Published in final edited form as:

Neurosci Lett. 2019 July 27; 706: 56–60. doi:10.1016/j.neulet.2019.04.055.

Spinal and afferent PKC signaling mechanisms that mediate chronic pain in sickle cell disease

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Abstract

Pain is the most characteristic feature of sickle cell disease (SCD). Patients with SCD live with unpredictable, recurrent episodes of acute painful crisis, as well as chronic unremitting pain throughout their lifetime. While most of the research and medical efforts have focused on treating vaso-occlusion crisis and acute pain, chronic pain remains a significant challenge faced by patients and physicians. Emerging evidence from human and animal studies has suggested the presence of a neuropathic component in SCD pain. New knowledge on the neurobiology of chronic pain in SCD has significant implications in unraveling the underlying mechanisms. This review focuses on the recent advances on the role of protein kinase C or PKC in promoting and maintaining chronic pain conditions. With a highlight of a specific PKC isoform, PKC δ , we aim to propose PKC as an essential regulator of chronic pain in SCD, which may ultimately lead to innovative therapeutic strategies for treating this devastating life-long problem in patients with SCD.

Keywords

PKC; pain; phosphorylation; sickle cell disease

2. Introduction

Sickle cell diseases (SCD) is a life-threatening hematological disorder that affects millions of people worldwide, particularly those with African, Spanish, Mediterranean, and Indian ancestry. Pain is a hallmark of SCD. Patients with SCD experience pain that is characterized as chronic baseline pain with intermittent episodes of acute pain crises [1]. Often described as deep, gnawing and throbbing, the crisis pain reflects vaso-occlusion, impaired oxygen supply, and infarction-reperfusion injury [2]. The unpredictable and recurrent episodes of acute pain can be worse than postoperative pain and as intense as terminal cancer pain, which require hospitalization for intensive treatment. Patients are also struggling with intractable chronic pain throughout the life long course of the disease [3].

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Chronic pain in SCD is a complex multidimensional disorder, which is beyond the pathophysiological basis for the disease itself. While early diagnosis, penicillin prophylaxis, blood transfusion, transcranial Doppler imaging, hydroxyurea, and hematopoietic stem-cell transplantation have dramatically improved the care and decreased disease severity, increased life expectancy and can even cure the disease [4], chronic pain is refractory to these interventions and persistent throughout a patient's life. Our laboratory has devoted extensive research efforts to better define the neuropathology of chronic pain in SCD. In this review, we focus on the role of protein kinase C (PKC) phosphorylation and signaling as a mechanism for pain in SCD. We attempt to highlight the most recent discoveries of PKC isoforms in chronic pain signaling, discuss the translational potential of PKC regulators for pain in SCD.

3. Protein kinase C (PKC) in pain signaling

PKC is a family of serine/threonine protein kinases that comprises ~2% of the human kinome [5]. Located on the tip of a branch of the AGC kinase group, PKC diverge into 10 family members (Figure 1A). All PKC isoforms share a highly conserved carboxyterminal that is linked by a hinge region to a divergent amino-terminal regulatory domain (Figure 1B). Inactive PKC is auto-inhibited by a pseudosubstrate sequence that is present in the regulatory domain and occupies the substrate binding pocket [6]. When the regulatory domain is recruited to plasma membranes through the binding to second messengers and/or allosteric effectors, auto-inhibition is relieved and the catalytic domain of PKC is exposed to exert its phosphorylation action on target substrates (Figure 1C). As a class of intensely regulated, typically membrane localized protein kinases, the PKC family is emerging as essential for diverse cellular processes, from cell differentiation and proliferation, to long-term potentiation of neuronal activity, by phosphorylating a wide variety of substrates [7].

Based on second messenger requirements, PKC isoforms are divided into three subfamilies: conventional (or classical), novel, and atypical PKC [8]. Conventional (c)PKCs contain the isoforms α , β I, β II, and γ . They are activated by signals of diacylglycerol (DAG) and Ca^{2+} . Novel (n)PKCs include the δ , θ , and η isoforms, which require DAG, but not Ca^{2+} for activation. On the other hand, atypical (a)PKCs including ζ and ι/λ isoforms are activated by mechanisms which are DAG and Ca^{2+} independent.

Upon activation, PKC isoforms are translocated to the plasma membrane by receptors for activated C-kinase (RACKs). They remain activated even after the original activation signal or the Ca^{2+} wave is gone [9]. The long-term activation provides them the capability to regulate multiple neuronal functions including development, differentiation, plasticity, and apoptosis. With the development of PKC isoform-selective inhibitors and the isoform specific knockout mice, the central role of PKC in nociceptive signaling has gained much better understanding in various pain paradigms including SCD pain.

3.1. Novel PKC

3.1.1. PKC δ —The predominant data for a role of PKC in sickle cell pain came from the study of PKC δ . PKC δ is member of novel PKC family that does not require Ca^{2+} for activation. PKC δ has been implicated to play a critical role in nociceptive pathways involved

in chronic pain. The first evidence came from our study, which identified the contribution of nociceptor PKC δ in peripheral neuropathy produced by paclitaxel [10]. In rat adult dorsal root ganglion (DRG) sensory neuron culture, paclitaxel was found to activate PKC δ in a time- and dose-dependent manner. Strikingly, it does not take much paclitaxel to activate PKC δ . Whereas most of neurotoxicity studies including those of apoptosis research have used 10–100 μ M of the drug, paclitaxel at 1–10nM was sufficient to induce prolonged activation of PKC δ in the nociceptors [10].

To substantiate the *in vitro* finding, the activation of PKC δ was further evaluated in a mouse model of paclitaxel induced peripheral neuropathy (PIPN) in which evoked hypersensitivity to heat, cold, and mechanical stimuli and non-evoked ongoing pain lasted for at least 30d [11]. Indeed, we found nociceptor PKC δ was still activated 20d after the last injection of paclitaxel. Spinal administration of PKC δ inhibitors effectively inhibited its activation in mice DRG neurons and attenuated the evoked pain manifestations in mice with PIPN. Furthermore, PKC δ was found to be important for spontaneous pain behaviors in PIPN. In the CPP paradigm, lidocaine or saline was each paired with a respective chamber. PIPN mice preferred the chamber where they received lidocaine, suggestive of ongoing spontaneous pain revealed by the non-rewarding lidocaine. When we replaced the pairing drug lidocaine with a PKC δ inhibitor, place preference was observed similar to that produced by lidocaine, indicative of spontaneous pain blockage by PKC δ inhibition. This was the first report that nociceptor PKC δ functions as a dual mediator of both spontaneous pain and evoked pain in peripheral neuropathy produced by paclitaxel.

In SCD, PKC δ was revealed to have functional relevance in pain development and maintenance. Taking advantage of the TOW SCD mice, for which we have carefully and fully characterized baseline pain phenotypes, we examined PKC δ -mediated mechanisms of chronic pain in SCD [12]. By analyzing PKC δ plasma membrane translocation, prominent activation of PKC δ was observed in the superficial laminae of the spinal cord dorsal horn in TOW mice, but not the control non-sickle littermate mice. Furthermore, hyperactivation of spinal PKC δ occurred exclusively in GABAergic inhibitory neurons (VGAT+), but not in glutamatergic excitatory ones (VGLUT2+) [12]. Since GABA transport activity could be blunted by PKC activation, it is possible that PKC δ may promote chronic pain in SCD through deactivation of inhibitory GABAergic mechanisms.

Mechanical allodynia and heat hyperalgesia in TOW mice were transiently, but effectively suppressed by a single intrathecal administration of PKC δ inhibitor. Inhibiting PKC δ , however, did not alter basal mechanical or thermal sensitivity in non-sickle control mice. Employing the CPP paradigm to determine the participation of PKC δ in ongoing pain, we found spinal PKC δ inhibition suppressed ongoing pain and produced CPP in TOW mice, but not control non-sickle mice. In addition to chemical inhibition, which may have selectivity issues *in vivo* especially at high doses, we further evaluated the functional involvement of PKC δ in SCD pain by neuron-specific silencing. Spinally delivered PKC δ siRNA in complex with RVG-9R, a chimeric peptide derived from rabies virus glycoprotein [13], selectively knocked down PKC δ in spinal neurons, attenuated spontaneous pain, mechanical allodynia, and heat hyperalgesia in TOW mice.

Another method to selectively target PKC δ can be accomplished by hematopoietic stem cell transplantation method (HSCT). In this case, sickle cell anemia can be generated in PKC δ -null or wildtype mice by HSCT. First, bone marrow cells were harvested from adult TOW sickle mice. PKC δ -KO mice or WT littermates were then subjected to myeloablative conditioning, followed by transplantation of bone marrow cells harvested from TOW mice. Typical hematologic defects and systematic signs of SCD were established in recipient PKC δ -KO and WT mice several weeks after HSCT when the recipient PKC δ -KO or WT mice no longer produced mouse hematocytes, but expressed exclusively human hemoglobins including the sickle β globin. Unlike transplanted PKC δ -WT mice, neither spontaneous pain nor evoked pain was detected in the mice lacking PKC δ despite the full establishment of disease phenotypes. This manipulation superimposed a TOW SCD model on the PKC δ -null or wildtype background, allowing complete deletion of PKC δ in the central nervous system and the separation of neuronal mechanisms of pain from that of the disease itself. These findings strongly indicated that spinal PKC δ is a critical mechanism for the generation and maintenance of both ongoing and evoked pain in SCD.

3.1.2. PKC ϵ —Besides PKC δ , PKC ϵ is another member of nPKC family with its role identified in a number of hyperalgesia conditions, such as ethanol withdrawal induced hyperalgesia [14] and thermal burn-induced mechanical hyperalgesia [15]. Epinephrine- or acetic acid-induced hyperalgesia was markedly attenuated in mice lacking the PKC ϵ gene [16]. In paclitaxel-induced peripheral neuropathy, PKC ϵ inhibitors effectively attenuated the hypersensitivity to mechanical and thermal stimuli. However, inhibiting spinal PKC ϵ did not attenuate or alter the spontaneous pain behaviors in these mice [10]

In the primary afferent nerve terminal, PKC ϵ can be translocated to the plasma membrane of nociceptors in response to inflammatory mediators such as bradykinin and substance P in inflammatory and neuropathic pain conditions [16, 17]. One of the most studied pathways by which PKC ϵ modulates nociception is through activation of the transient receptor potential vanilloid receptor 1 (TRPV1). The capsaicin receptor, TRPV1, is essential for thermal and inflammatory pain. In SCD, TRPV1 has been reported to mediate mechanical pain [18]. Indeed, application of capsaicin to the hind paw of rats increased PKC ϵ immunoreactivity in afferent fibers entering the dorsal horn of the spinal cord [19]. PKC ϵ subsequently phosphorylated TRPV1 on Ser502 and Ser800, which potentiated capsaicin-induced depolarization in afferent neurons [20, 21]. PKC ϵ -mediated TRPV1 activation may have significant implications in SCD pain. Previous study demonstrated that BERK mice with severe SCD exhibited robust behavioral hypersensitivity to mechanical stimuli. The persistent hypersensitivity to mechanical touch was further exacerbated when hypoxia was used to induce acute RBC sickling [18]. Pharmacologic inhibition of TRPV1 channel by the antagonist A-425619 attenuated mechanical sensitivity at the primary afferent terminals and isolated DRG neurons, and partially reversed the mechanical hypersensitivity in SCD pain [18]. Therefore, PKC ϵ can potentially contribute to the mechanical hypersensitivity in SCD by serving as an upstream regulator of TRPV1.

3.2. Conventional PKC

3.2.1. PKC α —The functional involvement of PKC α in pain has been well documented. Painful diabetic neuropathy is accompanied by increased phosphorylation of PKC α [22]. PKC α could activate TRPV1 independent from the binding of TRPV1 ligand in DRG neurons [19, 23]. Experiments in cell lines co-expressing TRPV1 with different sets of PKC isoforms showed that acute phorbol 12, 13-dibutyrate (PDBu)-induced activation of TRPV1 requires PKC α , but not PKC ϵ [23]. However, TRPV1 phosphorylation site(s) for PKC α remain unknown. Moreover, the activated PKC α could enhance Ca²⁺ influx into the pre-synaptic terminal by phosphorylation of voltage gated calcium channels [24], which potentiates the release of neurotransmitters from the presynaptic terminal.

Unlike TRPV1, transient receptor potential melastatin 8 (TRPM8) is desensitized by PKC α and/or β I [25]. Such negative regulation of TRPM8 by PKC has been shown to occur indirectly via PKC α / β I-mediated activation of phosphatase calcineurin through an indirect modulation [26]. TRPM8, the receptor for cool temperatures (8–26°C) and menthol, has significant implications for pain in SCD. Patients with SCD have enhanced pain sensitivity to cold environments [27, 28]. A previous study showed that BERK sickle mice had a marked increase in mild cold sensitivity measured by a temperature preference assay [29]. In this test, two plates were independently programmed to different temperatures (23°C *vs.* 30°C). BERK mice spent significantly less time on the plate set to 23°C than that at 30°C. In contrast, control mice spent an equal amount of time on each plate. However, the same study did not detect changes in the expression of TRPM8 between sickle and non-sickle mice at the transcriptional level, the question whether TRPM8 mediates cold allodynia in SCD remains open. It would be interesting to elucidate the possibility of PKC α -mediated TRPV1/TRPM8 signaling in SCD pain.

3.2.2. PKC β II—There has been no direct evidence for the involvement of PKC β II in SCD pain, although this PKC isoform has been previously reported to be a critical regulator of nociceptor excitability [30]. TRPV1 was found to be co-expressed with PKC β II in a subset of rat DRG sensory neurons. In these neurons, TRPV1 can bind directly to PKC β II, leading to the activation and translocation of PKC β II. Activated PKC β II, in turn, phosphorylated TRPV1 at Thr705, significantly increased the responsiveness of TRPV1 during basal condition. Therefore, DRG neurons expressing TRPV1-PKC β II complexes were constitutively hypersensitive in response to thermal and chemical painful stimuli.

The evidence of PKC β II involvement in nociceptive signaling was further strengthened by the recent findings from PIPN [10]. Of the conventional PKC isoforms expressed in rat DRG neurons (α , β I, β II), only PKC β II was activated by paclitaxel. It is worth noting that only very little paclitaxel (1–10nM) is needed to activate PKC β II in the nociceptors. There was a strong association between the predominate activation of PKC β II in isolectin B4 (IB4)-negative peptidergic cells and CGRP release induced by paclitaxel. In agreement with the *in vitro* observation, evoked sensory hypersensitivity and non-evoked ongoing pain were effectively blocked by inhibiting PKC β II in mice with PIPN.

3.2.3. PKC γ —There is a large body of literature suggesting the importance of PKC γ in central sensitization. PKC γ is expressed in a subset of excitatory interneurons in lamina II of the spinal cord [31, 32]. Its immunoreactivity was reported to be increased after peripheral nerve injury and inflammation [33]. The anatomical location of PKC γ in the spinal cord facilitates its function in central sensitization, though it should be noted that PKC γ is not expressed in the primary afferent sensory neurons. Studies in PKC γ knockout mice further linked PKC γ with inflammatory and neuropathic pain. In null mutants of PKC γ , nerve injury-induced allodynia and thermal hyperalgesia were largely absent while acute pain processing was left intact [31]. In addition, the same group reported reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC γ mutant mice [34]. However, another study performed similar experiments using the PKC γ mutant mice obtained from a different source (Jackson Laboratory), but did not find any differences in tolerance development between wildtype and PKC γ mutant mice [35]. PKC γ containing glutamatergic interneurons receive input from a subset of TPRV1 positive primary afferents, and can modulate lamina V transmission neurons [36]. This provides a bridge from peripheral stimuli to ascending transmission neurons. In addition, activation of PKC γ phosphorylates Src. Phosphorylation of the N-methyl D-aspartate receptor (NMDAR) NR2B subunit by Src increases channel kinetics and open time through alleviation of the voltage-dependent Mg²⁺ blockage on NMDAR [37, 38]. Thus, PKC γ functionally potentiates NMDA receptor activity in the spinal cord.

3.3. Atypical PKC

PKM ζ is a unique gene product from the PKC ζ gene that lacks a regulatory region and is therefore autonomously active at the synapse following translation [39]. In addition to numerous studies on its functionality in long-term potentiation (LTP) for learning and memory, these same synaptic mechanisms may be responsible for pathological pain conditions [40, 41]. Several interesting reports have implicated a role of PKM ζ in pain; however, almost all of the evidence was based on inhibiting PKM ζ using zeta inhibitory peptide (ZIP), which may not be relevant to PKM ζ (See below). In these studies, the development and maintenance of a sensitized state promoting persistent pain requires PKM ζ in the spinal cord [42].

Although ZIP was thought to be a pseudosubstrate peptide inhibitor that selectively inhibits PKM ζ , this may not be the case [43, 44]. The definitive evidence came from transgenic mice research. Two independent research groups produced PKM ζ conventional and conditional knockout mice. These mice lacking PKM ζ showed normal behaviors and in baseline tests of motor function and sensory perception, and had no deficits in learning and memory. Surprisingly, ZIP was still active in PKM ζ null mice, reversing LTP, indicating that the effects of ZIP are independent of PKM ζ [45, 46]. There were no significant differences in the development of postsurgical pain among wild-type, heterozygous, and homozygous conventional PKM ζ knockout mice, suggesting that PKM ζ is not required for the development of postsurgical pain after plantar incision [47]. At this point, it is not clear whether other types of persistent pain can be modulated by PKM ζ . However, those data in the literature that were obtained by employing ZIP in the studies should not be attributed to PKM ζ .

4. Summary

It is increasingly recognized that protein phosphorylation constitute an important post-transcriptional regulatory mechanism in pain signaling pathways. Activation of multiple PKC isoforms has been implicated in the development of peripheral and/or central sensitization in persistent pain conditions (Figure 2). We review some of the most recent studies on their essential roles of PKC isoforms in neuropathic pain induction and/or maintenance. Knowledge obtained from these studies will have significant impacts on understanding the neurobiology of pain in SCD. Specific PKC isoform-mediated mechanisms deserve immediate and comprehensive investigations in chronic pain associated with SCD.

Given the complicity of pain transmission system, results from such studies need to be interpreted in the context of the specific pain model system, intervention approaches, and pain evaluation endpoint. First, we should keep in mind that many chemical PKC inhibitors used in these studies can inhibit more than one isoform or affect other kinases [48]. Although RACK peptide-, antibody-, and gene-based inhibitors offer more selective inhibition, caution should be exercised when extrapolating the findings beyond the cellular system, as effective application of these tools *in vivo* need to be further validated. Mouse gene deletion (“knockout”) studies have provided much needed selectivity in studying PKC. However, this method has its own drawbacks. Interpretation of results can be compromised by unmatched genetic backgrounds in mice with different genotypes or adaptive changes associated with gene manipulation [49]. We are very excited about the perspective of employing HCST in studying pain in SCD, as the approach can overcome these potential problems in global and conditioned gene knockout models. In addition, activation of PKC integrates multiple signaling pathways. For example, there are positive feed-forward loops involving PKC and CaMKII in neuropathic pain [50]. Therefore, the potential interactions and crosstalks among these kinases need to be taken into consideration. More importantly, one difficult future task is to identify the exact cellular targets (at receptors/ion channel levels or intracellular effectors) that these PKC isoforms act on in SCD pain.

While important advances have been made to offer a cure of SCD, such as gene therapies, there are numerous challenges associated with these curative treatments including chronic pain outcome that remains inadequately addressed. Mechanism-based therapeutic strategies could lead to more precise treatment and management of SCD pain. We envision that the advances in understanding the neurobiology of SCD pain will provide unprecedented opportunities to substantially improve the quality of life for patients with SCD.

Acknowledgments

Funding sources: This work was supported by the National Institute of Health grants K99 HL133590 to YH and R35 HL140031 to ZJW.

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Highlights

The neurobiological mechanisms underlying chronic pain in sickle cell disease (SCD) is poorly understood.

Recent studies on protein kinase C (PKC) began to shed lights on cellular phosphorylation and signaling mechanisms for chronic pain.

With a highlight of PKC δ , this review summarizes the findings on PKC isoforms and proposes specific PKC isoforms as essential regulators of chronic pain in SCD.

Figure 1. The protein kinase C (PKC) family

A. Human kinome (Illustration reproduced courtesy of Cell Signaling Technology, Inc., www.cellsignal.com), shows the position of PKC isoforms on the branch of AGC kinases (red circle). B. The PKC family members can be divided into three structurally and functionally distinct subgroups according to their regulatory domains. C. A cPKC requires the binding of DAG to the C1 domain of the regulatory region, the binding of calcium to the C2 domain of the regulatory region, and the interaction with its membrane anchoring protein, RACK for activation.

Figure 2. PKC is positioned to play a central role in nociceptive signaling.

Receptor-dependent PLC β activation results in hydrolysis of PIP₂ to DAG and IP₃. IP₃ triggers the release of Ca²⁺ from the endoplasmic reticulum (ER). The rise in DAG and cytosolic Ca²⁺ levels leads to the activation of PKC and its translocation from the cytosol to the plasma membrane. At presynaptic terminals, PKC phosphorylates several nearby transient receptor potential channels (TRPs). In postsynaptic neurons, PKC potentiates the NMDA response by reducing Mg²⁺ blockage.