Protein Kinase C ε and γ: Involvement in Formalin-Induced Nociception in Neonatal Rats

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ABSTRACT

The central nervous system undergoes dynamic changes as it matures. However, until recently, very little was known about the impact of these changes on pain and analgesia. This study tested the hypothesis that the ε and γ isozymes of protein kinase C (PKC) contribute to formalin-induced nociception in an age-dependent manner. Expression of ε and γ PKC and the contributions of these isozymes in formalin-induced nociception was examined in postnatal day 7, 15, and 21 rats. εPKC expression in dorsal root ganglion neurons and γPKC expression in lamina II of the spinal cord increased from the first to the third postnatal week. Coupling immunohistochemical and Western analysis, translocation of εPKC followed intraplantar formalin in all ages. In contrast, formalin-induced γPKC translocation was observed only in postnatal day 21 rats. Behaviorally, intrathecal administration of the εPKC-specific inhibitor (εV1-2) attenuated phase 1 and phase 2 formalin behaviors at all ages. In contrast, intrathecal administration of the γPKC-specific inhibitor (γV5-3) attenuated only phase 2 responses in postnatal day 15 and 21 rats. Functionally, inhibition of εPKC decreased capsaicin-stimulated release of glutamate and calcitonin gene-related peptide in spinal cords isolated from postnatal day 7 rats. These results suggest that εPKC age independently mediates inflammatory pain produced by intraplantar formalin. In contrast, γPKC contributes to formalin-induced nociception in an age-dependent manner. Identifying the molecular mechanisms responsible for age-specific patterns of nociception is necessary for the rational development of novel therapeutic strategies for treating pediatric pain.

There is an increasing recognition for the need to adequately assess and treat pain in infants and children. To do this requires understanding how development may influence pain processing. For instance, intraplantar formalin has been shown to elicit age-dependent pain behaviors in neonatal rats (Teng and Abbott, 1998). In postnatal day 21 (P21) rats, intraplantar formalin produces a similar biphasic behavioral pattern as that observed in adult rats. In adult rats, phase 1 of the formalin response is characterized by the activation of primary afferents (Puig and Sorkin, 1996) and the release of excitatory neurotransmitters into the dorsal horn of the spinal cord (Skilling et al., 1988). Activation of descending inhibitory pathways follows, reducing spontaneous pain behaviors (quiescent phase). Phase 2 follows and is mediated by ongoing primary afferent activity (Skilling et al., 1988; Taylor et al., 1995; Puig and Sorkin, 1996) as well as by increased sensitivity of spinal responses to excitatory input (Dickenson and Sullivan, 1987; Coderre et al., 1990). P15 rats also exhibit a biphasic response but it is shorter in duration than that observed in P21 and adult rats. In contrast, P7 rats are 4-fold more sensitive to the nociceptive effects of intraplantar formalin compared with adult rats and exhibit a monophasic response pattern (Teng and Abbott, 1998).

Nonspecific inhibitors of protein kinase C (PKC) attenuate phase 2 formalin-induced pain behaviors in adult animals (Yashpal et al., 1995). Increased activation and translocation of PKC in dorsal horn neurons has been reported after peripheral administration of formalin (Yashpal et al., 1995). PKC is an important family of signal transduction molecules that function to phosphorylate a wide range of intracellular proteins. The PKC gene family has been divided into three groups of isozymes based on sequence homology and biochemistry: calcium- and diacylglycerol (DAG)-dependent PKC isozymes (α, βI, and γ), Ca²⁺-independent but
DAG-dependent PKC isozymes (δ, ε, η, and θ), and Ca²⁺- and DAG-independent PKC isozymes. In adult rats, γPKC has been identified in the superficial laminae of the dorsal spinal cord (Polgar et al., 1999) whereas εPKC has been identified in adult rat primary afferents that terminate in the superficial dorsal horn (Khasar et al., 1999). In addition to the presence of ε and γPKC in anatomical sites important for pain processing, a functional role for these two isozymes in pain processing in adult rodents has been identified using knockout mouse technology (Malmberg et al., 1997; Khasar et al., 1999).

Expression and activity of some PKC isozymes are developmentally regulated (Hashimoto et al., 1988; Akinori, 1998). The impact of age-dependent changes in PKC isozymes on pain processing is not known. PKC translocation requires the binding of PKC to isoyme-selective docking proteins, receptors for activated C kinase (RACK) (Mochly-Rosen and Gordon, 1998). Isozyme-selective PKC inhibitor peptides compete with activated PKCs for binding to RACK, thus preventing translocation and activity of the corresponding PKC isozymes (Mochly-Rosen and Gordon, 1998). Linking of isozyme-specific PKC inhibitor peptides to cell-permeable carrier peptides (e.g., derived from Tat, antenapedia, or even a poly arginine) enables efficient transfer of inhibitor peptides into cells and tissue (Chen et al., 2001). Upon entering the cell, the disulfide bond linking carrier to peptide is reduced, releasing the inhibitor peptide inside the cell to interact with its corresponding RACK (Chen et al., 2001). In the present study, isozyme-specific peptide inhibitors were used to determine whether intratral formalin induces translocation of ε and γPKC in an age-specific manner and whether this translocation mediates nociception.

**Materials and Methods**

**Animals.** P7, P15, and P21 male and female Sprague-Dawley rats (Simenson Laboratories, Gilroy, CA) were housed with their dam in a 12/12-h light/dark cycle (lights on at 7:00 AM) with food and water available ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Stanford University. Efforts were made throughout the experiment to minimize animal discomfort and to reduce the number of animals used.

**Peptide Synthesis.** All peptides were synthesized at Stanford's Protein and Nucleic Acid facility and conjugated to Tat, amino acids 47-57 [YGRKKRRQRRR] via a cysteine-cysteine bond at their N termini. The ePKC antagonist eV1-2 [RAVSLKPT] and the γPKC antagonist γV5-3 [CRLVLASC] were used at >90% purity.

**Experimental Design.** Spinal drug delivery in neonatal rats poses a unique challenge based on the size of the neonatal rats and the rapid elongation of the vertebral column and spinal cord during the first few postnatal weeks. These factors make insertion of a spinal catheter prohibitive or at the very least extremely difficult. Thus, we administered PKC peptide inhibitors reversibly linked to Tat carrier protein (5 μl in P7 rats, 10 μl in P15 and P21 rats) via direct lumbar puncture. The volume necessary to deliver the peptides to the lumbar spinal cord was determined previously by using Evan’s blue dye to determine that the spread of fluid reached the lumbar spinal cord. The increasing length and volume of the spinal cord between P7 and P21 required a larger volume of the same concentration to be delivered in the older rat. Thus, although twice as much compound is being delivered in P21 rats compared with P7 rats the increase in the surface area being covered by that volume ensures that the same anatomical region of spinal cord is seeing equivalent concentrations of drug (Marsh et al., 1999). Intrathecal drug delivery was completed by inserting a sterile 29-gauge 3/10-ml insulin syringe between the S1 and L6 vertebrae in rats anesthetized with halothane.

Tat-conjugated peptide inhibitors specific to the ε (eV1-2) and γ (γV3-5) isoforms of PKC, Tat carrier alone, or saline vehicle were administered 15 min before intraplantar formalin (1% in 10 μl in P7 pups, 2.5% in 10 μl in P15 and P21 pups; n = 6–10/group) or saline. Peptides were administered at 10, 50, or 100 pmol/5 μl. Rats were placed in Plexiglas observation boxes (3.5 inches in width × 6 inches in length × 8 inches in height), and behaviors were observed for 1 h. In all experiments, animals were maintained at nesting temperature with a heating pad placed below the observation chambers. The time sampling method for behavioral observation was used, in which the observer rapidly records the behavior of the animals every 2 min (Teng and Abbott, 1998). A score of 1 is given if the animal is licking, shaking, or elevating its hindpaw. A 6-min period of observation provides a maximum pain score of 3 for each animal. All studies were completed with the experimenter blinded to treatment group.

**Immunohistochemistry.** P7, P15, and P21 rats were administered 50 pmol/5 μl of Tat carrier, eV1-2, or γV3-5 intrathecally 15 min before intraplantar formalin (1% in 10 μl in P7 rats, 2.5% in 10 μl in P15 and P21 rats) or saline as described above in the behavioral experiments. At 5 and 10 min postformalin or saline, rats were deeply anesthetized and underwent transcardiac perfusion (n = 3/treatment/time). Lumbar spinal cord and L5 dorsal root ganglia (DRG) were harvested and fixed in 4% paraformaldehyde for 3 h. Tissue was cryoprotected in 30% sucrose at 4°C for a minimum of 48 h. Sections were freeze-mounted in OCT embedding medium on cork blocks for cryostat sectioning. For spinal cord sections, free-floating immunohistochemistry was performed on 30-μm L–L5 spinal sections. DRG were cut on a cryostat at 10 μm and slide mounted. Slides were heated overnight at 32°C. Immunohistochemistry was performed using an avidin-biotin complex technique as described previously (Sweitzer et al., 1999). Rabbit polyclonal antibodies to γPKC (1:500; cPKCγ and sc-211) or ePKC (1:1000; nPKCs and sc-214) from Santa Cruz Biotechnology (Santa Cruz, CA) were used.

Immunohistochemistry was scored blinded to experimental conditions. ePKC immunoreactive DRGs and γPKC immunoreactive lumbar spinal cords were imaged with a digital camera, and the images were transferred to a computer for analysis of cell counts and/or densitometry. All data represents the average from at least three tissue sections/animal with a minimum of three animals per group. Mean density of ePKC immunoreactivity per DRG section was calculated using ImageJ software. Density was normalized to tissue staining levels in a age-matched naive rat using the following calculation: TD−NORM = TD−NORM. TRT−NORM = TRT−NORM. TD is the staining density in the tissue from the treated rat, and NORM is the staining density in the tissue from normal naive rats. TD represents the total staining density of the tissue, and BD is the background staining density.

**Western Analysis.** Identical to the immunohistochemical analysis, P7 and P21 rats were administered 50 pmol/5 μl of Tat carrier, eV1-2, or γV3-5 intrathecally 15 min before intraplantar formalin (1% in 10 μl in P7 rats, 2.5% in 10 μl in P21 rats) or saline. At 5 and 10 min postformalin or saline, left lumbar spinal cord and left L4–L6 DRG were harvested and frozen on dry ice after CO₂ asphyxiation and decapitation (n = 3/time/treatment). DRG and spinal cord tissues were homogenized and sonicated in lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, and 1% Triton X-100) supplemented with protease inhibitors (Complete Mini; Roche Diagnostics, Mannheim, Germany). Proteins were quantitated using the BCA protein assay (Pierce Chemical, Rockford, IL), and 12 or 18 μg of protein (DRG or spinal cord, respectively) in SDS loading buffer was resolved on 7.5% SDS polyacrylamide gels. After protein transfer, polyvinylidene difluoride membranes were blocked in TWEEN 20/PBS containing 5% powdered milk for 1 h at room temperature. Membranes were then blotted with nPKCs (1:200; sc-214), or cPKCγ (1:200; sc-211) (Santa Cruz Biotechnology) overnight at 4°C in TWEEN 20/PBS containing 5% powdered milk. Membranes were probed with c antibodies to the corresponding RACK (Chen et al., 2001). In the present study, isozyme-specific peptide inhibitors were used to determine whether intratral formalin induces translocation of ε and γPKC in an age-specific manner and whether this translocation mediates nociception.
were then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2500) (Jackson Immunoresearch Laboratories Inc., West Grove, PA), and bands were detected using enhanced chemiluminescence detection (Amersham Biosciences UK, Ltd., Buckinghamshire, Little Chalfont, UK). Membranes were then blotted with β-actin (1:5000; Sigma-Aldrich) for 2 h at room temperature in Tween 20/PBS containing 5% powdered milk. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:2500) (Cell Signaling Technology Inc., Beverly, MA) and bands were detected using enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Buckinghamshire, England).

Calcitonin Gene-Related Peptide (CGRP) and Glutamate Release Assays. Whole spinal cords from P7 rats were isolated and maintained in oxygenated artificial cerebrospinal fluid for 1 h before experimental manipulation. Release assays using whole isolated spinal cords were only studied in P7 rats. Our previous experience suggests limited viability of this whole cord preparation prepared from older rats. The dose of capsaicin required to elicit maximal release of CGRP was determined by incubating the spinal cords in 800 μl of artificial cerebrospinal fluid to serve as baseline control release. A second 10-min incubation was done in the presence of 0, 2.5, 25, and 100 μM capsaicin (n = 4/treatment). To determine the dependence on extracellular calcium isolated cords were maintained in artificial cerebrospinal fluid lacking Ca2+ throughout the equilibration period as well as during the baseline control release and capsaicin (25 μM)-stimulated release.

The broad-spectrum PKC inhibitor GF109203X (1.2 μM; Sigma-Aldrich), the calcium-dependent PKC inhibitor Go6976 (1 μM; Calbiochem, San Diego, CA), the εPKC-specific peptide inhibitor (εV1-2; 25.6 pmol), Tat carrier (25.6 pmol), or saline vehicle was introduced in 800 μl of artificial cerebrospinal fluid for 10 min (n = 4/treatment). This fraction was collected and served as baseline control release. The concentration of PKC inhibitors is based on other studies in this laboratory showing efficacy at this concentration in preventing naloxone precipitated hyperresponsiveness of the slow ventral root potential after morphine exposure in P7 spinal cord (Sweitzer et al., 2004). Capsaicin (25 μM) was introduced in the presence of either εV1-2, Tat carrier, or saline vehicle and incubated with the isolated cords for 10 min.

Analysis of CGRP by Enzyme-Linked Immunosorbent Assay. Samples were analyzed using the rat CGRP enzyme immunoassay kit (SIP-Bio, Massy Cedex, France). Each sample was performed in duplicate as directed in the instructions. Briefly, each plate was washed three times with 300 μl of wash buffer before adding 100 μl of either sample, standard, or buffer to each well. In addition, each well received 100 μl of anti-CGRP acetylcholinesterase tracer. Samples were incubated overnight at 4°C with agitation. The next day, samples were washed three times with 300 μl of wash buffer, before adding 200 μl of Ellman’s reagent. Absorbances were recorded at 405 nm at 30 min. CGRP concentrations were determined by reference of the sample absorbances to the standard curve generated off the recombinant rat CGRP supplied with the kit.

Analysis of Glutamate by High-Performance Liquid Chromatography. Methods were derived from Graser et al. (1985). Samples (20 μl) were reacted with 1.0 μl of o-phenyldiaide/3-mercaptoprionic acid reagent (Sigma-Aldrich) for derivitization. After 1 min, a 20-μl sample was injected into the high-performance liquid chromatograph, which uses a reversed-phase C18 column (3.9 × 150 mm; Waters, Bedford, MA). Glutamic acid was quantitated at absorbance of 340 nm (Waters 2487). The samples were separated with a mobile phase gradient using the following solutions: 12.5 mM NaPO4, pH 7.2, and a 50% (v/v) mixture of 12.5 mM NaPO4 and acetonitrile. The glutamate peak occurs during the first 5 min after sample application when the mobile phase is isocratic phosphate buffer. The column was flushed by using a steep gradient (ending in 50% acetonitrile) before the next sample. This procedure allowed for the detection of glutamate at a sensitivity of 0.5 to 2.0 ng/sample. A dose-response curve was generated on each day of sampling. A PC-based data acquisition and analysis system (Millennium; Waters) was used to quantify glutamate based on the area under the curves.

Statistical Analysis. Behavioral data were analyzed for significance by analysis of variance for repeated measures followed by a post hoc Bonferroni analysis. Glutamate and CGRP release data were analyzed for significance by one-way analysis of variance followed by a post hoc Bonferroni analysis. Glutamate and CGRP release data were analyzed by t test. P values <0.05 were considered significant. All statistical analysis was done with GraphPad Prism version 3.02 (GraphPad Software Inc., San Diego, CA; www.graphpad.com).

Results

Developmental Changes in εPKC and γPKC Expression. Expression of εPKC expression in the L5 DRG increased with maturation. Approximately 41% of DRG cells expressed εPKC in P7 rats. The number of εPKC-positive

Fig. 1. Expression of εPKC in L5 dorsal root ganglion increased with postnatal maturation. In the absence of primary antibody, no appreciable staining is observed (A). Both small- and medium-diameter cell bodies showed εPKC-ir in P7 (B) and P15 (C) rats. In P21 rats, εPKC-ir was observed in both large- (large arrow), medium-, and small (small arrow)-diameter cell bodies (C). Scale bar, 100 μm.
TABLE 1
Age-specific ePKC expression in L5 dorsal root ganglion neurons and γPKC expression in lumbar spinal cord

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Dorsal Root Ganglion (% ePKC IR Cells)</th>
<th>Lumbar Spinal Cord (No. γPKC IR Cell Soma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lamina II</td>
</tr>
<tr>
<td>7</td>
<td>41.1 ± 1.8**,**†††</td>
<td>9 ± 1.5**,**†††</td>
</tr>
<tr>
<td>15</td>
<td>53.0 ± 1.8</td>
<td>17 ± 2.6**,**†††</td>
</tr>
<tr>
<td>21</td>
<td>60.3 ± 5.1</td>
<td>34 ± 2.5</td>
</tr>
</tbody>
</table>

IR, immunoreactive.
** Significantly different from postnatal day 15, P < 0.01.
††† Significantly different from postnatal day 21, P < 0.001.

PKC Isozymes and Formalin Nociception

The number of γPKC-positive neuronal somata and the location of these cells within the spinal cord changed with age. Within the dorsal horn, γPKC-positive cell somata increased in number and became localized to lamina II interneurons with postnatal age (Fig. 2, A–C; Table 1). In P7 rats, few cell somata stained positive for γPKC in lamina II, although fiber-like γPKC-ir within this lamina was observed (data not shown). In contrast, within the ventral horn, the number of γPKC-positive neurons decreased with postnatal maturation (Fig. 2, D–F; Table 1). Ventral horn γPKC-ir was predominantly nuclear in P7 rats. In P15 and P21 rats, γPKC-ir was both nuclear and cytoplasmic in ventral horn motor neurons (Fig. 2, D–F).

Translocation of ε and γPKC after Intraplantar Formalin and Prevention by Isozyme-Specific Inhibitors.

Using densitometry, decreased ePKC-ir in DRG was observed at 5 and 10 min postformalin at all three ages (Table 2; Fig. 3B). Intrathecal administration of the ePKC translocation inhibitor eV1-2 (50 pmol/5 μl) prevented the loss of ePKC-ir after formalin (Table 2; Fig. 3C). At postnatal day 21, a decrease in ePKC-ir was observed in both small- and large-diameter primary afferent cell bodies after formalin (Fig. 3B). Inhibition of γPKC translocation with γV3-5 did not alter ePKC-ir at any age (data not shown). By Western analysis, intraplantar formalin did not alter ePKC protein levels in DRGs from P7 and P21 rats (Fig. 3).

Intraplantar formalin did not alter γPKC-ir in the ventral horn at any postnatal age (data not shown). No change in the intensity, number of positive staining cells or gross intracellular localization of γPKC-ir within the dorsal horn was observed at 5 or 10 min postformalin in P7 and P15 rats. In contrast, increased γPKC-ir in lamina II of the lumbar spinal cord was observed at 10 min postformalin in P21 rats (Fig. 4C; Table 3). The increase in γPKC-ir was prevented by intrathecal administration of γV3-5 (50 pmol/5 μl) (Fig. 4D). Interestingly, in P21 rats, eV1-2 attenuated the formalin-induced increase in lamina II γPKC-ir (Fig. 4E). By Western analysis, intraplantar formalin did not alter γPKC protein levels in the lumbar spinal cord from P7 and P21 rats (Fig. 4G).

Modulation of Formalin-Induced Nociception by ePKC: Controls.

Intraplantar saline produced mild and transient behaviors that were more marked in P7 (Fig. 5A) rats than in P15 and P21 rats (Fig. 5, B and C, respectively). Intrathecal administration of eV1-2, γV5-3, or Tat carrier 15 min before intraplantar saline did not alter saline-associated responses (Fig. 5, A–C). Tat carrier administration did not alter age-specific formalin responses compared with saline vehicle (Fig. 5, D–F).

Modulation of Formalin-Induced Nociception by ePKC and γPKC.

Intrathecal administration of eV1-2 dose dependently attenuated phase 1 and phase 2 behaviors in all ages (Fig. 6). Statistical analysis of eV1-2 antinociception as a function of phase of the formalin response is shown in Fig. 6D. In P7 rats the age-specific monophasic formalin response was artificially split into three phases for behavioral comparison with older rats that display the stereotypic phase 1, quiescence and phase 2 formalin response. Furthermore, for all ages phase 2 was defined as beginning at 18 min and

![Fig. 2](image-url) Age-specific expression of γPKC in the lumbar spinal cord. In lamina II, the number of γPKC-ir neuronal cell somata increased with postnatal maturation with few apparent at P7 (A) compared with P15 (B) or P21 (C). In contrast, γPKC-ir in the ventral horn decreased with postnatal maturation with fewer γPKC-ir motor neurons at P21 (F) compared with P15 (E) or P7 (D). Scale bar, 30 μm (A–C) and 100 μm (D–F).
ending when behaviors significantly higher than baseline behaviors ceased. In P7 rats, 50 or 100 pmol/5 μl of eV1-2 produced a biphasic behavioral profile and attenuated behaviors in all phases of the formalin response (Fig. 6A). In P15 rats, all doses of eV1-2 attenuated phase 2 behaviors, whereas only the highest dose attenuated phase 1 behaviors (Fig. 6B). In P21 rats, all doses of eV1-2 attenuated phase 2 behaviors, and all but the lowest dose attenuated phase 1 behaviors (Fig. 6C).

In contrast to ePKC, inhibition of γPKC translocation dose dependently attenuated only phase 2 formalin responses (Fig. 7). Statistical analysis of γV3-5 antinociception as a function of phase of the formalin response is shown in Fig. 7D. Attenuation of phase 2 behaviors was age-dependent. In P7 rats, γV3-5 did not alter formalin-induced pain behaviors at any dose examined (Fig. 7A). In P15 rats, the two higher doses of γV3-5 attenuated phase 2 behaviors (Fig. 7B). In P21 rats, all doses of γV3-5 examined attenuated phase 2 behaviors (Fig. 7C).

**Table 2**

Translocation of ePKC in L5 dorsal root ganglion neurons from P7, P15, or P21 rats after intraplantar formalin or saline

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Normal</th>
<th>5-min Formalin</th>
<th>10-min Formalin + Tat Carrier</th>
<th>10-min Formalin + eV1-2</th>
<th>10-min Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>100 ± 13.2</td>
<td>42.5 ± 10.7**</td>
<td>22.5 ± 4.9***</td>
<td>23.1 ± 7.6**</td>
<td>96.5 ± 7.5***</td>
</tr>
<tr>
<td>15</td>
<td>100 ± 4.9</td>
<td>25.7 ± 5.1***</td>
<td>20.9 ± 4.5***</td>
<td>27.7 ± 6.4***</td>
<td>87.1 ± 8.7****</td>
</tr>
<tr>
<td>21</td>
<td>100 ± 10.8</td>
<td>14.8 ± 2.5***</td>
<td>20.7 ± 3.9***</td>
<td>35.4 ± 5.2*</td>
<td>81.4 ± 14.2*</td>
</tr>
</tbody>
</table>

* ** Significantly different from normal, P < 0.05, 0.01, and 0.001, respectively.
† †† ††† Significantly different from 10-min formalin + Tat carrier, P < 0.05, 0.01, and 0.001, respectively.
¶¶ ¶¶¶ Significantly different from 10-min formalin, P < 0.01 and 0.001, respectively.
mate release by 20% and CGRP release by 55% (Table 5). At the end of the release assays, electrophysiological recording of the nociceptive related slow ventral root potential (Feng and Kendig, 1996) in the isolated spinal cords showed the cords remained viable at the conclusion of the experiment (data not shown).

**Discussion**

Inhibition of εPKC translocation in P7, P15, and P21 rats and γPKC translocation in P15 and P21 rats attenuated formalin-induced nociception. The εPKC inhibitor εV1-2 attenuated phase 1 and phase 2 behaviors, whereas the γPKC inhibitor γV5-3 only attenuated phase 2 behaviors. These behavioral findings correlate well with the age-independent expression and translocation of εPKC in DRG and the age-dependent expression and translocation of γPKC in the lumbar spinal cord after formalin. Furthermore, the current study specifically identifies a role for εPKC within primary afferents in capsaicin-stimulated release of glutamate and CGRP.

**Developmental Regulation of ε and γPKC Expression.** Similar to findings reported by Beland and Fitzgerald (2001), there were few large-diameter cells in P7 DRG. This supports the postulate that differentiation of primary afferent subtypes occurs after the first postnatal week. Expression of εPKC in the DRG is developmentally regulated with increasing expression across maturation. In the present study, large-diameter cells were observed in P21 rats, and
many of these cells were εPKC-ir. In adult rats, 90% of dorsal root ganglion cells express εPKC (Khasar et al., 1999), suggesting that much of the maturation of εPKC in primary afferents occurs during the second and third postnatal weeks.

Embryonic and postnatal γPKC expression and activity differ between spinal cord and brain (Hashimoto et al., 1988; Akinori, 1998). Studies in the rodent brain suggest that γPKC is expressed only postnatally and that kinase activity increases with maturation (Hashimoto et al., 1988). In contrast, γPKC-ir is evident prenatally in neuronal somata in the dorsal and ventral horns of the spinal cord (Akinori, 1998). Similar to the findings by Akinori (1998), the present study reports a decrease in γPKC-ir cells with age; by postnatal day 21, only large motor neurons and lamina II interneurons are γPKC-ir. By adulthood, only 30% of ventral horn motor neurons show γPKC-ir (Akinori, 1998), suggesting that like εPKC, maturation of γPKC in the lumbar spinal cord occurs during the first few postnatal weeks.

In P7 rats γPKC-ir in lamina II of the dorsal horn was fiber-like with few γPKC-ir neuronal somata. The fiber-like appearance of γPKC-ir suggests an association of γPKC with neurofilaments and thus may be in an immature form. Alternatively, this fibrillar location of γPKC may mediate cellular functions that are distinct from those associated with responses to pain in older rats. It has been suggested that γPKC may be involved in synaptogenesis and the development of postsynaptic functions (Akinori, 1998). This highlights the importance of developing and using PKC isozyme-specific inhibitors to avoid the confounding developmental compensation issues surrounding traditional gene knockout technology.

Translocation of ε and γPKC after Formalin. Decreased εPKC-ir in the dorsal root ganglion and increased
γPKC-ir in the dorsal horn of the spinal cord were observed after intraplantar formalin. We speculate that these two opposite observations both reflect translocation. A decline or an increase in immunoreactivity in fixed tissue is likely to reflect activation-dependent changes in the accessibility of the antibodies to the activated enzyme. The commercial antibodies are generated against a small antigenic determinant at the C terminus of the enzyme; when the enzyme is translocated and associated with its RACK (Johnson et al., 1996; Csukai et al., 1997), and/or when the antigenic site is phosphorylated (Keranen et al., 1995), binding of the antibodies can be altered. Furthermore, the decline in immunoreactivity was not due to degradation of the enzyme (as indicated by Western blot analysis of cell lysates), indicating that use-dependent degradation of the enzyme did not occur due to the noxious stimulus. Importantly, the decline in immunoreactivity of ePKC was dependent upon its translocation; in the presence of the selective ePKC translocation inhibitor eV1-2 (Johnson et al., 1996; Nowak et al., 2003), the decline was inhibited. In contrast, in the presence of the Tat carrier, or the selective translocation inhibitor of γPKC γV5-3, the activation-induced decline in immunoreactivity was not altered. Similarly, increases in γPKC-ir were selectively blocked by the γPKC-specific inhibitor in the absence of protein expression changes by Western blot analysis.

**Inhibition of Pain: ePKC.** Blocking ePKC translocation attenuated both phase 1 and 2 of the formalin response in P7, P15, and P21 rats. The current findings suggest that activation of ePKC in primary afferents precedes and possibly induces γPKC activation in lamina II of the spinal cord. That activation of ePKC as a primary event in phase 1, from which γPKC activation and phase 2 behavioral responses result, is suggested by several findings. First, the anatomical location of these two distinct PKC isozymes supports the following cascade of events: activation of ePKC in primary afferents, followed by γPKC in dorsal horn interneurons. Second, inhibition of ePKC attenuates both phase 1 and phase 2 pain behaviors, whereas inhibition of γPKC only alters phase 2 behaviors. Furthermore, inhibition of ePKC prevented translocation of both e and γPKC, whereas inhibition of γPKC only modified γPKC translocation. The independence of ePKC actions from γPKC signaling is further illustrated in P7 rats, which are sensitive to anti-γPKC therapy but not to inhibition of ePKC.

We have shown ePKC-ir in small- and medium-diameter dorsal root ganglion cells, suggesting expression in nociceptors. In addition, we show that ePKC mediates the release of the excitatory neurotransmitter glutamate and the neuromodulatory transmitter CGRP in isolated spinal cords stimulated with capsaicin to activate VR1-expressing primary afferents. This suggests that ePKC is also expressed in the central terminals of small- and medium-diameter dorsal root ganglion cells and involved in the release of the pronociceptive peptide CGRP. Furthermore, it has been reported that ePKC may regulate depolarization and firing in dorsal root ganglion neurons through regulation of tetrodotoxin resistant sodium currents (Khasar et al., 1999). These findings expand on previous in vitro studies demonstrating enhanced

![Fig. 6. Inhibition of ePKC translocation with eV1-2 dose-dependently and age-independently attenuated phase 1 and phase 2 formalin behaviors in P7 (A), P15 (B), and P21 (C) rats (n = 8–10/group). D, statistical analysis of eV1-2 antinociception as a function of phase of the formalin response.](https://jpet.aspetjournals.org/cgi/content/figure/6/6/623/DC1/1)
presynaptic release of neuropeptides (Barber and Vasko, 1996; Frayer et al., 1999) and increased depolarization and firing of dorsal root ganglion neurons (Zhou et al., 2001) after nonspecific activation of PKC. Thus, we conclude that PKC, and specifically ePKC, modulates release of excitatory neurotransmitters and neuromodulatory peptides from central terminals of primary afferents.

Inhibition of Pain: γPKC. The present study provides direct evidence that selective inhibition of γPKC in vivo attenuates phase 2 formalin-induced pain behaviors in an age-specific manner. These findings expand on earlier studies in adult rats showing that nonspecific inhibitors of PKC attenuate phase 2 formalin responses (Yashpal et al., 1995) and increase γPKC immunoreactivity in lamina II after formalin (Malmberg et al., 1997) and that adult mice deficient in γPKC have decreased formalin-induced spontaneous pain behaviors (Malmberg et al., 1997). In our study, inhibition of γPKC translocation did not alter γPKC-ir or formalin-induced spontaneous pain behaviors in P7 rats compared with P21 rats. It is possible that in P7 rats, formalin induces changes in γPKC-ir at later times than were examined in this study. Alternatively, small changes in γPKC-ir may not be detectable by light microscopy. Regardless of the limitations of the techniques used in the present study, there are clearly differences in the time course and magnitude of translocation and these differences are age-dependent. Furthermore, these findings suggest phase 2 formalin responses may be independent of γPKC in P7 rats.

In P15 and P21 rats, central sensitization may be mediated presynaptically or postsynaptically on interneurons. PKC has been reported to increase the release of glutamate and aspartate in the spinal cord (Gerber et al., 1989). We have

TABLE 4
Capsaicin-stimulated release of CGRP from spinal cords isolated from P7 rat Data represented as average P8 CGRP/ml ± S.D. (n = 4/group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-Cap</th>
<th>Post-Cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cap, capsaicin</td>
<td>2.3 ± 0.7</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>0.5 mM Capsaicin</td>
<td>5.2 ± 1.4</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>5 mM Capsaicin</td>
<td>12.4 ± 2.8</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>25 mM Capsaicin</td>
<td>31.7 ± 4.8</td>
<td>12.2 ± 1.6</td>
</tr>
<tr>
<td>100 mM Capsaicin</td>
<td>51.1 ± 10.2</td>
<td>25.1 ± 10.2</td>
</tr>
<tr>
<td>Cap, capsaicin:</td>
<td>*P &lt; 0.05, ***P &lt; 0.001, significant from control precapsaicin release.</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 5
Capsaicin (25 μM)-stimulated release of glutamate and CGRP from spinal cords isolated from P7 rats was attenuated by eV1-2 Data represented as average ± S.D. (n = 4/group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glutamate in 20-μl sample</th>
<th>CGRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Cap</td>
<td>Post-Cap</td>
<td>Pre-Cap</td>
</tr>
<tr>
<td>GF109203X</td>
<td>0.9 ± 2.0</td>
<td>20.1 ± 5.0*</td>
</tr>
<tr>
<td>Go6976</td>
<td>0.8 ± 1.7</td>
<td>28.0 ± 7.0</td>
</tr>
<tr>
<td>Saline</td>
<td>5.26 ± 0.33</td>
<td>12.2 ± 0.3</td>
</tr>
<tr>
<td>Tat carrier</td>
<td>4.03 ± 0.44*</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>PKC e inhibitor</td>
<td>1.87 ± 1.15*</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>Cap, capsaicin:</td>
<td>nd</td>
<td>1.3 ± 1.0</td>
</tr>
</tbody>
</table>

Cap, capsaicin; nd, below the level of detection. *P < 0.05, ***P < 0.001, significant from saline vehicle. **P < 0.05, ***P < 0.001, significant from Tat carrier.
presented evidence in this study that ePKC in primary afferents modulates the release of excitatory glutamate and CGRP, providing a mechanism for presynaptic modulation of spinal interneurons. Postsynaptically, yPKC is expressed in excitatory interneurons in lamina II of the dorsal horn (Polgar et al., 1999), and PKC has been shown to alter N-methyl-
D-aspartate-induced currents in the spinal cord (Chen and Huang, 1992; Martin et al., 2001). More recently, it has been reported that N-methyl-
D-aspartate antagonism blocks formalin induced PKC translocation in the superficial dorsal horn (Chen and Huang, 1992; Yashpal et al., 2001) further supporting presynaptic modulation of postsynaptic neurons containing PKC.

Conclusions

Very young infants experience both noxious and some innocuous stimuli as a physiologically disturbing event (Fitzgerald et al., 1989; Fitzgerald and Anand, 1993). Furthermore, early painful experiences may predispose infants and children to a later enhancement in both physiological and cognitive/emotional responses to noxious insult (Taddio et al., 1997; Alvares et al., 2000; Ruda et al., 2000; Lidow et al., 2001). Thus, it is imperative to understand age-specific regulation of pain pathways for the rational development of nonopioid analgesics for managing pain in infants and children. The present study provides evidence that pharmacological manipulation of selective PKC isozymes may offer several attractive therapeutic targets for the management of pain in pediatrics and that therapies will need to be tailored to the different roles played by PKC isozymes at different ages.

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