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Involvement of protein kinase C in 5-HT-evoked thermal hyperalgesia and spinal fos protein expression in the rat

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Abstract

The present study was designed to characterize nociceptive response induced by 5-hydroxytryptamine (5-HT) and to investigate effects of inhibition of protein kinase C (PKC) in the periphery on noxious stimulus-evoked activity of the secondary neurons in the spinal cord. Subcutaneous injection of 5-HT (50 μg) and α-methylserotonin (α-m-5-HT, 5-HT_{2A} receptor agonist, 50 μg) into the unilateral hindpaw evoked significant decreases in paw withdrawal latency (PWL). The 5-HT-induced hyperalgesia was abolished by ketanserin (5-HT_{2A} antagonist, 10 μg, intraplantarly or i.pl.), but not by WAY100635 (5-HT_{1A} antagonist, 100 μg, i.pl.). 5-HT and α-m-5-HT also evoked numerous expressions of c-Fos-like immunoreactivity (c-fos-LI) in the ipsilateral dorsal horn (predominantly laminae I–II) of the lumbar spinal cord. However, treatment with 8-OH-DPAT (5-HT_{1A} receptor agonist, 100 μg, i.pl.) elicited only moderate thermal hyperalgesia and very limited expression of spinal c-fos-LI. Intraplantar chelerythrine (2, 6 or 10 μg), a PKC inhibitor, dose-dependently attenuated the hyperalgesia evoked by α-m-5-HT. Chelerythrine (10 μg, i.pl.) also completely prevented the development of hyperalgesia evoked by 5-HT but not by 8-OH-DPAT. Furthermore, pretreatment with chelerythrine significantly inhibited the expressions of c-fos-LI evoked by α-m-5-HT in laminae I–VI and by 5-HT in laminae I–II. These results demonstrate that PKC activation was involved in the development of nociceptive responses elicited by 5-HT and activation of peripheral 5-HT_{2A}, but not 5-HT_{1A}, receptors. The study also provides evidence at a cellular level that inhibition of PKC in the periphery suppresses the 5-HT-evoked neuronal activity in the central nervous system.
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Keywords: Protein kinase C (PKC); Peripheral pain mechanism; Hyperalgesia; 5-HT; Spinal cord

1. Introduction

Persistent pain and hyperalgesia are seen in the clinic more often than acute pain and are mostly associated with release of inflammatory factors. 5-Hydroxytryptamine (5-HT) is one of these endogenous factors and is released from platelets, mast cells and endothelial cells into a wound site in response to inflammation and injury (Lehtosalo et al., 1984; Rowley, 1956). This chemical is a potent pro-inflammatory and pro-nociceptive agent as it excites nociceptive afferents (Beck and Handwerker, 1974) and causes pain sensation in humans when applied to blister base or skin (Armstrong et al., 1953; Jensen et al., 1990a, b; Lindahl, 1961; Orwin and Fozard, 1986; Richardson et al., 1985; Schmelz et al., 2003). Furthermore, 5-HT enhances nociceptive responses induced by mechanical stimulus (Vinegar et al., 1989) and other inflammatory mediators, such as prostaglandin E_2, bradykinin, noradrenaline, histamine and substance P in animals (Hong and Abbott, 1994; Khalil and Helme, 1990) and humans (Babenko et al., 1999; Bleehen and Keele, 1977; Jensen et al., 1990a,b; Sicuteri et al., 1965). However, the cellular mechanisms of signal transduction in nociceptor sensitization and pain evoked by 5-HT are not fully understood.

Abbreviations: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin; α-m-5-HT, α-methylserotonin; c-fos-LI, c-Fos-like immunoreactivity; DMSO, dimethylsulphoxide; i.pl., intraplantar or intraplantarly; PBS, phosphate-buffered saline; PKC, protein kinase C; PWL, paw withdrawal latency.

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5-HT may act on multiple subtypes of 5-HT receptors in the periphery to elicit nociceptive response, including 5-HT_{1A} (Taiwo et al., 1992), 5-HT_{2A} (Abbott et al., 1996; Okamoto et al., 2002; Pertsch et al., 1993) and 5-HT_3 receptors (Zeitz et al., 2002), etc. It appears that 5-HT_{2A} receptors that are located on the peripheral sensory terminals (Carlton and Coggeshall, 1997; Pierce et al., 1996) are important in inducing pain and hyperalgesia. It has been demonstrated that 5-HT-evoked nociceptive responses can be mimicked and abolished by 5-HT_{2A}, but not by 5-HT_{1A} and/or 5-HT_3, receptor agonists and antagonists, respectively (Abbott et al., 1996; Doi-Saika et al., 1997; Grubb et al., 1988; Pertsch et al., 1993; Tokunaga et al., 1998). Intracellular transduction pathway following activation of 5-HT_{2A} receptors may possibly involve an increase in protein kinase C (PKC) as this type of receptors is coupled through G proteins to the stimulation of phospholipase C pathway leading to downstream of PKC activation (Ananth et al., 1987; Greene et al., 2000; Miller and Gonzalez, 1998). The present study examined whether PKC in the periphery was involved in nociceptive responses evoked by 5-HT and activation of peripheral 5-HT_{1A} and 5-HT_{2A} receptors. Particularly, the study investigated effects of peripherally administered 5-HT and PKC inhibitor on the secondary neurons in the spinal cord using c-Fos-like immunoreactivity (c-fos-LI) as a marker of neuronal activation.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, 230–300 g (Fuzhou Animal Center, China), were housed three per cage in the colony room on a 12:12 light–dark cycle with lights on from 0700 to 1900 h and maintained in a controlled environment with food and water ad libitum. All experiments were performed in a soundproof room during the light cycle. Rats were acclimatized to the experimental room and habituated to handling and behavioral testing for 30 min per day for 3 days and also for 30 min immediately prior to the experiment to minimize stress-induced pain suppression. The behavioral studies were conducted using blind testing protocols.

The animals were used only once and always carefully handled throughout the experiments. All experiments were carried out in accordance with ethical guidelines recommended by the International Association for the Study of Pain for experimental pain in conscious animals (Zimmermann, 1983) and were approved by the Animal Care and Use Committee of the University. Efforts were made to result in the least behavioural stress and to reduce the number of animals used.

2.2. Behavioral assays

Rats were placed in a device that held the body without restraining the head or legs. Noxious heat stimulation was carried out by immersing the hindpaw up to the ankle joint into a slowly stirred water bath at 47.5 °C while gently holding the site proximal to the stimulated part with a hand (Abbadie et al., 1994; Buritova and Besson, 2000; Wisden et al., 1990). The time that elapsed before the rat withdrew its paw was recorded as the paw withdrawal latency (PWL). A 20-s cut-off time was used to preclude possible damage to the paw. The water temperature was adjusted to 47.5 °C as this temperature produced an average baseline PWL of approximately 7 s in naive rats. The PWL for any test time point was measured two times at 4-min intervals and the mean values were calculated. Vehicle control and other experiments in this laboratory (Jiang et al., 2006; Wei et al., 2005) show that 4-min interval was reasonable to make repeated measurements.

5-HT and its analogs and vehicles were injected subcutaneously into the hindpaw. Fifty microliters of 5-HT antagonists or vehicle were administered 10 min before 5-HT and its analogs. Intraplantar (i.pl.) injections of drugs were performed on non-anesthetized rats (Gulwadi et al., 2002; Perrot et al., 2001). Briefly, rats were placed in a cylinder with only the hindpaw free for injection: the i.pl. injections were given rapidly (5 s), and rats were allowed to recover in their cages for 10 min before nociceptive testing. To examine the effect of PKC inhibitor, chelerythrine, on the nociceptive responses evoked by 5-HT and its analogs, 50 μl of chelerythrine or vehicle was injected intraplantarly 5 min before the algogens. The PWL was measured every 10 min after drug administration up to 1 h.

2.3. Immunohistochemistry for c-Fos protein

To analyze the induction of c-Fos-like immunoreactivity (c-fos-LI), rats were only treated with vehicles or drugs without any heat stimulation and deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p., Shenggong Chemicals, Shanghai, China) 1 h after vehicle or drug injection. The animals were perfused through the heart with 200 ml of phosphate-buffered saline (PBS; 0.05 M; pH 7.4), followed by 500 ml of fresh cold fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The lumbar spinal cord (L4-5) was removed from the vertebral canal and post-fixed in the same fixative at 4 °C for 4 h. The tissue was then cryoprotected in 30% sucrose in PBS for at least 2 days at 4 °C and then kept in PBS at 4 °C until sectioning.

Transverse sections were cut at 40 μm on a sliding microtome and collected at intervals of 120 μm in cold PBS. Sections were processed by a free-floating slice immunohistochemistry procedure. The sections were rinsed twice in PBS followed by a 5-min 0.3% hydrogen peroxide treatment and washed four times in PBS. The sections were blocked to prevent non-specific binding of antibody by a 30-min treatment in a blocking solution comprised of 2% normal rabbit serum and 0.3% Triton X-100 in PBS. The sections were incubated for 20 h at 4 °C with rabbit anti-c-fos polyclonal antibody (1:5,000 in PBS containing 2% normal rabbit serum; Santa Cruz Biotechnology, CA). The tissue was then washed three times in PBS and transferred to a goat anti-rabbit biotinylated secondary IgG complex (1:200 in 10% goat serum in PBS; Vector, Burlingame, CA) for 2 h at room temperature followed by exposing to avidin–biotin horseradish peroxidase complex (1:100; Vectastain ABC-Elite kit, Vector Burlingame, CA) for

1 h at room temperature. After the final wash with PBS, the chromogen was developed with 0.01% hydrogen peroxide and 0.05% diaminobenzidine. The sections were thoroughly rinsed with phosphate buffer, mounted on gelatin-subbed slides. After bidistilled water rinses to take off the excess stain, the sections were dehydrated in a series of graded alcohols. Finally, the slides were air-dried and coverslipped.

Specific staining was abolished by omission of primary antiserum in two rats. In this case no immunolabelling was observed.

2.4. Counting of c-Fos-like immunoreactive nuclei

Sections were visually scanned and photographed using a bright-field microscope. The individual sections were printed and overlaid with an acetate sheet on which the distribution of c-*fos* immunoreactive neurons was then plotted. For the quantification of fos-labeled neurons, each section of the dorsal horn was divided into three regions of interest according to the cytoarchitectonic organization of the spinal cord (Molander et al., 1984): superficial layer (laminae I–II), nucleus proprius (laminae III–IV) and deep dorsal horn (laminae V–VI). Six sections with highest density of c-fos-LI from each animal were selected for quantification of c-fos-LI. The terms fos-LI and fos-positive neurons are meant to be synonymous with c-fos-labeled neuronal nuclei. All c-fos-LI neurons in the defined areas on both sides were counted manually by an observer blinded to the treatment conditions. For each rat, the number of c-fos-LI neurons within each region was determined by averaging the counts made in the six sections and expressed as mean ± S.E.M. (standard error of the mean) in all the rats in that treatment group and subjected to analysis of variance (ANOVA).

2.5. Data analysis

Data were expressed as mean ± S.E.M. Differences between the groups were examined for statistical significance using one-way analysis of variance, followed by Tukey's test for multiple comparisons. The unpaired *t*-test (two-tailed) was also used to detect the difference between the drug-treated and vehicle groups. The Mann–Whitney *U*-test was used for nonparametric data. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

2.6. Drugs and method of administration

5-HT (5-hydroxytryptamine hydrochloride), α-m-5-HT (α-methylserotonin maleate salt, 5-HT_{2A} agonist), 8-OH-DPAT ((±)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide, 5-HT_{1A} agonist), ketanserin (tartrate salt, 5-HT_{2A} antagonist), WAY-100635 (maleate salt, 5-HT_{1A} antagonist) and chelerythrine chloride (PKC inhibitor) were purchased from Sigma (St. Louis, MO, USA). 5-HT, α-m-5-HT, 8-OH-DPAT and WAY-100635 were dissolved in sterile saline and prepared at concentrated solution as stocks. Chelerythrine and ketanserin stock solutions were prepared in 50% DMSO (dimethylsulphoxide, Shenggong, Shanghai, China). All stocks were stored at −20 °C and diluted to the working concentration with saline immediately before the experiment. The concentration of DMSO in the vehicle used to inject ketanserin and the highest dose of chelerythrine was 2%. Drug dose was selected based on the dose–response curves generated in previous studies (Abbott et al., 1996; Souza et al., 2002; Wei et al., 2005).

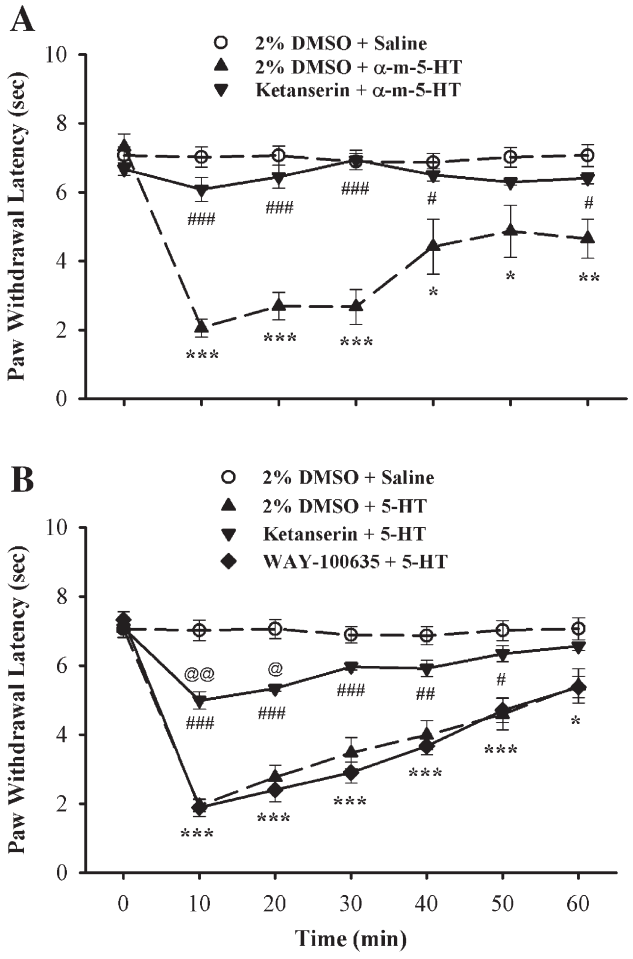

Fig. 1. 5-HT- and α-m-5-HT-induced thermal hyperalgesia. 2% DMSO or ketanserin (10 μg) or WAY100635 (100 μg) was administered subcutaneously into the hindpaw 10 min before the intraplantar injection of α-m-5-HT (A) and 5-HT (B). Each symbol represents the mean ± S.E.M. of paw withdrawal latency. Values that significantly differ between the groups treated with 5-HT or α-m-5-HT and control (2% DMSO plus saline) are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Values that significantly differ between the groups treated with 5-HT or α-m-5-HT in the absence and presence of ketanserin are indicated by # ($P<0.05$), ## ($P<0.01$) or ### ($P<0.001$). The symbol @ represents $P<0.05$ and @@ represents $P<0.01$ between group of ketanserin plus 5-HT and control (2% DMSO plus saline). $N=6–8$ in each group.

3. Results

3.1. Receptor mediation in 5-HT-induced hyperalgesia

Subcutaneous injection of saline in the hindpaw following similar pretreatment of 2% DMSO (10 min apart) did not alter the thermal nociceptive paw-withdrawal threshold compared with baseline (Fig. 1A, $n=7$). Injection of α-m-5-HT (50 μg) in the hindpaw following pretreatment with 2% DMSO produced

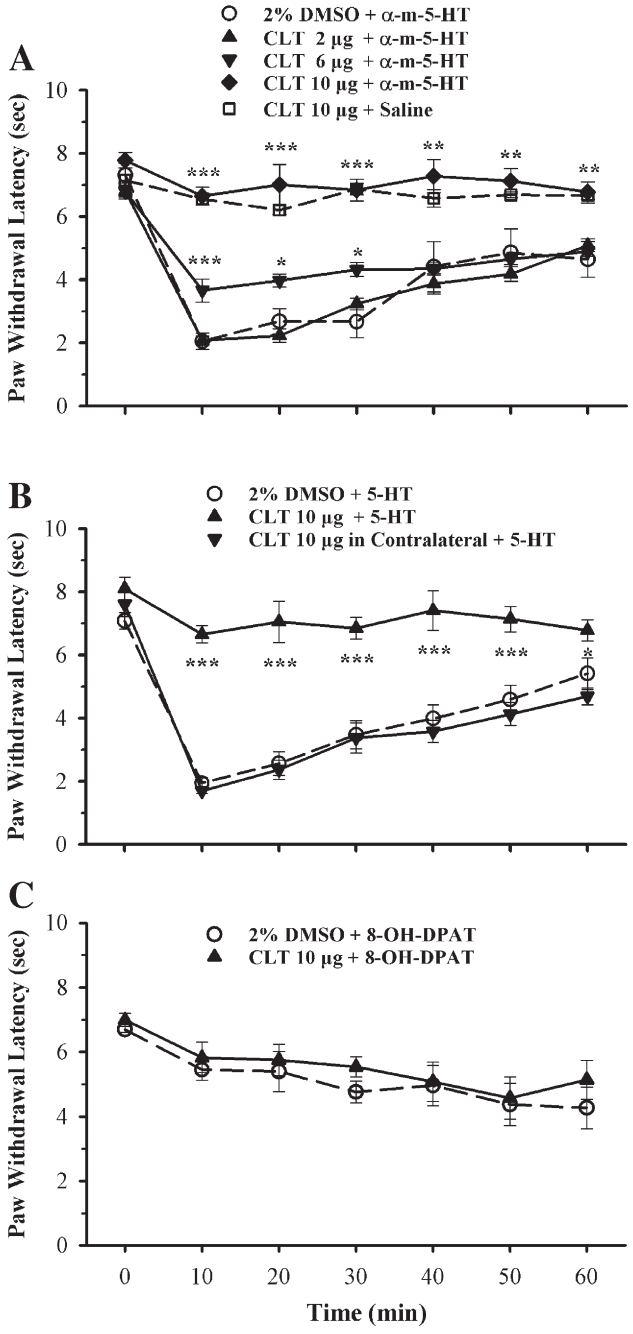

Fig. 2. Effects of chelerythrine on thermal hyperalgesia evoked by 5-HT and its analogs. Chelerythrine or vehicle (2% DMSO) was administered subcutaneously into the hindpaw 5 min before the intraplantar injection of α-m-5-HT (A), 5-HT (B) and 8-OH-DPAT (C). Each symbol represents the mean±S.E.M. of the paw withdrawal latency. Values that significantly differ between the groups treated with chelerythrine plus 5-HT or its analogs and 2% DMSO plus 5-HT or its analogs are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). $N=6$–8 in each group.

hyperalgesia as determined by reduction in the thermal nociceptive PWL. The decrease appeared and peaked at 10 min (the earliest observing time) after administration with a value of 72% of baseline. The thermal hyperalgesia gradually decayed after the peak and lasted at least 1 h (Fig. 1A, $n=8$). However, after pretreatment with ketanserin (10 μg, at −10 min), administration of α-m-5-HT (50 μg) did not change the PWL (Fig. 1A, $n=6$). The PWL in this group was not significantly different from control group (2% DMSO plus saline).

Administration of 5-HT (50 μg) 10 min after treatment with 2% DMSO in the hindpaw also profoundly decreased the PWL. The decrease occurred and peaked at 10 min after administration with a value of 73% of baseline. This response lasted at least 1 h (Fig. 1B, $n=8$) compared with control. However, 5-HT (50 μg) induced a very mild decrease in the PWL (25–30%) following pretreatment with ketanserin (10 μg, −10 min; Fig. 1B, $n=7$) and this decrease persisted for only 20 min ($P<0.05$–0.01 vs. 2% DMSO plus saline). On the other hand, pretreatment with WAY100635 (selective 5-HT_{1A} receptor antagonist, 100 μg; Fig. 1B, $n=8$) did not change 5-HT-induced hyperalgesia. Decrease in the PWL in this group was not significantly different from control group at any tested time point.

3.2. Effects of chelerythrine on hyperalgesia evoked by 5-HT, α-m-5-HT and 8-OH-DPAT

To investigate the involvement of PKC in the peripheral processing of nociceptive response evoked by activation of 5-HT_{2A} receptors, chelerythrine (2, 6 and 10 μg, i.pl., −5 min, $n=8$ each) was administered at the same paw lately injected with α-m-5-HT (50 μg). Fig. 2A shows that chelerythrine dose-dependently suppressed the thermal hyperalgesia evoked by α-m-5-HT. Chelerythrine at a dose of 10 μg completely prevented the development of the α-m-5-HT-evoked hyperalgesia ($P>0.05$ vs. 2% DMSO plus saline). When 10 μg of chelerythrine was injected, subsequently administered 5-HT (50 μg, i.pl.) also failed to evoke hyperalgesia (Fig. 2B, $n=8$, $P>0.05$ vs. 2% DMSO plus saline). Administration of chelerythrine (10 μg, i.pl.) with subsequent treatment of saline did not alter thermal paw-withdrawal threshold (Fig. 2A, $n=6$). To determine whether the effect of chelerythrine was produced via local or systemic PKC inhibition, the same dose of this agent (10 μg) was injected into the hindpaw that was contralateral to the paw injected with 5-HT (50 μg, $n=7$). Fig. 2B illustrates that pretreatment with chelerythrine in the contralateral paw failed to produce inhibition on the 5-HT-induced hyperalgesia.

To determine whether PKC activation is involved in 5-HT_{1A} receptor-mediated response, effect of 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist, was examined. As shown in Fig. 2C, 100 μg of 8-OH-DPAT ($n=8$) elicited a moderate decrease in the PWL. The PWL significantly decreased at 10 min following the administration with a value of 19% of baseline ($P<0.05$). The PWL then maintained at approximately 20–35% of reduction level ($P<0.05$–0.001 vs. vehicle treatments) throughout the experiment. Although 8-OH-DPAT only elicited a very moderate decrease in the PWL, pretreatment with the PKC inhibitor did not change the 8-OH-DPAT-evoked response. Fig. 2C illustrates that in the presence of chelerythrine (10 μg), 8-OH-DPAT (100 μg, $n=8$) still caused a decrease in the PWL which was not significantly different from that induced by 2% DMSO plus 8-OH-DPAT.

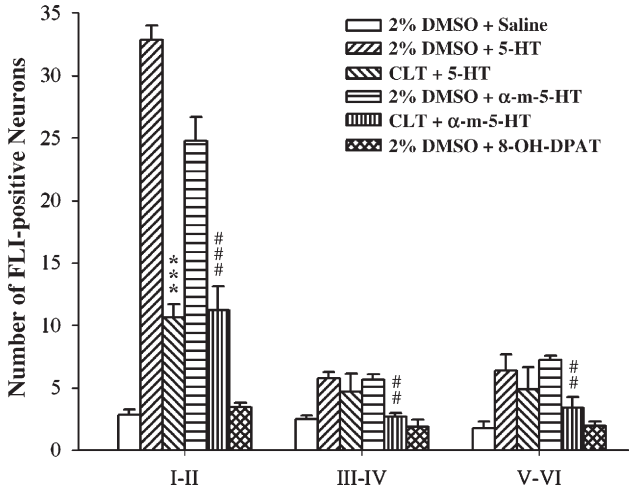

Fig. 3. Histograms presenting the laminar distribution of neurons with c-fos-like immunoreactive nuclei in the L4-5 segments 1 h after intraplantar administration of the chemicals. Data are expressed as the mean±S.E.M. of fos-positive neurons in the superficial laminae (I–II), nucleus proprius (laminae III–IV) and deep dorsal horn (laminae V–VI) on the side of the spinal cord ipsilateral to the site of drug injection. The number of animals in each treatment group was four. *Represents statistical analysis between chelerythrine plus 5-HT and 2% DMSO plus 5-HT (***$p<0.001$), and # represents comparison between chelerythrine plus α-m-5-HT and 2% DMSO plus α-m-5-HT (## $p<0.01$; ### $p<0.001$).

3.3. Effects of chelerythrine on c-fos-LI evoked by 5-HT, a-m-5-HT and 8-OH-DPAT

In all c-fos-LI experiment, 2% DMSO (vehicle for 10 μg of chelerythrine) or chelerythrine was administered i.pl. 5 min before 5-HT and its analogues or saline to assess the effects of the PKC inhibitor on the expressions of algogen-evoked c-fos-LI.

Very few c-fos-LI expressions (6.9±0.3, $n=4$, Figs. 3 and 4) were elicited in the dorsal horn of the lumbar spinal cord in the rats that received injection of saline in the unilateral hindpaw. However, c-fos-LI expression was greatly enhanced in ipsilateral L4-5 segments following administration of 5-HT (50 μg, i.pl.) while little increase was observed in the side contralateral to the site of injection. Most fos-LI-labeled neurons were located in the superficial laminae (I–II) and only a few were distributed in the nucleus proprius (laminae III–IV) and deep dorsal horn (V–VI). Mean count of the fos-LI neurons was 32.9±1.2 in laminae I–II which accounted for 73% of the total number of labeled neurons in the dorsal horn per section. The numbers of the c-fos-LI neurons were 5.8 ±0.6 (13%) in laminae III–IV and 6.4±1.3 (14%) in laminae V–VI (Figs. 2 and 3, $n=4$). Injection of α-m-5-HT (50 μg, i.pl.) also elicited significant c-fos-LI expressions in L4-5 segments which displayed a distribution pattern similar to that evoked by 5-HT. Mean numbers of the fos-LI-labeled neurons were 24.8±1.9 (66% of the total c-fos-LI counts), 5.7±0.4 (15%) and 7.3±0.3 (19%) in laminae I–II, III–IV and V–VI, respectively ($n=4$, Figs. 3 and 4). However, administration of 8-OH-DPAT at a dose of 100 μg only resulted in minimal expressions of fos-LI neurons in the spinal dorsal horn in L4-5 segments (7.3±0.4), which were not significantly different from saline-treated group (Figs. 2 and 3, $n=4$).

Pretreatment with 10 μg of chelerythrine (i.pl.) markedly reduced the expression of c-fos-LI in laminae I–II evoked by injection of 5-HT (50 μg, i.pl.). Compared with the group treated with 2% DMSO plus 5-HT, the number of the fos-LI-labeled neurons was reduced by 68±3% (Figs. 3 and 4, $n=4$, $P<0.001$). However, the expression of the fos-LI neurons in laminae III–VI was not significantly affected compared with 5-HT group without the PKC inhibitor. Chelerythrine (10 μg, i.pl.) also greatly inhibited the α-m-5-HT (50 μg, i.pl.)-evoked expression of spinal c-fos-LI neurons. The inhibition occurred throughout all laminae in the dorsal horn. The fos-LI-labeled neurons were reduced by 55±8, 52±5 and 53±12% in laminae I–II, III–IV and V–VI, respectively. Statistical analysis revealed that all of these reductions were significant compared with the group treated with 2% DMSO plus α-m-5-HT (Figs. 3 and 4, $n=4$, $P<0.01$–0.001).

4. Discussion

The present study demonstrated that i.pl. injection of both 5-HT and α-m-5-HT produced reversible thermal hyperalgesia in the hindpaw. This is compatible with our previous observations showing that i.pl. injection of 5-HT or α-m-5-HT induces spontaneous pain-related behaviors (Abbott et al., 1996; Hong and Abbott, 1994). It is well known that 5-HT is a major component of inflammatory factors (Foon et al., 1976; Vinegar et al., 1987) and, therefore, is pro-nociceptive. Increase of peripheral 5-HT is involved in the etiology of somatic (Abbott et al., 1997; Okamoto et al., 2002; Pertsch et al., 1993; Wei et al., 2005) and visceral pain (Espejo and Gil, 1998). This is in contrast to the effects of 5-HT in the CNS. Serotonergic pathway in the CNS is involved in the descending inhibition of nociception that originates in the spinal cord (Aimone et al., 1987; Cannon et al., 1982; Kwiat and Basbaum, 1992; Zemlan et al., 1980) and functions to activate endogenous opioids (Vaz et al., 1996). The use of 5-HT reuptake inhibitors produces antinociception by an increase of 5-HT content in the CNS (Oliva et al., 2002).

The present study also demonstrated that ketanserin, the most selective 5-HT_{2A} receptor antagonist to date (Hoyer et al., 2002), almost completely abolished the thermal hyperalgesia produced by α-m-5-HT and 5-HT while WAY100635, a selective high-affinity antagonist at 5-HT_{1A} receptors (Fletcher et al., 1996; Forster et al., 1995), did not alter the 5-HT-evoked response at a maximal effective dose (Wei et al., 2005). These results indicate that the 5-HT-evoked thermal hyperalgesia is basically, if not completely, mediated by 5-HT_{2A} receptors. It has been recently demonstrated that systemic injection of the 5-HT_{2A} receptor antagonists, sarpogrelate and ketanserin, reversed mechanical hyperalgesic in a neuropathic pain rat (Nitanda et al., 2005). It would be interesting to investigate the effects of 5-HT_{2A} receptor blockade on mechanical or other nociceptive stimulation in inflammatory models in the future. However, 5-HT_{2A} receptors do not mediate normal sensation as systemic injection of ketanserin (10 mg/kg, i.p.) (Nitanda et al., 2005) or topical application of 5-HT_{2A} antagonists (ketanserin and sarpogrelate) does not affect the thermal (Hong et al., in

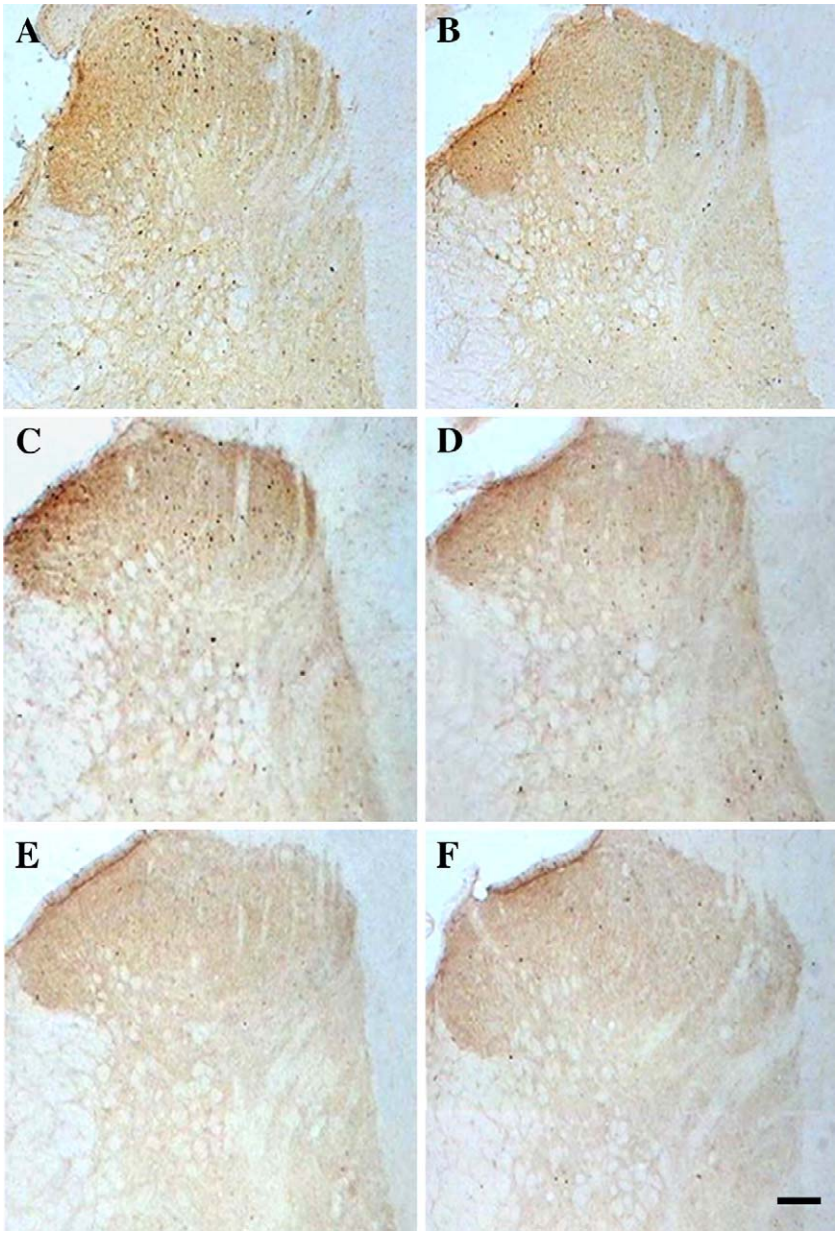

Fig. 4. Photomicrographs of transverse sections of the lumbar spinal cord illustrating the distribution of fos-labeled neurons in the dorsal horn of the lumbar spinal cord in rats treated i.pl. with (A) 2% DMSO plus 50 μg of 5-HT, (B) 10 μg of chelerythrine plus 50 μg of 5-HT, (C) 2% DMSO plus 50 μg of α-m-5-HT, (D) 10 μg of chelerythrine plus 50 μg of α-m-5-HT, (E) 2% DMSO plus saline and (F) 2% DMSO plus 100 μg of 8-OH-DPAT. Chelerythrine or vehicle was administered subcutaneously into the hindpaw 5 min before the intraplantar injection of 5-HT or its analogs. In all of the panels, the side of the spinal cord is ipsilateral to the site of drug or vehicle injection. Scale bar, 100 μm.

press) or mechanical (Nitanda et al., 2005) sensation in the normal hind limb, indicating that there is no tonic action of 5-HT2A receptors in thermal and mechanical sensation under normal conditions. Based on the result that i.pl. 8-OH-DPAT produced a mild decrease in the PWL and ketanserin did not completely abolish the 5HT-evoked hyperalgesia, 5-HT_{1A} receptors may play a minor role in 5-HT-evoked thermal hyperalgesia. It seems that the activation of 5-HT_{1A} receptors is probably much more involved in mechanical than thermal sensation (Aley and Levine, 1999).

Furthermore, our study showed that the selective PKC inhibitor, chelerythrine (Green and Cottrell, 1997) prevented the development of the 5-HT- and α-m-5-HT-evoked hyperalgesia. The effects of chelerythrine were specific as the PKC inhibitor exerted a dose-dependent inhibition on the α-m-5-HT-evoked hyperalgesia but failed to alter the 8-OH-DPAT-evoked response. The effects of chelerythrine were ascribed to its local, but not systemic, action because subcutaneous administration of chelerythrine in the non-inflamed (contralateral) paw failed to produce any antinociceptive effect. These results indicate that PKC activation is involved in the signal transduction pathway of 5-HT-evoked responses. Now, our results indicated that PKC is involved in the hyperalgesic response produced by the activation of 5-HT_{2A}, but not 5-HT_{1A},

receptors. It has been demonstrated that activation of 5-HT_{1A} receptors involves an increase of cAMP (Taiwo et al., 1992) and PKA (Aley and Levine, 1999).

Our behavioural results are supported by the determination of c-fos protein expression, a functional marker identifying the activity in the spinal dorsal horn neurons receiving nociceptive inputs from primary afferents (Harris, 1998). One of advantages of c-fos determination is to reflect the level of cellular activity of different neuronal populations of the spinal cord in the conscious animal. The present study showed that i.pl. injection of 5-HT and α-m-5-HT elicited large numbers of c-fos-LI neurons in the ipsilateral dorsal horn of the lumbar spinal cord, indicating the central processing of nociceptor sensitization evoked by these pro-nociceptive agents. The expressions of c-fos-LI evoked by 5-HT and α-m-5-HT were mainly located in the superficial laminae (approximately two-thirds of the total c-fos-LI neurons) where nociceptive primary afferents terminate and where the majority of nociceptive neurons are located (Millan, 1999). It should be noticed that the distribution of c-fos-LI expression following the treatment with α-m-5-HT was similar to those seen after 5-HT injection. However, administration of 8-OH-DPAT, a 5-HT_{1A} receptors agonist, at a large dose (Abbott et al., 1996) resulted in very little expression of c-fos-LI in the spinal dorsal horn which was in accordance with that seen in a previous study (Doi-Saika et al., 1997).

Importantly, i.pl. administration of chelerythrine greatly decreased 5-HT- and α-m-5-HT-evoked c-fos-LI expressions. Moreover, the decreases of the c-fos-LI neurons occurred predominantly in the superficial (I–II) laminae of the spinal dorsal horn. To our knowledge, this is the first study to demonstrate that inhibition of PKC in the periphery suppresses production of noxious stimulus-induced neuronal activity at the level of the spinal dorsal horn. It was noticed that although pretreatment with 10 μg of chelerythrine completely prevented the development of the 5-HT- and α-m-5-HT-evoked hyperalgesia, the same treatment only partially suppressed the evoked c-fos-LI expressions in laminae I–II. A dissociation between "pain" and c-fos-LI expression has been reported (Harris et al., 1995; Presley et al., 1990). For example, significant levels of c-fos-LI expression are still seen in the superficial laminae of the spinal cord in the rats that display no pain-related behavior to a paw injected with formalin following administration of morphine (Presley et al., 1990). The present study also showed that chelerythrine reduced the expression of c-fos-LI in laminae III–VI in α-m-5-HT group but not in 5-HT group. These results indicate that intracellular signaling system mediating the 5-HT- and α-m-5-HT-evoked responses may not be identical in the neurons of laminae III–VI where multiple subtypes of 5-HT receptors may mediate the 5-HT-evoked response. These might include 5-HT_{1A} and 5-HT_3 receptors (Parada et al., 2001; Taiwo et al., 1992; Zeitz et al., 2002) that may involve different cellular mechanisms, such as cAMP (Taiwo et al., 1992) and PKA (Aley and Levine, 1999), etc. The results in the present study showing that ketanserin did not completely block the 5-HT-evoked hyperalgesia also support this notion.

The results that inhibition of PKC attenuated the α-m-5-HT-evoked hyperalgesia and the spinal c-fos-LI were supported by the findings that the 5-HT_{2A} receptors are coupled through G proteins to phospholipase C which produces diacylglycerol leading to the activation of PKC (Ananth et al., 1987; Greene et al., 2000; Miller and Gonzalez, 1998). Therefore, PKC must be one of the key downstream effector molecules that mediate the nociceptor sensitization produced by 5-HT that activates 5-HT_{2A} receptors. The involvement of PKC in the 5-HT-evoked hyperalgesia was in good agreement with previous studies showing that the inhibition of PKC in the periphery greatly attenuates the nociceptive responses produced by formalin (Souza et al., 2002), carrageenan and the nerve growth factor (NGF; Khasar et al., 1999), all of which have been demonstrated to release 5-HT (Abbott et al., 1996; Lewin et al., 1994; Vinegar et al., 1987). Therefore, it is likely that the activation of PKC due to 5-HT's action on the 5-HT_{2A} receptors at least partially contributes to peripheral nociceptive processing in the formalin, carrageenan and NGF pain models.

A growing body of evidences indicates that activation of PKC in the periphery is involved in inflammatory and nociceptive processes. Treatment with phorbol esters leads to nociceptor excitation (Leng et al., 1996; Schepelmann et al., 1993) and its sensitization to thermal (Leng et al., 1996) or mechanical stimulation (Schepelmann et al., 1993). Importantly, the inhibition of PKC attenuates or abolishes pain-related or hyperalgesia response in the widely used pain models, such as alcoholic neuropathy (Dina et al., 2000), carrageenan (Aley et al., 2000) and formalin (Souza et al., 2002). Furthermore, PKC has been demonstrated to contribute to bradykinin-evoked response in sensory neurons (Cesare et al., 1999) and hyperalgesia evoked by inflammatory factors, such as epinephrine (Khasar et al., 1999), NGF (Khasar et al., 1999), prostaglandin E2 (Gold et al., 1998), bradykinin (Souza et al., 2002) and 5-HT, all of which are released or synthesized in response to inflammation or injury. It appears that PKC activation may in fact be the common cellular pathway that ultimately leads to the nociceptor sensitization and establishment of hyperalgesia or painful conditions. To take all these together, it is proposed that the enzyme PKC in the periphery can be targeted as a novel therapy for treatment of pain of peripheral origin with limited central side-effects.

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