

# Involvement of Subtypes $\gamma$ and $\epsilon$ of Protein Kinase C in Colon Pain Induced by Formalin Injection

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## Key Words

Visceral pain · Protein kinase C · Formalin · Colon · Wide dynamic range neuron

## Abstract

Protein kinase C (PKC) has been widely reported to participate in somatic pain; however, its role in visceral pain remains largely unclear. Using a colon inflammatory pain model by intracolonic injection of formalin in rats, the present study was to examine the role of PKC in visceral pain and determine which subtypes may be involved. The colon pain behavior induced by formalin injection could be enhanced by intrathecal pretreatment with a PKC activator (PMA), and alleviated by a PKC inhibitor (H-7). Wide dynamic range (WDR) neurons in the L6-S1 spinal dorsal horn that were responsive to colorectal distension were recorded extracellularly. It was found that neuronal activity was greatly increased following formalin injection. Microdialysis of PMA near the recorded neuron in the spinal dorsal horn facilitated the enhanced responsive activity induced by formalin injection, while H-7 inhibited significantly the enhanced response induced by formalin injection. Western blot analysis revealed that membrane translocation of PKC- $\gamma$  and PKC- $\epsilon$ , but not other

subtypes, in the spinal cord was obviously increased following formalin injection. Therefore, our findings suggest that PKC is actively involved in the colon pain induced by intracolonic injection of formalin. PKC- $\gamma$  and PKC- $\epsilon$  subtypes seem to significantly contribute to this process.

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## Introduction

Visceral pain is one of the most common symptoms and frequent reasons for seeking medication. However, compared with somatic pain, the underlying mechanisms of visceral pain remain less understood. Studying the related signal transduction would provide some valuable insights into the visceral pain. Among those nociceptively activated molecules, protein kinase C (PKC) is one of the most important signal molecules involved in the modulation of pain transmission. PKC has been well demonstrated by many studies on its modulation of so-

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matic pain [1–7]. However, it is unclear whether it also participates in visceral pain. PKC belongs to a family of phospholipid-dependent serine/threonine kinases, and molecular cloning analysis has demonstrated that PKC family of isoenzymes consists of at least 11 related proteins, which are divided into four subclasses: conventional PKC (cPKC) subtypes ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), novel PKC (nPKC) subtypes ( $\eta$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ ), the atypical PKC subtypes ( $\tau$ / $\lambda$ ,  $\zeta$ ), and the PKC-related kinases [8]. These individual subtypes differ in tissue expression, subcellular distribution, substrate specificity and activating cofactor requirements, suggesting that their cellular functions may differ. Therefore, the systematic analysis of PKC subtypes involved in visceral pain is quite necessary.

Since the distal gastrointestinal tract has been a major focus of visceral inflammatory pain for animal studies [9–11], we used a colonic inflammatory pain model induced by intracolonic injection of formalin [12] to investigate the effects of PKC activation or inhibition on the colon pain behaviors and responsive activity of spinal dorsal horn neurons. We also identified the subtypes PKC involved in this type of visceral pain by analyzing the membrane translocation of PKC after formalin injection.

## Materials and Methods

### *Animal Preparation*

All animals were acquired from the Animal Center of Capital Medical University, Beijing, China. Adult male Wistar rats (weighing 200–250 g) were housed individually with food and water available ad libitum and kept under controlled conditions of temperature (23–25°C) and light/dark cycle (12 h/12 h). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University and were consistent with the International Association for the Study of Pain guidelines for Pain Research in conscious animals [13]. All efforts were made to minimize the number of animals used and their sufferings.

For the behavioral test, rats were randomly divided into six groups: (1) intracolonic (i.c.) injection of formalin; (2) i.c. injection of formalin and intrathecal (i.t.) administration of saline; (3) i.c. injection of formalin and i.t. administration of 1,(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7); (4) i.c. injection of formalin and i.t. administration of phorbol-12-myristate-13-acetate (PMA); (5) i.c. injection of saline; (6) i.c. injection of saline with i.t. administration of PMA.

For electrophysiological recordings, the rats were divided into four groups: (1) i.c. formalin injection (F); (2) i.c. formalin injection with saline microdialysis (F+saline); (3) i.c. formalin injection with PMA microdialysis (F+PMA); (4) i.c. formalin injection with H-7 microdialysis (F+H-7).

For Western blots, animals were divided into 3 groups: (1) naive rats (N); (2) i.c. injection of saline (S); (3) i.c. of formalin (F).

### *Intrathecal Catheter Implantation*

Rats were initially anesthetized with 4% isoflurane. An adequate level of anesthesia was then maintained using 2% of isoflurane. A polyethylene catheter (PE-10, Baxter Healthcare Corporation, Deerfield, Ill., USA) was inserted through the atlanto-occipital membrane and guided through the subarachnoid space to the lumbar enlargement [14]. After surgery, rats were given an antibiotic (gentamicin 2 mg i.m., Elkins-Sinn, N.J., USA) and individually housed. All animals were given a minimum of 5 days to recover before experimental procedures began. Those with free activities and no weight loss were used in the experiments.

### *Drug Treatments*

All drugs were administered through intrathecal catheter. For behavior testing, each group received 20  $\mu$ l of their respective solution: sterile saline; PKC activator PMA (100 ng, Sigma, USA, in 5% DMSO); PKC inhibitor H-7 (200  $\mu$ g, Sigma, in saline). The solutions were administered 30 min before formalin injection followed by a 10- $\mu$ l flush of saline. The doses of PMA and H-7 were chosen based on their effects on somatic pain [15].

### *Behavior Test*

The visceral pain was slightly modified from Miampamba et al. [12]. Before initiating the visceral pain, each animal was allowed 20 min to be habituated to its surroundings. Anesthesia was then achieved by using isoflurane. The animal was suspended by its tail, and the rectum was gently emptied with a cotton-capped stalk. Then a colonoscope was inserted through the anus. The colonoscope was made of a plastic tube a little larger than the diameter of the colon with a lateral slot used to puncture the intestinal wall under visual control. Then 100  $\mu$ l of 5% formalin was injected into the colon using an 85-mm-long needle, at about 35 mm from the anal margin. The solution was supplemented with 1% Evans blue to control the full submucosal injection of formalin. Those with luminal leakage occurred at the time of injection and the injection that had been trans-mural at the end of the test was excluded from the experiments. Animals were then placed into the transparent cages and were allowed to recover freely from anesthesia, at which point the observation began.

It has been reported that after formalin injection, four main behaviors can be observed: (1) abdominal licking and nibbling, which stands for the least painful suffering and is marked as L; (2) body stretching, i.e. backward extension of the hind limbs, marked as B; (3) contraction of the flanks, sometimes evolving to a stretching attitude, marked as C, and (4) body contractions, involving curving of the back with occasional spasms running along the flank, marked as W [12]. The pain scores were calculated according to the formula revised from Miampamba et al.:  $S = 1L + 2B + 3C + 4W$ . In this formula, L, B, C and W represent the numbers of according behaviors during the time period. The animals' behaviors were recorded continuously for 120 min and calculated for each of the successive 15-min periods.

### *Electrophysiological Recordings*

Rats were deeply anesthetized with 4% sodium pentobarbital (Beijing Chemical Corporation, 50 mg/kg) and maintained with supplementary intravenous doses of 5–10 mg/kg/h. The trachea was cannulated for artificial ventilation with room air. The rat was initially paralyzed with pancuronium bromide (Organon, USA, 0.3 mg/kg i.v.) and paralysis was maintained at a dose of

0.2–0.3 mg/kg/h. Mean arterial blood pressure was monitored continuously and was maintained at >80 mm Hg. Core body temperature was kept at 37°C by a body temperature controller (CMA/150, Sweden). The lumbosacral spinal cord was exposed by laminectomy (L6–S2) and the rat was suspended in a stereotaxic frame by thoracic vertebra and ischia clamps. The skin was retracted laterally and tied to make a pool for mineral oil. The dura was carefully removed and warm (37°C) mineral oil was applied to keep the spinal cord from drying. A tungsten microelectrode (3–5 MΩ) was inserted into the spinal dorsal horn at L6–S1. Wide dynamic range (WDR) neurons were identified according to their responses to the colorectal distension of the colon at different intraluminal pressures. Once a neuron was confirmed to be responsive, a fiber connected with a microdialysis machine (CMA 102, Sweden) was guided through L6–S1 and left in the spinal cord. After these, a colonoscope was inserted into the colon, and 100 μl of 5% formalin was injected as in the behavior study. During the process of searching for the cell and recording the control reaction, saline was used for the microdialysis. After a cell was identified, the solution was switched to PMA (10 μg/ml) or H-7 (5 mM) and administered at the rate of 5 μl/min. The concentration was chosen because the passage rate for the drugs in a microdialysis fiber is about 1–2%. The recording electrode was kept within 750 μm to the microdialysis fiber. Neuronal signals were recorded and analyzed with a PowerLab system (AD Instrument Limited, Australia). The firing frequency was analyzed in units of 15 min. The firing frequency of a neuron before formalin injection was normalized as 100% and the following discharges were expressed as the percentage of baseline.

#### SDS-PAGE and Western Blot

The rats were sacrificed by overdose of 4% sodium pentobarbital (100 mg/kg) at the time points (30, 60 and 120 min) accordingly after formalin injection. The spinal cords were then hydro-extruded with cold saline and L6–S1 segments were identified and dissected. Immediately thereafter, tissues were homogenized at 4°C in 100 μl (10 μl/mg tissue) buffer A (50 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 5 mg/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mM KF, 50 μM okadaic acid, 5 mM sodium pyrophosphate) and centrifuged at 30,000 g for 30 min at 4°C for the collection of cytosolic fractions (the supernatant). The pellet fractions were solubilized in 100 μl buffer B (buffer A mixed with 0.5% Nonidet P-40), sonicated and centrifuged again. The resulting supernatants were taken as particulate fractions. Protein concentration within each sample was determined using a BCA kit (Pierce, Rockford, Ill., USA) before equivalent loading into wells of 10% SDS-PAGE gels for electrophoresis. Protein was transferred to nitrocellulose membranes (Schleicher and Schell, USA). The membrane was washed for 10 min with TTBS (20 mM Tris-Cl, pH 7.5, containing 0.15 M NaCl, and 0.05% Tween-20) followed by the blocking solution with 10% nonfat milk in TTBS. Membranes were subsequently incubated in the PKC rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., USA) at 1:1,000 dilutions for 3 h at room temperature, respectively, and were again followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, USA) as second antibodies at 1:5,000 dilutions for 1 h. The membranes were washed three times (each for 10 min) in TTBS after the incubation with the primary or second-

ary antibodies. Finally, membranes were incubated in Supersignal West chemiluminescent reagents (Pierce) to obtain a signal for exposure to radiographic film. Immunoblot images were scanned and densitometric analysis was performed (ImageQuant, Amersham Biosciences, Piscataway, N.J., USA).

#### Data Analysis and Statistics

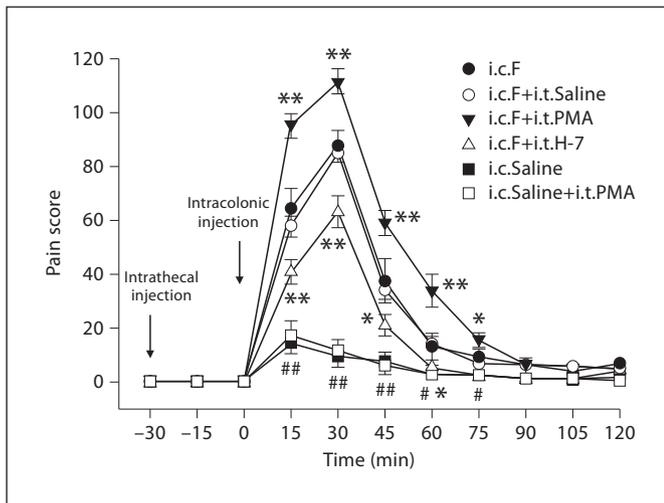
All data were expressed as means ± SEM, and statistical analysis was conducted with one way ANOVA followed by post hoc fisher's PLSD unless specifically noted. For electrophysiological data, one-way repeated measures ANOVA was adopted to compare the difference among different time points in formalin group. For the Western blot data, a quantitative analysis for the immunoblot bands was performed by using the GelDoc-2000 Imagine System (Bio-Rad Inc., USA). The membrane translocation was calculated by the ratio of membrane protein to the total protein.  $p < 0.05$  was regarded as significant.

## Results

### Effects of PKC Activation and Inhibition on Colon Pain Behaviors

Behavioral observations were started with testing the effect of intracolonic injection of saline. Mild pain behaviors (abdominal licking and nibbling) were seen after the injection and lasted for about 15 min. Pain behaviors became almost indiscernible at 60 min (fig. 1). The effect of PMA pretreatment was further tested in rats that received intracolonic saline injection. Intrathecal pretreatment with PMA produced no obvious pain behaviors (fig. 1). Further, PMA pretreatment did not produce a significant effect on the pain behavior induced by intracolonic saline injection (fig. 1,  $n = 12$ ,  $p > 0.05$ ).

Intracolonic formalin injection produced strong pain behaviors, characterized by body stretching, contraction of the flanks and body contraction, which reached the peak at 30 min and lasted about 60 min. Pain behaviors then declined, characterized by abdominal licking and nibbling. Two hours after formalin injection, pain-related behaviors could not be discerned. Pain scores of formalin group (i.c.F) were  $64.61 \pm 7.11$  at 15 min and  $88.17 \pm 5.34$  at 30 min. In contrast, the pain scores for control group (i.c. saline) were just  $14.83 \pm 4.54$  and  $9.33 \pm 3.89$ , respectively, in the same periods of time ( $n = 12$ ,  $p < 0.01$ ). After 30 min, the pain scores gradually declined. However, the pain score ( $8.83 \pm 3.37$ ) still remained at a relatively high level, significantly different from the saline group at 75 min after formalin injection ( $2.57 \pm 1.07$ ,  $n = 12$ ,  $p < 0.05$ ). Thereafter, pain behaviors had no significant difference when compared with the saline groups (fig. 1).



**Fig. 1.** Time course of behavioral changes was shown by the mean pain scores after intracolonic (i.c.) injection of formalin or saline. Total pain behaviors during the period of 120 min were observed and scored in the time unit of 15 min. Saline, PKC activator (PMA) or PKC inhibitor (H-7) was administered intrathecally 30 min before intracolonic injection of formalin or saline. Pain scores were increased following i.c. formalin injection (filled circle, i.c.F) and reached the peak at 30 min followed by a gradual decline from 30 to 120 min after formalin injection. Intrathecal (i.t.) administration of saline (open circle, i.c.F+i.t.Saline) did not affect the pain scores induced by formalin, while i.t. administration of PMA (triangle down, i.c.F+i.t.PMA) significantly enhanced the pain behaviors caused by formalin. H-7 (triangle up, i.c.F+i.t.H-7) significantly alleviated the pain induced by formalin (vs. i.c.F or i.c.F+i.t.Saline). Note that i.t. administration of PMA in the presence of intracolonic saline (i.c.Saline+i.t.PMA, open square), not formalin, injection did not significantly induce pain behaviors. \*\*  $p < 0.01$ , \*  $p < 0.05$ : significant difference when compared with group i.c.F+i.t.Saline or group i.c.F. ##  $p < 0.01$  and #  $p < 0.05$ : difference between i.c.Saline and i.c.F.  $n = 12$  for each group. The two arrows indicate the time of i.t. administration and i.c. injection, respectively.

Intrathecal pretreatment with PMA significantly enhanced the formalin-induced behaviors, shown by the obvious increase in pain scores. Pain scores were  $95.17 \pm 4.49$  (compared with group i.c.F,  $n = 12$ ,  $p < 0.01$ ) 15 min after formalin injection. This facilitation of pain behaviors by PMA lasted as long as 75 min after formalin injection (vs. group i.c.F,  $n = 12$ ,  $p < 0.05$ ). There was no statistically significant difference in pain scores among groups of formalin inflamed rats with and without PMA treatment 75 min after formalin injection. A control experiment was done by pretreating with saline intrathecally and no significant effect on the formalin-induced pain was seen (line with empty circle, fig. 1).

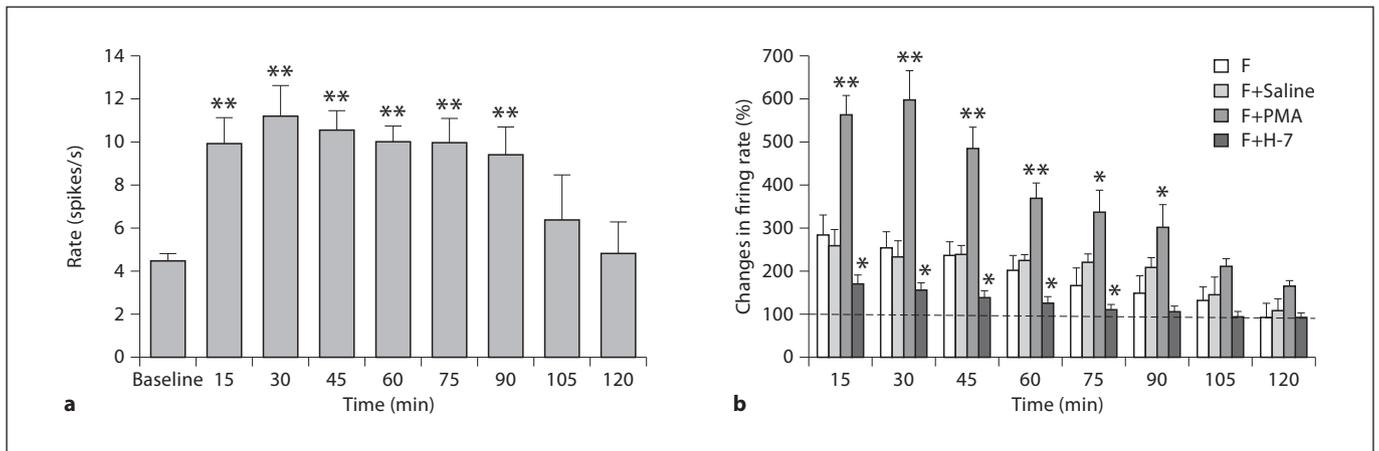
Pretreatment with a PKC inhibitor, H-7, decreased significantly the pain behaviors induced by formalin injection. At 15 min after formalin injection, H-7 decreased the pain scores ( $40.83 \pm 4.54$ ), which were significantly lower than in the colon-inflamed rats with intrathecal saline pretreatment ( $64.61 \pm 7.11$ ,  $n = 12$ ,  $p < 0.01$ ) (fig. 1). The inhibition of pain behaviors induced by H-7 lasted about 75 min, and no significant difference was discerned in pain scores between the i.c.F and i.c.F+i.t.H-7 groups starting from 75 min after formalin injection. All these data demonstrate that formalin-induced consistent colon pain involves the activation of PKC.

#### *Changes in Dorsal Horn Neuronal Activity Induced by Formalin Injection and Effects of PKC Activation and Inhibition*

A total of 24 WDR neurons were recorded in 24 rats. Six rats were included in each group. All neurons recorded were 100–500  $\mu\text{m}$  below the dorsal surface of the spinal cord. Fifteen minutes after formalin injection, the average discharge rate was  $10.13 \pm 1.66$  Hz, more than 2-fold compared with baseline ( $4.48 \pm 0.32$  Hz,  $n = 6$ ,  $p < 0.01$ ). After that, the firing frequency decreased gradually, but remained significantly higher compared with baseline at 90 min after formalin injection. Beyond 90 min, no significant difference was discerned in the firing frequency compared with the baseline (fig. 2a).

Microdialysis pretreatment with PMA significantly increased the firing frequency to  $563.4 \pm 44.60\%$  at 15 min after formalin injection, while the frequency of those subjected to intracolonic injection of formalin only was  $283.75 \pm 46.1\%$ . The enhancement by PMA reached a peak ( $577.1 \pm 42.3\%$ ) at 30 min after formalin injection, and remained at an enhanced level during a period of 30–90 min. After that, the PMA effect was minimal and no significant difference was discerned compared with those pretreated with saline (fig. 2b). The average discharge rate after microdialysis pretreatment with H-7 was lowered to  $169.7 \pm 20.5\%$  at 15 min after formalin injection, which was significantly decreased compared with those ( $283.75 \pm 46.1\%$ ) pretreated with saline microdialysis at the same time point. At 30 min after formalin injection, the firing rate was  $155.40 \pm 8.70\%$  significantly lowered than those ( $254.0 \pm 37.4\%$ ) pretreated with saline at the same time point (fig. 2b). Starting from 75 min, no difference was discerned in the H-7 group when compared with those subjected to saline microdialysis.

A comparison between the F and F+saline groups showed no significant difference at all time points, dem-



**Fig. 2.** Time course of intracolonic injection of formalin induced spinal WDR neuronal firing. **a** Grouped results showed that the discharge frequency significantly increased 15 min after formalin injection and lasted for 90 min compared with baseline ( $n = 6$ ,  $p < 0.01$ ). **b** Changes in neuronal firing after formalin injection and the effect caused by application of PMA, H-7 or saline by microdialysis. The discharge frequency before formalin injection was set as 100%. There was no significant difference in the en-

hanced activity of neurons between groups of formalin injection only (F) and formalin injection with saline pretreatment (F+Saline). PMA enhanced significantly the firing of WDR neurons during the period of 90 min after formalin injection (F+PMA); while H-7 markedly inhibited the enhanced discharge for about 75 min (F+H-7). \*  $p < 0.05$  and \*\*  $p < 0.01$ : compared with F+Saline or F.  $n = 6$  for each group.

onstrating that microdialysis did not significantly affect the neuronal activities.

#### *Changes in Membrane Translocation of PKC- $\gamma$ and PKC- $\epsilon$ Subtypes in the Course of Colon Pain*

Because the behavior test shows that the maximum pain scores appeared at 30 min and a significant decline was evident at 60 min after formalin injection, the spinal cords were sampled for Western analysis at 30, 60 and 120 min to measure the changes in membrane translocation of PKC subtypes. Of the four cPKC subtypes (PKC- $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), PKC- $\gamma$  witnessed an obvious change induced by formalin injection. Its membrane translocation was significantly increased 30 min after formalin injection compared with naïve rats ( $37.1 \pm 3.2\%$  vs.  $22.4 \pm 5.1\%$ ,  $n = 8$ ,  $p < 0.05$ ; fig. 3a). No statistically significant difference was discerned 60 and 120 min after formalin injection compared to the control groups (naïve and intracolonic saline injection). There were no significant changes in subtypes  $\alpha$ ,  $\beta$ I and  $\beta$ II after formalin injection (fig. 4a–c).

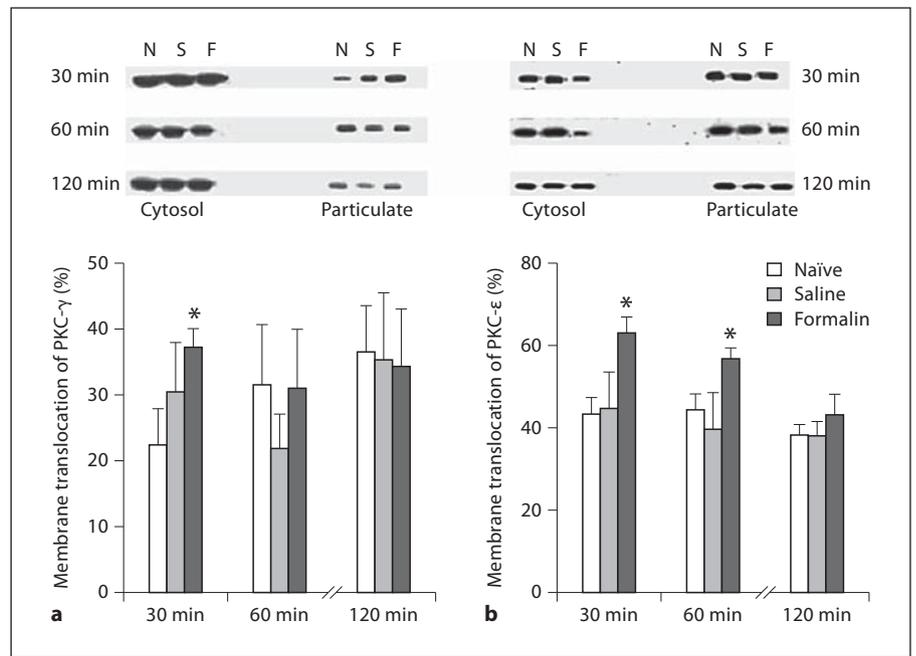
For nPKC subtypes, the membrane translocation of nPKC- $\epsilon$ , but not of nPKC- $\theta$ ,  $\eta$  or  $\delta$ , was significantly increased ( $63.5 \pm 4.7\%$ ) at 30 min after formalin injection when compared to the groups with naïve and intracolonic saline injection ( $43.2 \pm 4.6\%$ ,  $n = 8$ ,  $p < 0.05$ ). Unlike the PKC- $\gamma$ , an increased expression of PKC- $\epsilon$  was

still discerned 60 min after formalin injection ( $57.2 \pm 3.5\%$  for group F vs.  $44.8 \pm 5.3\%$  for group N,  $n = 8$ ,  $p < 0.05$ ; fig. 3b).

#### **Discussion**

The present study is the first to provide direct evidence that PKC in the spinal cord participates actively in the colon pain induced by intracolonic injection of formalin, and that the subtypes involved are PKC- $\gamma$  and PKC- $\epsilon$ , which are activated in the form of membrane translocation.

Several types of colon pain models have been used for studying the mechanisms of visceral pain. The models of colon inflammatory pain are induced by intracolonic instillation of a variety of inflammogens [9, 10, 16, 17], and are well accepted to be a means for experimental research of visceral pain. Among those inflammogens, formalin administration has been proved to reliably evoke the colon pain [18–20]. In our experiments, we found that the pain behavior reached a peak at 30 min after formalin injection. Ninety minutes after formalin injection, no obvious difference was observed when compared to the control group. These are consistent with the report by Miampamba et al. [12] and supported by our electrophys-



**Fig. 3.** Changes in membrane translocation of cPKC- $\gamma$  and nPKC- $\epsilon$  in the spinal cord after intracolonic injection of formalin. **a** Upper panel: Western blot analysis of expression of cPKC- $\gamma$  membrane translocation at 30, 60 and 120 min after formalin injection. Left, the expression of PKC- $\gamma$  in the cytosol of spinal cord. Right, the expression of PKC- $\gamma$  in the particulate. Lower panel: quantitative analysis indicated that levels of cPKC- $\gamma$  membrane translocation increased 30 min after formalin injection. **b** Upper panel:

Western blot analysis of expression of nPKC- $\epsilon$  membrane translocation at 30, 60 and 120 min after formalin injection. Left: expression of PKC- $\gamma$  in the cytosol of spinal cord. Right: expression of PKC- $\gamma$  in the particulate. Lower panel: quantitative analysis indicated that the levels of PKC- $\epsilon$  membrane translocation increased at 30 and 60 min after formalin injection. \*  $p < 0.05$  versus naïve or saline,  $n = 8$  for each group. N = Naïve rats; S = i.c. injection of saline; F = i.c. injection of formalin.

iological data in which WDR neurons were seen to be sensitized after formalin injection. Therefore, an enhanced response of pain behavior after formalin injection was closely correlated with the increased firing of WDR neurons in the spinal dorsal horn. After inflammation by formalin, peripheral recruitment of receptors on colon nociceptors can produce nociceptive influxes [12, 21, 22], which will drive afferent nociceptive axonal terminals in the spinal dorsal horn to robustly release glutamate [23–25]. The binding of glutamate to N-methyl-D-aspartate (NMDA) receptors mediates calcium flux, initiating the activation and relocation of PKC to the cell membrane [26, 27]. Furthermore, there is evidence showing that upon persistent nociceptive stimulation PKC can phosphorylate NMDA receptors, thus potentiating their channel activities [28]. The enhanced function of NMDA receptors, combined with the excessive glutamate release from the central terminals of nociceptive neurons after peripheral inflammation, provides a positive feedback loop for the central sensitization. In addition, nociceptive

stimulation drives primary afferents in the spinal dorsal horn to release substance P that binds to neurokinin 1 receptors contributing to sensitization of WDR neurons via the PKC-dependent pathway [29, 30].

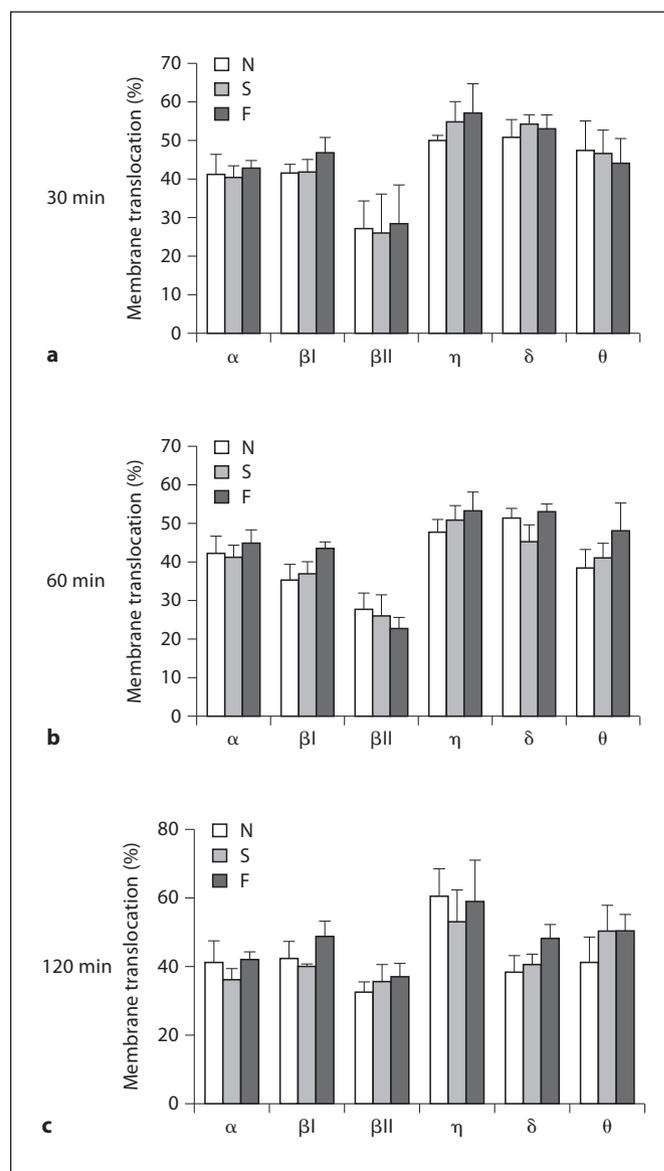
On the other hand, sensitization of primary afferent neurons in dorsal root ganglia has also been suggested to contribute to the induction and development of visceral pain, which is characterized by an enhanced excitability of dorsal root ganglia neurons under the conditions of visceral pain [31–33]. All of these cases of neuroplasticity in the course of central and peripheral sensitization would help explain the behavioral changes after intracolonic injection of formalin.

In our experiments, intrathecal administration of H-7 significantly alleviated the pain behavior and inhibited the sensitization of WDR neurons induced by formalin injection. H-7 has been widely used as a PKC inhibitor [34–36], and its  $K_i$  value for inhibiting PKC is about  $6 \mu\text{M}$  [37], while the  $K_i$  value for inhibiting  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases is  $97 \mu\text{M}$  [38]. The concentra-

tion of H-7 applied in our study was about 30–50  $\mu\text{M}$ ; thus, the dose we chose was more likely to inhibit PKC rather than other protein kinases. The result that application of PMA enhances pain, together with the fact that microdialysis of PMA could further increase the firings of neurons in the spinal dorsal horn after formalin injection provided further evidence for the involvement of PKC in the colon pain. PMA has been adopted in numerous studies as a PKC activator [39–41]. The PMA concentration we chose in the experiments was approximately 8 nM, which is believed to specifically activate PKC [42], and a similar dose was adopted in a report by Codderre [43]. In addition, PMA administered 30 min before intracolonic injection (saline or formalin) did not produce any obvious visceral pain behaviors. This supports the view that the activation of PKC per se in the spinal cord would not evoke colon pain, but its activation could be critical for the induction and development of colon pain induced by formalin.

So far, there is still a lack of detailed information regarding which subtypes of PKC are involved in the colon pain induced by intracolonic application of formalin. PKC- $\gamma$  is fundamental for the development of neuropathic pain [1]. Additionally, evidence supports the idea that PKC- $\gamma$  also participates in inflammatory pain [44]. There is one report about the activation of PKC- $\gamma$  in visceral hyperalgesia [45], and here we also showed that PKC- $\gamma$  was upregulated in membrane translocation in the colon pain model. This shows clearly that PKC- $\gamma$  can also be activated by intracolonic nociceptive stimulation.

In our experiments, PKC- $\epsilon$  was activated, as shown by the increased membrane translocation after intracolonic application of formalin, and the time course of PKC- $\epsilon$  membrane translocation was consistent with the behavioral changes. The coincidence of the time course indicates that the activation of PKC- $\epsilon$  should be closely related with the initiation and development of colon pain. Indeed, PKC- $\epsilon$  has been shown to contribute to acute and chronic somatic hyperalgesia induced by several inflammatory mediators such as carrageenan [46] and formalin [44] or inflammatory mediators such as bradykinin, tumor necrosis factor- $\alpha$  and prostaglandin  $E_2$  [47–49]. Our experiments provide evidence showing that PKC- $\epsilon$  was also involved in colon pain. It is highly suggested that intracolonic application of formalin damages the tissue, which releases inflammatory mediators like bradykinin that activates G-protein-coupled receptors, kinin  $B_2$ , to stimulate phospholipase C (PLC) signaling in primary afferent neurons [50]. PLC has been proven to be an upstream signal for PKC- $\epsilon$  activation in both acute and chronic in-



**Fig. 4.** No changes in membrane translocation for PKC isoforms ( $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ ,  $\eta$ ,  $\delta$  and  $\theta$ ) in the spinal cord after intracolonic injection of formalin. Quantitative analysis showed no changes in membrane translocation for the PKC isoforms mentioned above at 30 (a), 60 (b) and 120 min (c) after formalin injection.  $n = 8$  for each group. N = Naïve rats; S = i.c. injection of saline; F = i.c. injection of formalin.

flammatory hyperalgesia [51]. Furthermore, activation of PKC- $\epsilon$  can phosphorylate the transient receptor potential vanilloid 1 that serves as a polymodal detector of pain-producing chemical and physical stimuli [52], contributing importantly to the development of visceral hyperalgesia.

However, the enhanced expression of PKC- $\gamma$  seems to be a short-lasting process compared with that seen in PKC- $\epsilon$ . Thus, we propose that PKC- $\gamma$  would play a role in the initiation of colon pain, while PKC- $\epsilon$  would be important for both the initiation and maintenance of colon pain.

PKCs subtypes, such as PKC- $\alpha$ ,  $\beta$ I and  $\beta$ II, have been shown to be involved in neuropathic pain [53, 54]. For the subtypes of nPKC, PKC- $\delta$  has been demonstrated to contribute to the thermal hyperalgesia induced by the interleukin-6/gp130 ligand-receptor complex [55]. In the present study, we also screened the changes in the PKC subtypes mentioned above following formalin injection. However, there were no significant changes in the expression of these subtypes. Therefore, various PKC subtypes may get involved in different types of pain, and this provides a possibility that individual treatment can be tar-

geted against certain PKC subtypes in a certain type of pain.

In summary, the findings from our study deliver strong evidence that PKC at the spinal cord level participates actively in the colon pain following colonic inflammatory stimuli. Among the subtypes, PKC- $\gamma$  and PKC- $\epsilon$  are indicated to be the prominent ones in the pathogenesis of formalin-induced colon pain.

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