered i.t. in a volume of 5 μl using a 50 μl Hamilton microsyringe. A flick of the tail was used as an indication that the needle had penetrated the dura.

2.3. Behavioral experiments

Mice were acclimatized for an initial 1 h in an individual plastic cage (22.0 cm × 15.0 cm × 12.5 cm) which also served as the observation chamber. The animals were challenged i.t. with morphine or M3G, and individually observed for 5 min except in the time course experiments. The observation of items of the induced behaviors was the total response time (s) of the following behaviors: hindlimb scratching, biting or licking of hindpaw. The latency to induce the first scratching behavior after i.t. injection of M3G was also recorded.

2.4. Drugs

The following drugs were used: M3G, naloxone hydrochloride, D(−)-2-amino-5-phosphonovaleric acid (D-APV), 3-((+)2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate (MK-801), ifenprodil (Sigma Chemical Co., St. Louis, MO, USA), L-NAME (Nakalai tesq, Kyoto, Japan), 1-(2-trifluoromethylphenyl) imidazole (TRIM), N^{ω}-propyl-L-arginine, W1400 (Tocris Cookson, Bristol, UK), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126), ODQ and KT5823 (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. Sendide and [D-Phe^7, D-His^9] substance P(6-11) were synthesized by solid phase methodology [23]. CP-99,994 (+)-[2S,3S]-3-(2-methoxy-benzylamino)-2-phenylpiperidine] and CP-100,263 (−)-[(2R,3R)-3-(2-methoxy-benzyl-amino-2-phentlpiperidine) were obtained through courtesy of Pfizer Pharmaceuticals. RP-67580 {2-[1-imino-2-(2-methoxyphenyl)rthyl-7,7-diphenyl-4-perhydroisoindolone(3a,7aR) was a generous gift from Rhone-Poulenc Rorer (Vitry sur Seine, France). RP-67,580, ifenprodil, L-NAME, TRIM, N^{ω}-propyl-L-arginine, W1400, and ODQ were dissolved in 50% dimethylsulfoxide (DMSO) for preparing 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. U0126 and KT5823 were 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 and substance P antiserum were dissolved in artificial CSF. Substance P antiserum was obtained from rabbits by repeated intradermal injection of SP, which was coupled to bovine serum albumin by glutaraldehyde as reported elsewhere [23]. The 5 μl of 6.71% DMSO used as vehicle control for i.t. injection gave no significant effect on M3G-induced behavior when compared to artificial CSF control. All antagonists were co-administered i.t. with M3G in a volume of 5 μl. Naloxone was injected subcutaneously in a volume of 0.1 ml/10 g body weight 15 min before i.t. injection of M3G. U0126 or antiserum against substance P was 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 whole spinal cord was taken by pressure expulsion with physiological saline. The dorsal part of lumbar spinal cord was dissected quickly on an ice-cooled glass dish for western blot analysis. L4–S1 lumbar spinal cord sections were dissected out and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 days and then transferred to maintenance solution of 15% sucrose in 0.01 M phosphate buffered saline, pH 7.4 at 4 °C for immunohistochemistry.

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 16,000 × *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, then electrophoresed in a 10% SDS-polyacrylamide gel (BIO-RAD, Hercules, CA) and 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 Phosohate-buffered saline (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl pH 7.5) with 0.1% (v/v) Tween 20 (T-PBS). All antibody applications were done 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 antibody 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 with the Dolphine-Chemi Image System (Wealtec). MagicMark western protein standard (Invitrogen) was simultaneously resolved on the gel, and the molecular weight of protein was estimated.

2.7. Immunohistochemical analysis

Lumbar spinal cord sections were embedded in Tissue Mount (Shiraimatsu Co., Ltd.) and stored at −80 °C. L4–L5 spinal sections were cut on a freezing microtome at either 20 μm thickness, collected in the maintenance solution, and stored until stained. The sections were incubated 0.5% H_2O_2 solution in T-PBS for 30 min and then blocked in 5% normal goat serum with T-PBS for 1 h. All antibodies were diluted in T-PBS. Sections were then incubated at room temperature for 2 h in anti-phospho-p44/42 MAP kinase (1:100 dilution in T-PBS; Cell Signaling Technology). Following the antibody incubation, the sections were rinsed with T-PBS, followed by incubation in a secondary biotinylated anti-rabbit IgG antibody for 2 h (1:2000 dilution in T-PBS; ABC kit; Vector Laboratories, Burlingame, CA). The sections were again rinsed with T-PBS, followed by incubation in the avidin–biotin complex (ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. They were then rinsed in T-PBS and then in phosphate buffer and stained with 3,3′-diaminobenzidine tetrahydrochroride (DAB, Sigma) solution (0.025% DAB in phosphate buffer containing 0.0005% H_2O_2; Sigma). A brown reaction product appeared after about 5–15 min, at which time the reaction was terminated by transferring the sections to phosphate buffer. Sections were mounted on glass slides, dried, dehydrated, coverslipped with NEW MX mounting medium (Matsunami-glass.co.jp) and observed for phospho-ERK staining.

2.8. Analyses of data

Statistical analyses of the results were performed using the Dunnett's test for multiple comparison, after analysis of variance (ANOVA). Differences were considered to be significant if $P < 0.05$. All values are expressed as mean ± S.E.M.

3. Results

3.1. Scratching, biting and licking induced by i.t. morphine and M3G

We first compared the potency of the behavioral activation of i.t. administered morphine and M3G (Fig. 1A and B). The behavioral response consisting scratching, biting and licking was induced dose-dependently by i.t. injection of M3G (1.0–3.0 nmol), which peaked at 0–5 min and disappeared afterward. The time course effect of M3G was similar to that of morphine. M3G was much more potent in inducing the behavioral response than morphine. The latency to elicit the first scratching after i.t. M3G (3.0 nmol) was 21.4 ± 3.2 s. Pretreatment with naloxone (1.0 and 4.0 mg/kg, s.c.), an opioid receptor antagonist, failed to reverse the behavioral response to i.t. M3G; the response time of scratching, biting and licking were unaffected by pretreatment with naloxone (saline plus M3G: 55.3 ± 3.2 s; naloxone 1.0 mg/kg plus M3G: 49.6 ± 3.2 s; naloxone 4.0 mg/kg plus M3G: 47.3 ± 4.7 s; $n = 10$).

3.2. Effects of tackykinin NK_1 receptor antagonists, and antiserum against substance P on i.t. M3G-induced behavioral response

M3G-induced behavioral response was inhibited dose-dependently by co-administration of peptidic antagonists for NK_1 receptors, sendide (0.125–0.5 pmol) and [D-Phe^7, D-His^9] substance P(6-11) (0.5–2.0 nmol) (Fig. 2A and B). The non-peptidic antagonists CP-99,994 (0.1–1.6 nmol) and RP-67,580 (0.5–2.0 nmol) also resulted in a dose-dependent inhibition of M3G-induced nociceptive response (Fig. 2C and D). Treatment with CP-100,263, an enantiomer of CP-99,994, did not prevent the behavioral response induced by i.t. M3G (Fig. 2C). Antiserum against substance P, injected i.t. 5 min prior to M3G, inhibited i.t. M3G-induced behavioral response in a dilution-related manner (Fig. 2E).

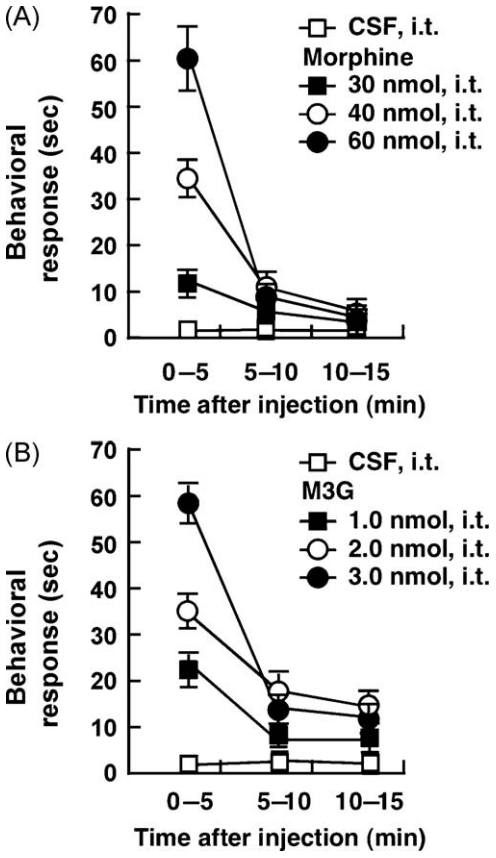

Fig. 1. Time course of scratching, biting and licking response induced by i.t. injection of morphine (A) or morphine-3-glucuronide (M3G) (B) in mice. The duration of scratching, biting and licking response induced by morphine or M3G was measured every 5 min for the 15-min period starting immediately after i.t. injection. Each value represents the mean ± S.E.M. of 10 mice in each group.

3.3. Effects of NMDA receptor antagonists on i.t. M3G-induced behavioral response

M3G-induced response was inhibited dose-dependently by co-administration of the competitive NMDA antagonists, D-APV (6.25–50.0 pmol) and CPP (3.125–25.0 pmol) (Fig. 3A and B). Similarly, MK-801 (74.1–250.0 pmol), a non-competitive NMDA antagonist, and ifenprodil (111.1–375.0 pmol), a competitive antagonist of the polyamine recognition site of NMDA receptor ion-channel complex, showed a dose-dependent inhibition of i.t. M3G-evoked nociceptive response (Fig. 3C and D).

3.4. Effects of NOS inhibitors on i.t. M3G-induced behavioral response

The non-selective NOS inhibitor L-NAME (20–80 nmol) produced a dose-dependent inhibition of i.t. M3G-induced behavioral response (Fig. 4A). TRIM (6.67–22.5 nmol), a relatively selective nNOS inhibitor, and N^{ω}-propyl-L-arginine (5.0–15.0 nmol), a

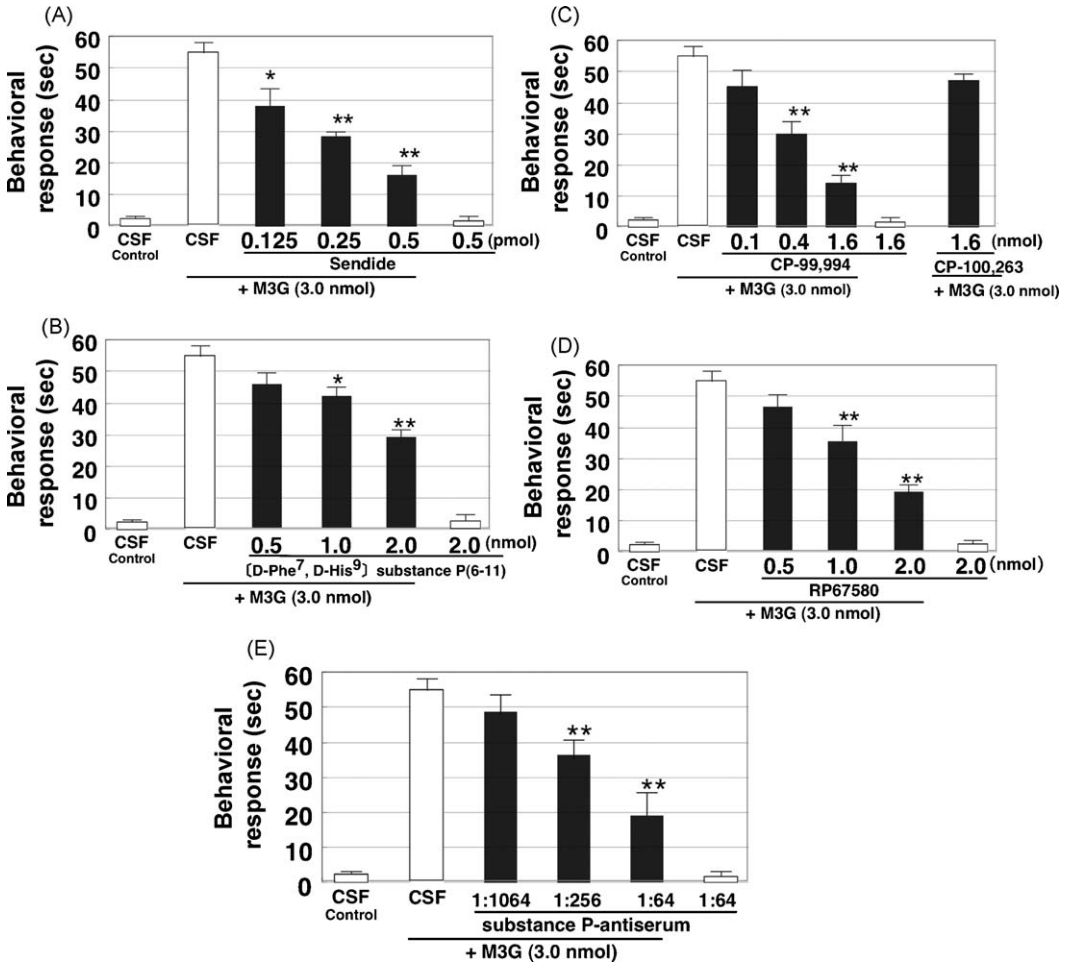

Fig. 2. Effects of sendide (A), [D-Phe^7, D-His^9] substance P(6-11) (B), CP-99,994 and CP-100,263 (C), RP67580 (D) and substance P-antiserum (E) on M3G-induced scratching, biting and licking response in mice. Each antagonist was co-administered i.t. with M3G in a total volume of 5 μl. The antiserum against substance P was pre-injected i.t. 5 min prior to i.t. injection of M3G (3.0 nmol). First (antiserum against substance P) and second (M3G) injections were done a volume of 5 μl, separately. The duration of scratching, biting and licking response was determined over a 5 min period starting immediately after i.t. injection. Each value represents the mean ± S.E.M. of 10 mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

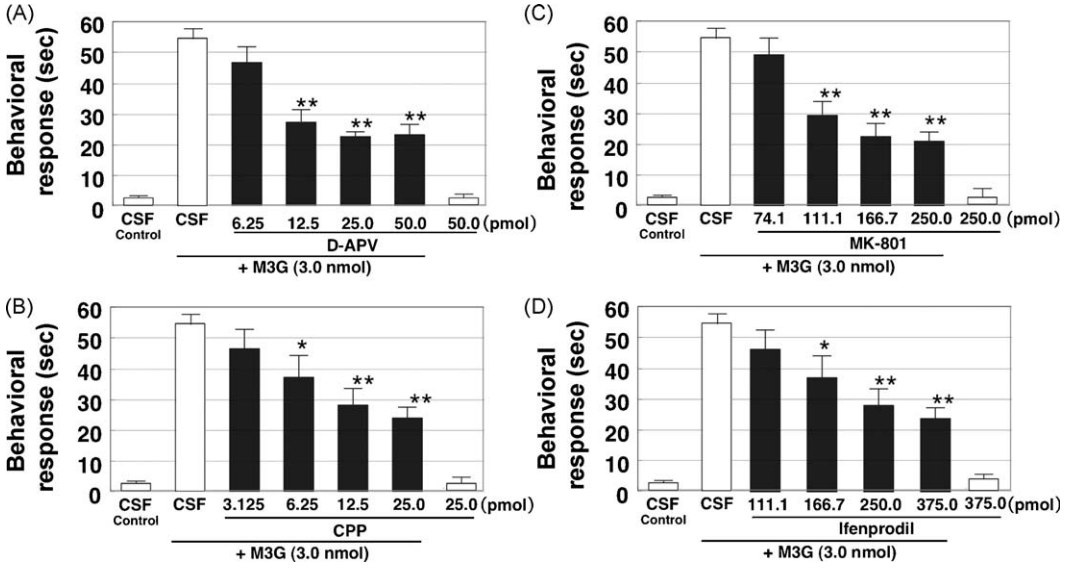

Fig. 3. Effects of D-APV (A), CPP (B), MK-801 (C) and ifenprodil (D) on M3G-induced scratching, biting and licking response in mice. Each antagonist was co-administered i.t. with M3G in a total volume of 5 μl. The duration of scratching, biting and licking response induced by M3G was determined over a 5 min period starting immediately after i.t. injection. Each value represents the mean ± S.E.M. of 10 mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

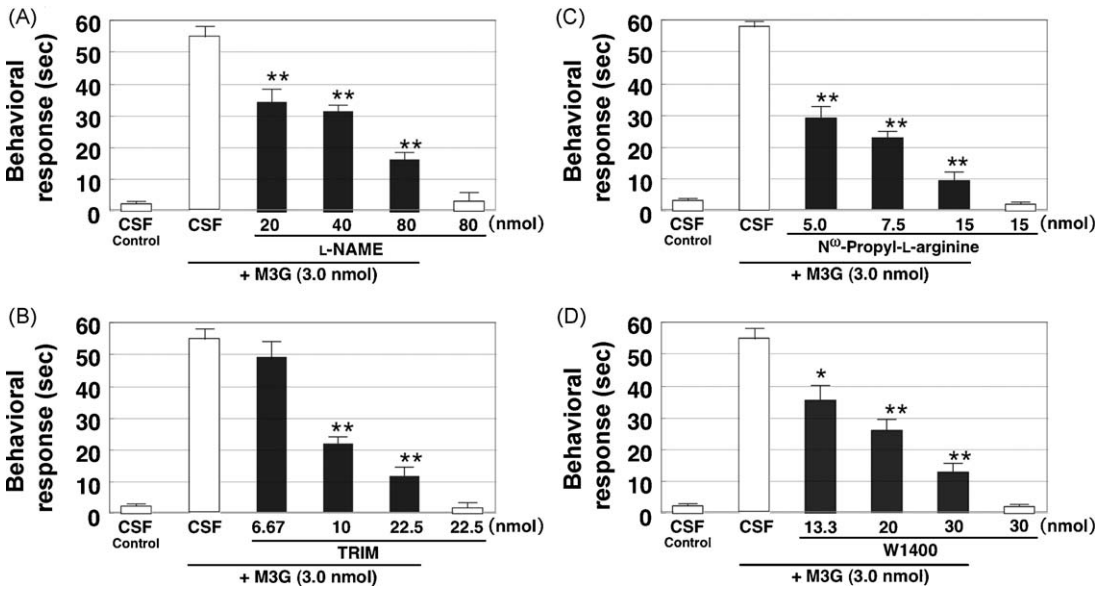

Fig. 4. Effects of L-NAME (A), TRIM (B), N^{ω}-propyl-L-arginine (C) and W1400 (D) on M3G-induced scratching, biting and licking response in mice. Each inhibitor was co-administered i.t. with M3G in a total volume of 5 μl. The duration of scratching, biting and licking response induced by M3G was determined over a 5 min period starting immediately after i.t. injection. Each value represents the mean ± S.E.M. of 10 mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

selective nNOS inhibitor, dose-dependently inhibited a series of scratching, biting and licking responses to i.t. M3G (Fig. 4B and C). The selective iNOS inhibitor W1400 (13.3–30 nmol) also attenuated a series of scratching, biting and licking response to i.t. M3G in a dose-dependent way (Fig. 4D).

3.5. Effects of a soluble guanylate cyclase inhibitor or a protein kinase G inhibitor on i.t. M3G-induced behavioral response

The soluble guanylate cyclase inhibitor ODQ (2.5–5.0 pmol) produced a dose-dependent inhibition of the behavioral response to i.t. M3G (Fig. 5A). KT-5823 (2.0–8.0 nmol), a protein kinase G inhibitor, also dose-dependently attenuated a series of scratching, biting and licking response evoked by i.t. M3G (Fig. 5B).

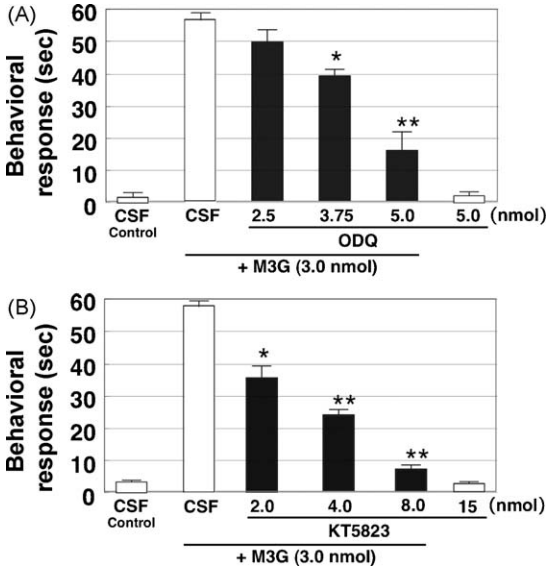

Fig. 5. Effects of ODQ (A) and KT5823 (B) on M3G-induced scratching, biting and licking response in mice. Each inhibitor was co-administered i.t. with M3G in a total volume of 5 μl. The duration of scratching, biting and licking response induced by M3G was determined over a 5 min period starting immediately after intrathecal injection. Each value represents the mean ± S.E.M. of 10 mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

3.6. Activation of ERK in dorsal spinal cord after i.t. injection of M3G

We further examined whether spinal ERK is activated by i.t. injection of M3G in the lumbar dorsal cord. In the immunohistochemical studies, increased numbers of phospho-ERK immunoreactive cells in the dorsal horn of the lumbar spinal cord were observed at 3 min post-i.t. M3G, as compared to post-i.t. artificial CSF (Fig. 6A and B). The activation of ERK in the lumbar dorsal cord was also quantified by Western blot assay (Fig. 6C and D). We compared the effect of M3G (2.0–3.0 nmol) with that of the artificial CSF-treated control in the lumbar dorsal cord extracted 3 min after i.t. injection. Western blot assay further revealed that 2.0 nmol of M3G increased significantly phospho-ERK2 expression, but had no significant effect on the phospho-ERK1 expression. A higher dose (3.0 nmol) of M3G showed a significant elevation of both phospho-ERK1 and phospho-ERK2 when compared to the artificial CSF-treated control (Fig. 6D). Numerous phospho-ERK immunoreactive cells were found in the superficial dorsal horn with only a few cells in the deep dorsal horn.

3.7. Effects of the MEK inhibitor on ERK activation in the dorsal spinal cord and on nociceptive response after i.t. injection of M3G

We tested whether upstream effectors of the ERK were necessary for M3G-induced nociceptive response because MEK (MAP kinase kinase) is required for ERK activation. Previous work has shown that the MEK inhibitor U0126 is able to block the high-dose i.t. morphine-induced nociceptive response [10]. The MEK inhibitor U0126 (5.0 nmol) reduced a significant ERK activation 3 min after i.t. M3G (3.0 nmol) (Fig. 7A). The i.t. injection of M3G alone or in combination with U0126 had no effect on the spinal total ERK expression (Fig. 7A). In the behavioral test, i.t. injection of U0126 (1.25–5.0 nmol) caused a dose-dependent inhibition of M3G (3.0 nmol)-induced nociceptive response when compared with the artificial CSF-treated controls (Fig. 7B). These results

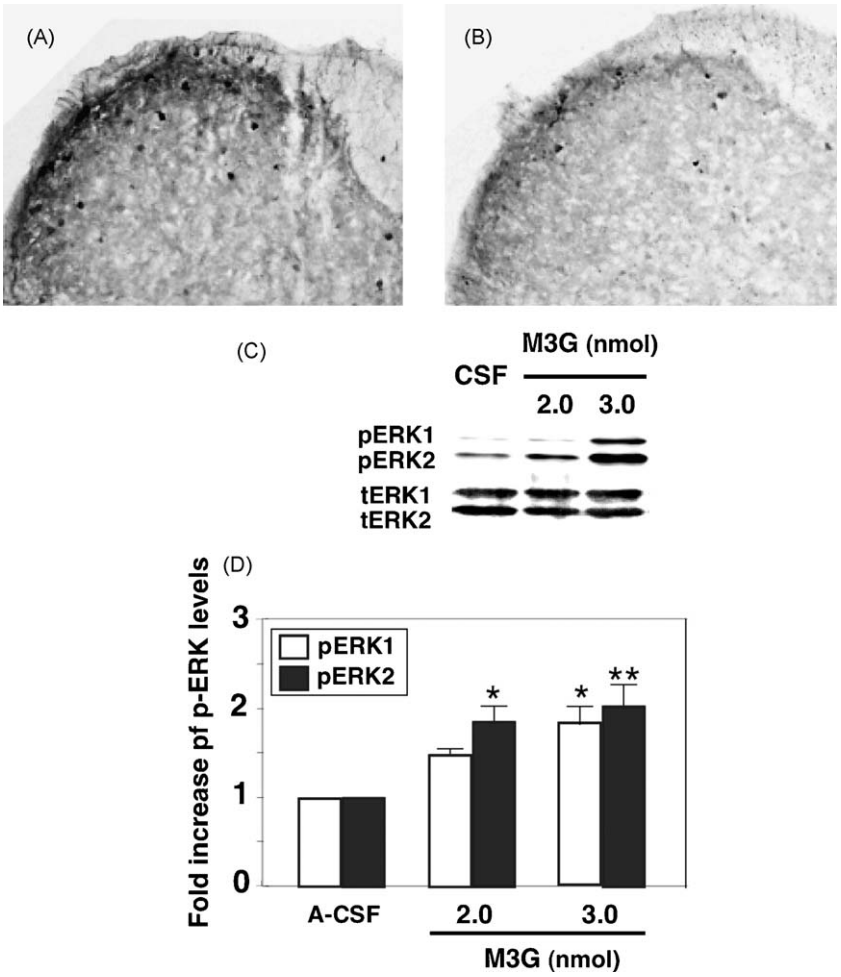

Fig. 6. Immunohistochemistry with phospho-ERK (pERK) antibody in the lumbar spinal cord. (A and B) An increased number of pERK immunoreactive cells are observed in the lumbar spinal cord 3 min after i.t. injection of M3G (A) as compared to A-CSF control (B). (C) Western blots of the dorsal spinal homogenates for pERK and total-ERK (tERK). Western blot analysis reveals a marked increase in the levels of both pERK1 (p44MAPK) and pERK2 (p42MAPK) in the dorsal spinal cord after i.t. injection of M3G. Dorsal spinal cord samples were taken 3 min after i.t. injection of M3G. tERK was used as a loading control. (D) Quantification of pERK1 and pERK2 levels in the dorsal spinal cord. Each value represents the mean ± S.E.M. of four mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

further confirm that activation of the ERK signaling pathway mediates i.t. M3G-induced nociceptive response.

3.8. Effects of the NOS inhibitors, the guanylate cyclase inhibitor and the PKG inhibitor on M3G-induced ERK activation

To test the hypothesis that nNOS and iNOS by increased releases of neurotrasmitters/neuromodulators from the primary afferents is necessary for upstream activators of phospho-ERK, N^{ω}-propyl-L-arginine, an nNOS inhibitor or W1400, an iNOS inhibitor was coinjected with M3G (3.0 nmol) was co-injected i.t. with M3G. Correlated with behavioral results (Fig. 4C), i.t. N^{ω}-propyl-L-arginine significantly inhibited the increase of phospho-ERK1 and 2 in the dorsal spinal cord of i.t. M3G-treated mice (Fig. 8A). The i.t. injection of the iNOS inhibitor W1400 at a dose of (30 nmol) that affected behavior significantly suppressed the increase of phospho-ERK2 in the dorsal spinal cord after M3G injection, whereas the phospho-ERK1 level was unchanged (Fig. 8B). A sample blot is shown in Fig. 8A and B. To further investigate whether spinal ERK activation by i.t. injection of M3G is specifically mediated via NO–cGMP–PKG signaling pathway, ODQ, a selective inhibitor of NO-sensitive soluble guanylate cyclase, or KT-5823, an inhibitor of PKG was co-injected i.t. with M3G (3.0 nmol). Both i.t. ODQ and KT-5823 were very effective in blocking the phospho-ERK1 and 2 in the dorsal spinal cord of i.t. M3G-treated mice (Fig. 8C and D).

4. Discussion

The present results demonstrate for the first time that i.t. injection of M3G could evoke a series of scratching, biting and licking indicative of nociceptive responses, and the activation of the NO–cGMP–PKG–ERK may contribute to M3G-induced nociceptive response in mice.

Behavioral studies have shown that i.t. morphine in a dose of 60 nmol into the spinal subarachnoid space can evoke a spontaneous nociceptive syndrome consisting of scratching, biting and licking which are independent of opioid mechanisms [1,24]. Through similar behavior response was also induced by i.t. injection of M3G, nociceptive behavioral response of M3G (3.0 nmol) was much more potent than that of morphine (60 nmol). This is consistent with previously reported results that i.t. M3G is more potent in evoking hyperalgesia and allodynia than morphine in rats [2,9]. Of particular interest in the present study is that the latency of scratching behavior induced by i.t. M3G was later (approximately 20 s) than that of substance P (8.8 ± 1.9 s), but not morphine (60 nmol); the response to i.t. morphine occurred in over 60 s following i.t. injection [1]. M3G concentration in the

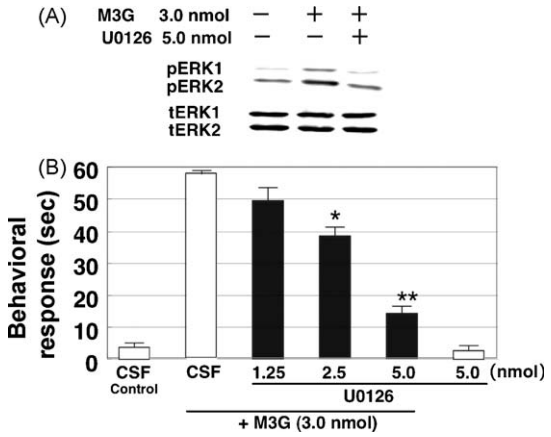

Fig. 7. Effect of U0126, a MEK inhibitor, on spinal ERK activation and nociceptive response induced by i.t. injection of M3G. (A) Western blots of the dorsal spinal homogenates for phospho-ERK (pERK) and total-ERK (tERK). Dorsal spinal cord samples were taken 3 min after i.t. injection of M3G. tERK was used as a loading control. U0126 (5.0 nmol) was given i.t. 5 min prior to i.t. M3G. This western blot is representative of four independent experiments. (B) Inhibition of M3G-induced behavioral response by U0126 (1.25–5.0 nmol). The duration of scratching, biting and licking response induced by M3G were determined over a 5 min period starting immediately after i.t. injection. Each value represents the mean ± S.E.M. of 10 mice in each group. Statistically significant difference compared with M3G alone is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$.

cerebrospinal fluid has been found to detect more than half of morphine administered systemically to rats and humans [8]. Furthermore, it is shown that morphine can be metabolized in vitro to its 3- and 6-glucronides by the human brain homogenate [25]. Taken together, it is therefore plausible that nociceptive responses evoked by high-dose i.t. morphine may result from an increasing accumulation of the morphine metabolite M3G in the spinal cord.

We have confirmed in the present study that there was a pharmacological characteristics of M3G-induced behavioral response; M3G-induced nociceptive response was inhibited by i.t. co-administration of peptidic and non-peptidic antagonists for NK_1 receptors, sendide, [D-Phe^7, D-His^9] substance P(6-11), CP-99,994 and RP-67,580, and i.t. pretreatment with substance P antiserum. M3G-induced scratching was similar to that seen after i.t. injection of substance P, which can be blocked by substance P antagonist [26]. These previously reported data led us to speculate that scratching behavior elicited by i.t. injection of M3G may be a syndrome indicating nociceptive stimulation as well as licking and biting of the hindquarters. i.t. co-administration of D-APV and CPP, competitive NMDA antagonists, MK-801, a non-competitive NMDA antagonist, and ifenprodil, a competitive antagonist of the polyamine recognition site of NMDA receptor ion-channel complex, inhibited M3G-induced nociceptive response. In view of these findings, it is possible to speculate that i.t. injection of M3G could release primary afferent neuromodulators/neurotransmitters such as substance P and glutamate, which could lead to the release of NO in the dorsal spinal cord. Indeed, i.t. injection of high-dose morphine in rats evokes a marked increase of glutamate release in the dorsal spinal cord extracellular fluid [4].

There is much evidence to suggest that NO is involved in the transmission of nociceptive information in the spinal cord [27]. Stimulation of primary afferent C-fibers with capsaicin or formalin could induce nociceptive responses with an increase of NO production in the spinal cord, which is blocked by L-NAME [1,4,28–30]. Development of hyperalgesia in models of chronic pain is also highly sensitive to NOS inhibitors, suggesting a pivotal role for NO in the transitions from acute to chronic pain. Our previous studies showed that the non-selective NOS inhibitors, L-NAME and TRIM or the selective nNOS inhibitor 7-nitroindazole, dose-dependently blocked high-dose i.t. morphine-induced nociceptive behavior [10]. Consistent with those results, besides L-NAME, TRIM and N^{ω}-propyl-arginine, the nNOS inhibitors dose-dependently reduced the nociceptive response to i.t. M3G. However, it should be noted that there was a difference of the inhibitory effect of W1400, an iNOS inhibitor, on high-dose morphine- and M3G-induced responses; W1400 inhibited M3G-induced response in a dose-dependent manner, wherease the iNOS inhibitor had a weak inhibition of morphine-induced response with a lack of dose-dependency [10]. The reason for this differential sensitivity of the iNOS inhibitor is unclear largely. These findings obtained by NOS inhibitors suggest that M3G could activate indirectly both iNOS as well as nNOS, possibly through an increased release of substance P and glutamate in the dorsal spinal cord.

Based on our previous results of high-dose i.t. morphine [1,4,10], a similar phenomenon could, in part, explain i.t. M3G-induced nociceptive response; Substance P and glutamate released from presynaptic sites in response to i.t. M3G could activate NK_1 and NMDA receptors, which could trigger a feed forward mechanism of stimulation of nNOS activity via mechanism largely dependent on Ca^{2+}. Increases in intracellular Ca^{2+} either by extracellular Ca^{2+} influx through NMDA receptor or Ca^{2+} channels as well as release from intracellular Ca^{2+} stores via production of inositol-1,4,5-triphosphate after activation of G-protein-coupled NK_1 receptors will result in activation of nNOS in the dorsal horn [27,31]. The iNOS has also been demonstrated to be related to inflammatory and neuropathic pain. For example, the iNOS is upregulated in the spinal cord following intradermal capsaicin or carrageenan injection and in models of spinal cord injury [32–34], and a selective inhibitor of iNOS can block hyperalgesia [33,35]. iNOS is induced by agents such as various proinflammatory cytokines, independent of intracellular Ca^{2+} and induces NO for long periods [36,37]. Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α have been implicated in pain facilitation in the spinal cord. IL-1β, IL-6, and TNF-α μRNA and protein expression are elevated in the spinal cord in response to peripheral nerve injury, spinal nerve injury, or subcutaneous formalin and zymasan [38]. Behavioral studies have demonstrated that increased expression of iNOS stimulated by proinflammatory pathway in glia contributes significantly to the development and maintenance of hyperalgesia [33,39]. Glial activation and cytokine production are found to be induced by NO as well as substance P and glutamate [40–43]. These convergent results provide considerable support to hypothesis that increased releases of substance P and glutamate from presynaptic sites in response to M3G might activate both NK_1 and NMDA receptors, which trigger nNOS activation via a mechanism largely dependent on Ca^{2+}, and iNOS activation via a mechanism of NO-mediated glial activation/cytokine release through neuron signals.

Multiple transmitter receptors are coupled to ERK activation in the spinal cord: ionotropic NMDA and non-NMDA glutamate receptors, metabotropic glutamate receptor subtypes 1, and G-protein coupled NK_1 receptor [20,44]. Extracellular Ca^{2+} appears essential for noxious stimulation-induced ERK [45]. Recent evidence suggests a role of ERK phosphorylation in the nociceptive processing in the spinal cord [19,20,44]. In the present study, we found that i.t. injection of M3G evoked a strong activation of spinal phospho-ERK, which correlates with M3G-induced nociceptive behavior. Thus, spinal ERK activation by M3G appears to be related to nociceptive processing. Furthermore, reduction of i.t. M3G-induced behavioral response by the MEK inhibitor U0126 was accompanied by decreased ERK phosphorylation in the lumbar spinal cord. These results suggest that the signal transduction pathway through MEK and phospho-ERK in the lumbar spinal cord can be activated after i.t. injection of M3G. Overall, the data seem to suggest that the changes in phospho-ERK in the lumbar spinal cord

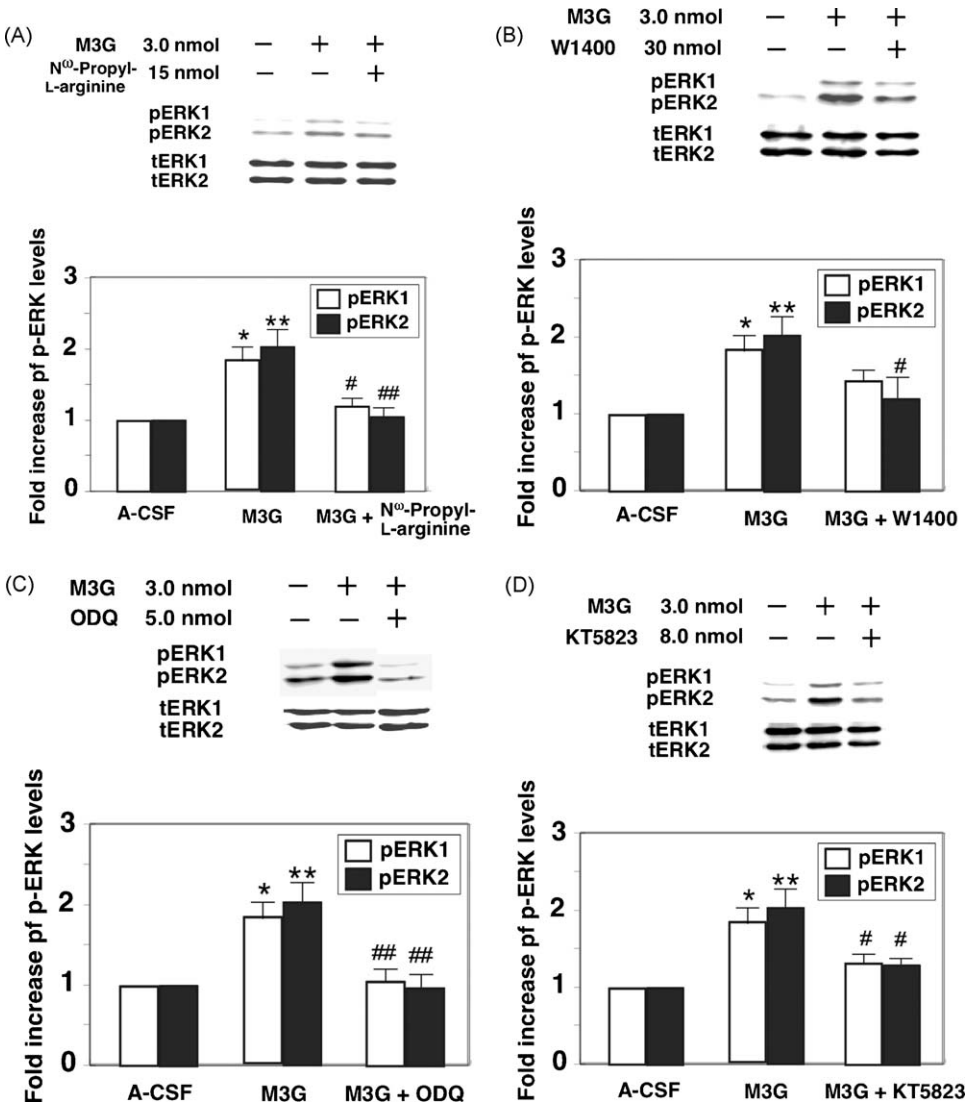

Fig. 8. Effect of N^{ω}-propyl-L-arginine (A), W1400 (B), ODQ (C), and KT5823 (D) on spinal ERK activation induced by i.t. injection of M3G. Western blots of the dorsal spinal homogenates for phospho-ERK (pERK) and total-ERK (tERK). Representative Western blots are shown on top, and quantification of pERK1 and pERK2 levels are shown in the bar graphs. Dorsal spinal cord samples were taken 3 min after i.t. injection of M3G. tERK was used as a loading control. N^{ω}-Propyl-L-arginine, W1400, ODQ, and KT5823 were co-administrated i.t. with M3G. Each value represents the mean ± S.E.M. of four mice in each group. Statistically significant difference compared with corresponding A-CSF controls is indicated by $^{**}P < 0.01$, $^{*}P < 0.05$. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$, compared to corresponding M3G alone.

might affect the sensitivity and consequently affect the nociceptive behavior.

Moreover, we have demonstrated that treatment with the nNOS inhibitor N^{ω}-propyl-L-arginine or the iNOS inhibitor W1400 could clearly block phospho-ERK. These results support a critical role of NO-ERK pathways in the establishment of M3G-induced nociception. Previous studies have shown that NO–ERK pathway activates in cGMP/PKG-dependent manner [46–48]. The content cGMP is higher in the spinal cord in chronic inflammatory and nerve-injury models [13,14]. Spinally delivered 8-bromo-cGMP, a membrane-permeable cGMP analog, facilitates nociceptive behavior [17,18]. PKG deficiency or spinally delivered PKG inhibitors reduce formalin-induced nociceptive behavior in rats or mice [12,15,17]. It is therefore likely that both cGMP and PKG act together to contribute to spinal nociceptive processing. NO-activated ERK in cGMP–PKG–MAPK pathway may also play an important role in long-term hyperexcitability of sensory neurons induced by peripheral nerve injury [49], and in the potentiation of long-term potentiation (LTP) in rat hippocampal and amygdala slices by 3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole (YC-1), a drug known to modulate the response of soluble guanyl cyclase to NO [50]. Results presented in this study show that the soluble guanylate cyclase inhibitor ODQ and the PKG inhibitor KT-5823 could clearly block ERK activation. These results raise the possibility that the NO–cGMP–PKG–ERK pathway in the spinal cord may be involved in the mechanism of M3G-induced nociceptive behavior.

Taken together with the results of the present study, it appears that M3G-induced ERK activation via the NO–cGMP–PKG mechanism translocates onto membrane and phospholylates ion channels or receptors, which increases membrane excitability and induces spinal neuronal sensitization. This may be mediated through phosphorylation of ion channels or receptors such as A-type potassium channel Kv4.2 [51,52], leading to increased neuronal excitability or to the trafficking of AMPA receptors from the cytoplasm to the subsynaptic membrane [51,53,54].

In conclusion, the current study suggests that nociception evoked by i.t. injection of M3G may be mediated by spinal NOS activity in the spinal cord. Further studies provide the first evidence of the clear relation between spinal NO–cGMP–PKG and

ERK activation in nociception evoked by i.t. M3G. This implies that inhibitors of the NO–cGMP–PKG–ERK pathway may provide a putative tool to control nociception associated with acutely administered high-dose morphine.

References

[1] Sakurada T, Wako K, Sakurada C, Manome Y, Tan-No K, Sakurada S, et al. Spinally-mediated behavioral responses evoked by intrathecal high-dose morphine: possible involvement of substance P in the mouse spinal cord. Brain Res 1996;724:213–21.

[2] Woolf FJ. Intrathecal high dose morphine produces hyperalgesia in the rat. Brain Res 1981;209:491–5.

[3] Yaksh TL, Harty GJ, Burton M, Onofrio BM. High doses of spinal morphine produce a nonopiate receptor-mediated hyperesthesia: clinical and theoretical implications. Anesthesiology 1986;64:590–7.

[4] Watanabe C, Sakurada T, Okuda K, Sakurada C, Ando R, Sakurada S. The role of spinal nitric oxide and glutamate in nociceptive behaviour evoked by high-dose intrathecal morphine in rats. Pain 2003;106:269–83.

[5] Boerner U, Abbott S, Roe RL. The metabolism of morphine and heroin in man. Drug Metab Rev 1975;4:39–73.

[6] Christensen CB, Jorgensen LN. Morphine-6-glucronide has high affinity for the opioid receptor. Pharmacol Toxicol 1987;60:75–6.

[7] Pasternak GW, Bodnar RJ, Clark JA, Intrurrisi CE. Morphine-6-glucuronide, a potent mu agonist. Life Sci 1987;41:2845–9.

[8] Smith MT. Neuroexcitatory effects of morphine and hydromorphine: evidence inmplicating the 3-glucuronide metabolites. Clin Exp Pharmacol Physiol 2000;27:524–8.

[9] Yaksh TL, Harty GJ. Parmacology of the allodynia in rats evoked by high dose intrathecal morphine. J Pharmacol Exp Ther 1988;244:501–7.

[10] Komatsu T, Sakurada C, Sasaki M, Sanai K, Tsuzuki M, Bagetta G, et al. Extracellular signal-regulated kinase (ERK) and nitric oxide synthase mediate intrathecal morphine-induced nociceptive behavior. Neuropharmacology 2007;52:1237–43.

[11] Qian Y, Chao DS, Santillano DR, Cornwell TL, Narin AC, Greengard P, et al. cGMP-dependent protein kinase in dorsal root ganglion: relationship with nitric oxide synthase and nociceptive neurons. J Neurosci 1996;16:3130–8.

[12] Schmidtko A, Ruth P, Geisslinger G, Tegeder I. Inhibition of cyclic guanosine 5'-monophosphate-dependent protein kinase I (PKG-I) in lumbar spinal cord reduces formalin-induced hyperalgesia and PKG upregulation. Nitric Oxide 2003;8:89–94.

[13] Garry MG, Richardson JD, Hargreaves KM. Carrageenan induced inflammation alters the content of i-cGMP and i-cAMP in the dorsal horn of the spinal cord. Brain Res 1994;646:135–9.

[14] Siegan JB, Hama AT, Sagen J. Alterations in rat spinal cord cGMP by peripheral nerve injury and adrenal medullary transplantation. Neurosci Lett 1996;215:49–52.

[15] Tao YX, Hassan A, Haddad E, Johns RA. Expression and action of cyclic GMP-dependent protein kinase Ialpha in inflammatory hyperalgesia in rat spinal cord. Neuroscience 2000;95:525–33.

[16] Tegeder I, Schmidtko A, Niederberger E, Ruth P, Geisslinger G. Dual effects of spinally delivered 8-bromo-cyclic guanosine mono-phosphate (8-bromo-cGMP) in formalin-induced nociception in rats. Neurosci Lett 2002;332:146–50.

[17] Tegeder I, Del-Turco D, Schmidtko A, Sausbier M, Feil R, Hofmann F, et al. Reduced inflammatory hyperalgesia with preservation of acute thermal nociception in mice lacking cGMP-dependent protein kinase I.. Proc Nati Acad Sci USA 2004;101:3253–7.

[18] Morita K, Kitayama T, Morioka N, Dohi T. Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice. Pain 2008;138:525–36.

[19] Ji RR, Baba H, Brenner GJ, Woolf CJ. Nociceptive specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 1999;2:1114–9.

[20] Karim F, Wang CC, Gereau RW. Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 2001;21:3771–9.

[21] Galan A, Lopez-Garcia JA, Cervero F, Laird JMA. Activation of spinal extracellular signaling-regulated kinase-1 and -2 by intraplantar carrageenan in rodents. Neurosci Lett 2002;322:37–40.

[22] Hylden JLK, Wilcox GL. Intrathecal morphine in mice: a new technique. Eur J Pharmacol 1980;67:191–4.

[23] Sakurada T, Manome Y, Tan-No K, Sakurada S, Kisara K, Ohba M, et al. A selective and extremely potent antagonist of the nerokinin-1 receptor. Brain Res 1992;593:319–22.

[24] Sakurada T, Watanabe C, Okuda K, Sugiyama A, Moriyama T, Sakurada C, et al. Intrathecal high-dose morphine induces spinally-mediated behavioral responses through NMDA receptors. Mol Brain Res 2002;98:111–8.

[25] Yamada H, Ishi K, Ishii Y, Ieiri I, Nishimoto S, Morioka T, et al. Formation of highly analgesic morphine-6-gluclonide following physiologic concentration of morphine in human brain. J Toxicol Sci 2003;28:395–401.

[26] Fasmer OB, Post C. Behavioral response induced by intrathecal injection of 5-hydrixytrypyamine in mice are inhibited by asubstance P antagonist, D-Pro^{2}, D-Trp^{7,9}-substance P. Neuropharmacology 1983;22:1397–400.

[27] Meller ST, Gebhart GF. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 1993;52:127–36.

[28] Wu J, Lin Q, McAdoo DJ, Willis WD. Nitric oxide contributes to central sensitization following intradermal injection of capsaicin. NeuroReport 1998;9:589–92.

[29] Sakurada T, Sugiyama A, Sakurada C, Tan-No K, Sakurada S, Kisara K, et al. Involvement of nitric oxide in spinally mediated capsaicin- and glutamate-induced behavioural responses in the mouse. Neurochem Int 1996;29:271–8.

[30] Sakurada C, Sugiyama A, Nakayama M, Yonezawa A, Sakurada S, Tan-No K, et al. Antinociceptive effect of spinally injected L-NAME on the acute nociceptive response induced by low concentrations of formalin. Neurochem Int 2001;38:417–23.

[31] Berridge MJ. Inositol trisphosphate and calcium signaling. Nature 1993;361:315–25.

[32] Wu J, Fang L, Lin Q, Willis WD. Nitric oxide synthase in spinal cprd cemtral sensitization following intradermal injection of capsaicin. Pain 2001;94:47–58.

[33] Osborne MG, Coderre TJ. Effect of intrathecal administration of nitric oxide synthase inhibitors on carrageenan-induced thermal hyperalgesia. Br J Pharmacol 1999;126:1840–6.

[34] Sakaue G, Shimaoka M, Fukuoka T, Hiroi T, Inoue T, Hashimoto N, et al. NF-Kappa B decoy suppresses cytokine expression and themal hyperalgesia in a rat neuropathic pain model. NeuroReport 2001;12:2079–84.

[35] Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF. The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 1994;33:1471–8.

[36] Xu J, Kim GM, Chen S, Yan P, Ahmed SH, Ku G, et al. iNOS and nitrotyrosine expression after spinal cord injury. J Neurotrauma 2001;18:523–32.

[37] Hamada Y, Ikata T, Katoh S, Tsuchiya K, Niwa M, Tsutsumishita Y, et al. Role of nitric oxide in compression injury of rat spinal cord. Free Radic Biol Med 1996;20:1–9.

[38] Wieseler-Frank J, Maier SF, Watkins LR. Central proinflammatory cytokines and pain enhancement. Neuro-signal 2005;14:166–74.

[39] Sung CS, Wen ZH, Chang WK, Ho ST, Tsai SK, Chang YC, et al. Intrathecal interleukin-1beta administration induces thermal hyperalgesia by activating inducible nitric oxide synthase expression in the rat spinal cord. Brain Res 2004;1015:145–53.

[40] Martin FC, Charles AC, Sanderson MJ, Merrill JE. Substance P stimulate IL-1 production by astrocyte via intracellular calcium. Brain Res 1992;599:13–8.

[41] Holguin A, O'connor KA, Biedenkapp J, Campisi J, Wieseler-Frank J, Milligan ED, et al. HIV-1 gp120 stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). Pain 2004;110:517–30.

[42] Guo W, Wang H, Watanabe M, Shimizu K, Zou S, LaGraize SC, et al. Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. J Neurosci 2007;27:6006–18.

[43] Watkins LR, Hutchison MR, Ledeboer A, Wieseler-Frank J, Milligan ED, Maier SF. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. Brain Behav Immun 2007;21:131–46.

[44] Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van DerMeerC. et al. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. J Neurosci 2004;24:8310–21.

[45] Lever IJ, Pezet S, McMahon SB, Malcangio M. The signaling components of sensory fiber transmission involved in the activation of ERK MAP kinase in the mouse dorsal horn. Mol Cell Neurosci 2003;24:259–70.

[46] Callsen D, Pfeilschifter J, Brune B. Rapid and delayed p42/p44 mitogen-activated protein kinase activation by nitric oxide: the role of cyclic GMP and tyrosine phosphatase inhibition. J Immunol 1998;161:4852–8.

[47] Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, et al. Nitiric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase 1/2 acdtivation in postcapillary endothelium. J Biol Chem 1998;273:4220–6.

[48] Yamazaki M, Chiba K, Mohri T, Hatanaka H. Activation of the mitogen-activated protein kinase cascade through nitric oxide synthesis as a mechanism of neuritogenic effect of genipin in PC12 cells. J Neurochem 2001;79:45–54.

[49] Sung YJ, Walters ET, Ambron RT. A neuronal isoform of protein kinase G couples mitogen-activated protein kinase nuclear important to axotomy-induced long-term hyperexcitability in Aplysia sensory neurons. J Neurosci 2004;24:7583–95.

[50] Chien WJ, Liang KC, Teng CM, Kuo SC, Lee FY, Fu WM. Enhancement of long-term potentiation by a potent nitric oxide-guanylyl cyclase activator 3-(5-hydroxymethyl-2-furyl)-1-benzyl imidazole. Mol Pharmacol 2003;63:1322–8.

[51] Hu HJ, Glauner KS, Gereau RW. ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K currents. J Neurophysiol 2003;90:1671–9.

[52] Morozov A, Muzzio IA, Bourtchouladze R, Van-strien N, Lapidus K, Yin D, et al. Rap1 couples camp signaling to a distinct pool of p42/44MAPK regulating excitability, synaptic plasticity, learning, and memory. Neuron 2003;39:309–25.

[53] Zhu JJ, Qin Y, Zhao M, Van-Aelst L, Malinow R. Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell 2002;110:443–55.

[54] Ji RR, Kohno T, Moore KA, Woolf CJ. Central sensitization and longterm potentiation-do pain and memory share similar mechanism? Trends Neurosci 2003;26:696–705.