Protein kinase C modulation of thermo-sensitive transient receptor potential channels: Implications for pain signaling

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Abstract

A variety of molecules are reported to be involved in chronic pain. This review outlines the specifics of protein kinase C (PKC), its isoforms and their role in modulating thermo-sensitive transient receptor potential (TRP) channels TRPV1-4, TRPM8, and TRPA1. Anatomically, PKC and thermo-sensitive TRPs are co-expressed in cell bodies of nociceptive dorsal root ganglion (DRG) neurons, which are used as physiological correlates of peripheral and central projections involved in pain transmission. In the past decade, modulation of painful heat-sensitive TRPV1 by PKC has received the most attention. Recently, PKC modulation of other newly discovered thermo-sensitive pain-mediating TRPs has come into focus. Such modulation may occur under conditions of chronic pain resulting from nerve damage or inflammation. Since thermo-TRPs are primary detectors of acute pain stimuli, their modulation by PKC can severely alter their function, resulting in chronic pain. Comprehensive knowledge of pain signaling involving interaction of specific isoforms of PKC with specific thermo-sensitive TRP channels is incomplete. Such information is necessary to dissect out modality specific mechanisms to better manage the complex polymodal nature of chronic pain. This review is an attempt to update the readers on current knowledge of PKC modulation of thermo-sensitive TRPs and highlight implications of such modulation for pain signaling.

Key words: Analgesia, transient receptor potential channels, protein kinase C, Anti-inflammatory, GPCR

DISCOVERY AND CLASSIFICATION OF THERMO-SENSITIVE TRP CHANNELS

Early studies on the physiology of photoreceptors involving Drosophila phototransduction identified a spontaneously occurring Drosophila mutant photoreceptor, named transient receptor potential (TRP). The name TRP was given to the mutant photoreceptor due to its unique phenotype where response to prolonged illumination declines rapidly to baseline during light stimulation with transient potential in comparison to a wild-type photoreceptor. The search for homologs of TRP among invertebrates and vertebrates led to the identification of a large and diverse family of membrane proteins, grouped under the umbrella of the TRP superfamily. Certain members of this superfamily respond to thermal, chemical, or mechanical stimuli or a combination of the three (polymodal). The TRP channels that exhibit a unique response to temperature have been given the name thermo-TRPs. These include members from the subfamily vanilloid TRPV (TRPV1, 2, 3, and 4), melastatin TRPM (TRPM2, 4, 5, and 8), and ankyrin transmembrane proteins TRPA (TRPA1). Between them, response to noxious heat is mediated by TRPV1 (~42°C) and TRPV2 (~52°C), innocuous warm temperature by TRPV3 (~39°C), TRPV4 (~34°C), and TRPM2 (~37°C), innocuous cool temperature by TRPM8 (~17°C), noxious cold...
by TRPA1 (~10°C), and increasing temperatures from ~15°C-35°C by TRPM4 and TRPM5.

Among all thermo-sensitive TRP channels, TRPV1-4, TRPM8, and TRPA1 are expressed in subsets of nociceptive dorsal root ganglion (DRG) neuron cell bodies including their peripheral and central projections. Discovery of this subset of thermo-TRPs as molecular targets to naturally occurring compounds, such as capsaicin, mustard oil, menthol, or camphor that elicit thermal or painful behavior, underlies the basis for sensory functions of nociceptive neurons. In addition to being detectors of thermal and chemical stimuli, some thermo-TRP members also detect painful mechanical stimuli. Evidence for expression or function of other thermo-sensitive TRP channels TRPM2, 4, and 5 among nociceptive DRG neurons has not yet been reported.

**EXPRESSION OF THERMO-TRPS IN NOCICEPTIVE DRG NEURONS**

**Nociceptive DRG neurons**

In order to understand the signaling pathways of pain transmission from peripheral sensory nerve endings to the spinal cord and brain, many studies have focused on characterization of subpopulations of DRG neurons that respond to painful stimuli. Nociceptive DRG neurons are also referred to as nociceptors. Two major categories of nociceptors are C fibers and Aδ fibers. C fiber nociceptors have small-diameter, thinly myelinated, slowly conducting axons. In contrast, Aδ fiber nociceptors have medium diameter, thinly myelinated axons with conduction velocities greater than those of C fibers. C fibers can be further divided into peptide (containing substance P [SP] or calcitonin gene-related peptide [CGRP]) and non-peptide (Isolectin B4 positive [IB4+]) subsets. Aδ fibers are further classified as type I and type II.

Type I Aδ fibers are high-threshold and rapidly conducting mechanoreceptors that are weakly responsive to noxious heat, cold, and chemical stimuli. Type II Aδ fibers are low-threshold, less rapidly conducting, and highly responsive to noxious heat. Cell bodies of C and Aδ neurons are located in the DRG, trigeminal ganglia, and nodose ganglia. While the peripheral projections from DRG neurons terminate in several tissues, such as the skin or viscera, the central projections mainly terminate in the dorsal horn laminae I and II.

**TRPV1-4 in nociceptive DRG neurons**

The cloning of TRPV1 led to the discovery of the molecular evidence for the response of some sensory neurons to capsaicin (the hot component of chili peppers), noxious heat, and acidic pH. TRPV1 is distinct in being a molecular target of capsaicin and also a target for hypersensitivity to heat during inflammation due to injury. TRPV1 has been characterized to be expressed in peptidergic (SP or CGRP) and non-peptidergic (IB4+) subpopulations of nociceptive C fiber terminals and cell bodies [Table 1]. True to its physiological response and detection of low to moderately painful heat, TRPV1 has also been suggested to be expressed in the type II subpopulation Aδ fiber terminals and cell bodies.

TRPV2, the second member of the thermo-TRPV subfamily, has been characterized to be expressed in the type I Aδ subpopulation of DRG neurons that have a higher threshold to heat than type II Aδ neurons expressing only TRPV1 [Table 1]. However, a TRPV2 knockout study has questioned the physiological role of TRPV2 in sensing high threshold, noxious heat. TRPV2 knockout mice could detect noxious heat beyond threshold temperatures for TRPV1. Furthermore, in the same study mice with a double knockout of both TRPV1 and TRPV2 genes, retained the acute response to noxious heat.

These observations in vivo possibly suggest the following: (i) heat in the noxious range may either activate other heat thermosensors TRPV3 and/or TRPV4; (ii) noxious heat can activate yet another undiscovered thermosensor; or (iii) what appears to be a response to noxious heat is actually a response to tissue damage at such high temperatures. Further studies with gene deletion of all four heat-sensitive thermo-TRPs (TRPV1-4) may provide an answer to the above-mentioned hypotheses. TRPV2 activation by stretch stimuli suggests that it may function as a mechanosensor in a subpopulation of type I Aδ DRG neurons. TRPV2 to stretch may be a mechanism that is necessary for axon outgrowth.

**Table 1: Distribution of TRPV1-4, TRPM8, and TRPA1 in nociceptive DRG neurons**

| Thermo-TRP | C fiber distribution | Aδ fiber distribution |
|------------|---------------------|----------------------|
| TRPV1      | Present in both peptidergic and non-peptidergic IB4+ subsets | Type II |
| TRPV2      | Absent              | Type I               |
| TRPV3      | Present, subsets not yet characterized | Putatively type I |
| TRPV4      | Present in peptidergic subsets | Present, subsets not yet characterized |
| TRPM8      | Present in peptidergic subsets | Present, subsets not yet characterized |
| TRPA1      | Present in peptidergic subsets | Present, subsets not yet characterized |
TRPV3, the third member of the thermo-TRPV subfamily, has been found to be expressed in subpopulations of TRPV1-positive nociceptive DRG neurons. TRPV3 has been shown to be expressed in the subpopulations of capsaicin sensitive, and hence TRPV1-positive C fiber cell bodies [38] [Table 1]. Sensitization of TRPV3 upon repeated application of higher threshold non-noxious heat suggests that it has a role in sensing pain due to heat stimuli.

TRPV4, the fourth member of the thermo-TRPV subfamily is expressed in subpopulations of peptidergic (SP or CGRP) C fiber cell bodies [39] [Table 1]. Functional studies in these subpopulations have suggested that TRPV4 has a role in facilitating mechanical hyperalgesia. Further studies are required to characterize TRPV4 expression in subpopulations of Aδ DRG neurons [Table 1].

**TRPM8 in nociceptive DRG neurons**

TRPM8 was cloned as a thermo-TRP channel that responds to menthol (the cooling ingredient of the plant mentha (eg, Mentha arvensis)) and non-noxious cool temperatures. TRPM8 is expressed in subpopulations of peptidergic (SP or CGRP) C fiber terminals and cell bodies [41,42] [Table 1]. TRPM8 is also expressed in Aδ fiber terminals and cell bodies [41,42] [Table 1]. A small percentage of co-expression of TRPM8 with TRPV1 has also been shown [43,44] [Table 1]. In summary, the nociceptive subpopulations of peptidergic or TRPV1-positive C fiber terminals and cell bodies could account for the role that TRPM8 plays in promoting cold allodynia or pain caused by either a combination of menthol and cold temperatures or only painfully cold temperatures [42,43].

Certain subpopulations of DRG neurons expressing TRPM8 cannot be identified by C fiber pain markers like pain peptides (SP or CGRP) or TRPV1 [41,43,44] Some medium diameter DRG neurons express TRPM8 alone but not the Aδ marker NF200 [41]. These subpopulations that are beyond the reach of pain markers suggest that there are distinct TRPM8 expressing neurons that may play a role in analgesia [42]. Further studies are needed to determine the pathophysiological significance of TRPM8 in DRG neurons with and without co-expression of conventional pain markers.

**TRPA1 in nociceptive DRG neurons**

TRPA1 was cloned based on its activation by noxious cold temperatures [18] and response to isothiocyanates (the pungent components of mustard oil) [46]. The specificity of TRPA1 towards mustard oil was established based on TRPA1 knockout study [47]. TRPA1 is critical for sensing pungent irritants in air pollutants that activate nociceptive neurons innervating respiratory airways [47]. TRPA1 has been shown to be expressed in subpopulations of peptidergic and TRPV1-positive C fiber cell bodies [40] [Table 1]. Based on its expression in nociceptive DRG neurons and activation by pungent chemical compounds, TRPA1 is a potential candidate for polymodal pain processing [42]. Although some studies have suggested TRPA1 as a putative noxious cold sensor [18,48-50] with a role in cold hyperalgesia or allodynia [51,52] there is debate over the role of TRPA1 in sensing noxious cold in vivo [47]. The role of TRPA1 in sensing noxious cold could not be confirmed in TRPA1 knockout mice [47], whereas partial loss in the response to noxious cold was noted in TRPM8 knockout mice [53]. Based on the current knowledge, it is difficult to clearly attribute the role of TRPA1 to either TRPA1 or TRPM8. A double knockout of TRPA1 and TRPM8 may hold the key to resolve this debate. Also, a TRPA1 knockout study [50] could not confirm initial reports on the direct role of TRPA1 in mechanosensation [54], suggesting an indirect mechanism by which mechanical stimuli excite TRPA1.

**Modulation of thermo-TRP channels in nociceptive DRG neurons is significant**

Modulation of thermo-TRPs expressed in nociceptive DRG neurons is of great significance, as it would alter the polymodal sensitivity of pain fibers and enhance pain under acute or chronic conditions of inflammation and nerve injury [55]. Chronic pain, either inflammatory or neuropathic, is currently undertreated. The symptoms can be polymodal in nature given the expression of polymodal sensors like some of the thermo-TRPs in the nociceptive DRG neurons. Thus, it is necessary to delineate mechanisms that are modality specific. From among a large variety of second messenger molecules that can modulate polymodal sensitivity of the thermo-TRP channels, this review hereafter will focus on protein kinase C, a potent signaling molecule in nociceptive DRG neurons that is involved in inflammatory and neuropathic pain. Since PKC has a family of 12 different isoforms, it is necessary to investigate the specificity or redundancy of these isoforms towards modulation of a modality specific to a thermo-TRP in subsets of nociceptive neurons. Such information may eventually lead to micro-management of clinical symptoms of inflammatory or neuropathic pain that can be polymodal and complex.

**Discovery and classification of PKC**

The covalent attachment of phosphate molecules to either seryl (S) or threonyl (T) residues of proteins was identified first by Lipmann and Levene at the Rockefeller Institute for Medical Research (Rockefeller University, New York) in 1932. Enzymes responsible for such protein modifications were named serine/threonine kinases. Of the protein kinases discovered between 1955 and 1980, a novel protein kinase was discovered in 1977, which was activated...
by calcium-dependent neutral protease (now known as calpain) in mammalian tissues.\[56] Proteolytic cleavage of the kinase generates two functional domains, one that is hydrophobic and the other that shows full catalytic activity, which is suppressed in the “proenzyme”. This “proenzyme” was named Protein Kinase C and is known to be activated in the presence of both Ca2+ and phospholipids. Studies on the activation mechanism of PKC have led to the critical finding that phosphatidylserine (PS), an anionic phospholipid in the membrane, diacylglycerol (DAG), one of the metabolites of membrane phospholipids, and Ca2+ are required for PKC activation.\[57]

A more detailed molecular understanding of PKC came after cloning and sequencing of the enzyme in the mid-1980s. Subsequently, several isoforms of PKC were completely sequenced.\[58-61] It is now known that the PKC family consists of at least 12 isoforms encoded by nine genes and categorized into three groups: (1) conventional PKCs (cPKC) (α, βI, βII, and γ), which are Ca2+-dependent and activated by both PS and the second messenger DAG; (2) novel PKCs (nPKC) (δ, ε, η, and θ), which are Ca2+-independent and regulated by DAG and PS; and (3) atypical PKCs (aPKC) (ζ and η), which are Ca2+-independent and do not require DAG for activation, although their activity is regulated by PS.\[62-67]

**Neuronal expression of PKC isoforms**

The isoforms α, βI, βII, γ, ε, δ, ζ, and their mRNAs have been identified in rat brain and spinal cord using Western and Northern blot analysis and in situ hybridization. Immunohistochemical analysis using isoform-specific antibodies have revealed differential distribution of PKC isoforms in the rat central nervous system (CNS). The γ isoform is apparently expressed solely in the rat brain and spinal cord and has not been found in any other tissues. In the rat brain, low amounts of the γ isoform are found at birth, which increases up to 2-3 weeks of age.\[69,70] The βI and βII isoforms generated by alternative splicing of a common primary transcript are expressed in different ratios and in different neurons at distinct stages of development.\[70-72] The isoform βI is already expressed at birth, mainly in the brain stem, and its expression gradually increases with age. The isoform βII is poorly expressed in the forebrain at birth but rapidly increases up to 2-3 weeks of age. The isoforms α and δ are universally distributed in all tissues and cell types that have so far been examined; however, the α isoform is distributed unevenly in the brain.\[73] The isoform ε is expressed mainly in the forebrain, spinal cord and sensory DRG neurons.\[74]

**Expression of PKC isoforms in nociceptive DRG neurons**

Using western blot of whole lysates of DRG and immunocytochemistry in DRG neurons, PKC isoforms βI, βII, ε, δ, and ζ were shown to be expressed in DRG neurons sensing painful heat.\[78] This study also showed that other isoforms α, γ, μ, ι, and θ were not expressed in all subsets of DRG neurons.\[78] In our studies, we confirmed these observations and additionally, we show that the PKC isoforms βI, βII, ε, δ, and ζ were indeed co-expressed with TRPV1, the noxious heat sensor in DRG neurons [Figure 1].

Immunoelectron microscope analysis revealed that each isoform is localized in distinct intracellular compartments in the CNS.\[74,76-79] The γ isoform is localized predominantly in the cytoplasm associated with most ribosomes, outer membranes of cell organelles and areas of weaker density in the nucleoplasm. In most CNS regions, the α isoform is present in the periphery of the perikarya and is also scattered sparsely in the perinuclear area. Similarly, isoform βII is also clustered in the cytoplasm adjacent to the plasma membrane, whereas isoform βI is concentrated around the trans-face of the Golgi complex and proximal dendrites. In the cell bodies of painful heat-sensing DRG neurons, an earlier study showed cell membrane localization of isoforms βI and βII and cytosolic localization of isoforms ε, δ, and ζ.\[75] In contrast, we found that isoforms βI and βII were not only localized to the cell membrane but also found in the cytosol [Figure 1]. However, we observed localization of other isoforms ε, δ, and ζ that was consistent with the earlier study (data not shown). Such discrepancy can be easily explained. The earlier study analyzed western blots from whole rat DRG lysates,\[79] whereas we used cytosolic fractions of rat DRG [Figure 2]. In addition, we confirmed both cell membrane and cytosolic expression of β isoforms by using heterologous expression of βI- and βII-EGFP constructs in DRG neurons [Figure 3]. Real-time translocation of βI-EGFP and βII-EGFP from cytosol to cell membrane in response to pain stimuli like capsaicin and ATP or phorbol ester PMA were also confirmed in our studies. In this review, we show a representative real-time translocation of βI-EGFP in a DRG neuron responding to capsaicin and phorbol ester PMA (Video files PKC beta 1 CAP and PKC beta 1 PMA). Further studies using such powerful in vitro assays are needed to clarify whether subsets of nociceptive DRG neurons that express multiple isoforms of PKC in either the cytosol or the cell membrane have distinct roles in pain signaling or whether there is a redundancy.

**Video**

A representative real-time translocation video showing the effect of capsaicin on PKCβI-EGFP (Video file PKC beta 1 CAP); effect of PMA on PKCβI-EGFP (Video file PKC beta 1 PMA) in a single cell body of a
**Method:** The primary antibodies obtained from Santa Cruz. Their dilutions were rabbit anti-PKC-βI (1/200), rabbit anti-PKC-βII (1/200), and goat anti-VR1 (1/100). Secondary antibodies were goat anti-rabbit-FITC (1/100) (Sigma), rabbit anti-goat biotinylated species (1/100) (Sigma), and Streptavidin Texas Red (1/100) (Amersham Biosciences). All antibodies were diluted in Tris buffered saline (TBS) containing 50 mM Tris and 150 mM NaCl at pH 7.5. Controls included (i) replacement of the primary antibody with normal rabbit serum or (ii) goat serum or (iii) omission of primary antibody. No staining was apparent minus primary antibody or with normal serum controls. All experiments were replicated three times and at least two cultures were examined for each PKC isoform. Cultures were fixed and permeabilized by incubating in cold (4°C) 3.5-4% paraformaldehyde in TBS for 20 min at room temperature (RT), washed three times in TBS, and blocked in blocking buffer 1 [1% bovine serum albumin (BSA), 1% fetal bovine serum (FBS) in TBS) for 1 h at RT. After washing in TBS, cultures to be double immunostained were incubated with the first primary antibody at RT for 1 h, washed in TBS, and incubated with the first secondary antibody (anti-rabbit FITC) for 1 h at RT; it was further incubated with the second primary antibody (goat anti-TRPV1) at 4°C overnight. After washing in TBS, the cultures were incubated in blocking buffer 2 (10 % BSA in TBS) for 10 minutes at RT, followed by TBS wash and a two-step incubation with second secondary antibody. The first step involved incubation of the cultures with rabbit anti-goat biotinylated species for 1 h at RT. In the second step, the cultures were washed as previously and incubated with Streptavidin Texas Red for 1 h at RT. After washing, coverslips were inverted on to microscope slides in a 25-μL fluorescence anti-fade solution (Vector Laboratories) and sealed with clear nail polish. Immunostained cultures were visualized by a confocal laser scanning microscope (CLSM) using Leica TCS SP2 System fitted with PL APO 100x/ 1.40-0.7 OIL objective). Sequential scans (FITC and Texas Red) were collected and merged and quantified using Leica TCS SP2 Software ver. 3.0. Fluorescence intensities of FITC and Texas Red were measured across the merged images of neurons as a function of distribution of the specific PKC isoforms and TRPV1, respectively, from the cytosol to the plasma membrane.

**Figure 1:** Confocal images of DRG neuron cell bodies' double immunostained for PKC isoforms and TRPV1. PKC isoforms are indicated by the green fluorescence of fluorescein isothiocyanate (FITC) and TRPV1 is indicated by the red fluorescence of Streptavidin Texas Red. Co-localization of PKC isoforms and TRPV1 is shown in overlay with yellow regions. Fluorescence intensity shown as traces represent localization of each PKC isoform (green trace) and TRPV1 (red trace) and is measured across an average of z-series cross-sections of the cell body of the neuron. Cytosolic and plasma membrane distribution of PKCβI and PKCβII are shown. Cytosolic distribution of PKCδ and PKCe are shown.

| PKC    | TRPV1 | Overlay | Fluorescence Intensity |
|--------|-------|---------|------------------------|
| βI     |       |         |                        |
| βII    |       |         |                        |
| ε      |       |         |                        |
| δ      |       |         |                        |

10 μm
and kept on ice for 1 h with intermittent vortex mixing. Homogenized cocktail and homogenized completely at 1,600 rpm in a homogenizer with an added protease inhibitor [10 mM Tris-HCl (pH 7.4), 1% NP40, 0.1% sodium deoxycholate (DOC), 0.1% SDS, 150 mM NaCl, 1 mM EDTA] supernatant was aspirated and DRG pellet re-suspended in RIPA Buffer thus obtained were separated by SDS-PAGE (7.5% polyacrylamide).

Assay. The remainder of the supernatant of the cell lysate was then heated for 5 min at 95°C in an SDS sample buffer and protein samples of supernatant was analyzed for total protein content using Protein Marker. Membranes (PVDF, Millipore) were probed with primary antibodies rabbit anti-PKC βI at 1:100 dilutions. Secondary antibody consisted of anti-rabbit HRP (1/5000) (Promega). Proteins were detected using the ECL system (Bio-Rad).

DRG neuron in the presence of 2 mM extracellular Ca²⁺. In Video β1 CAP, 100 nM capsaicin-induced reversible translocation; and in Video β1 PMA, 20 μM PMA induced a sustained translocation. Method: One-day old DRG cultures were transiently transfected with PKCβI-EGFP (Clontech Laboratories, Inc., USA) using the Effectene Transfection Reagent (QIAGEN) protocol for 12-well culture plates. After 48 h of transfection, the neurons were used for real-time PKC translocation experiments visualized with Confocal Laser Scanning Microscope (TCS SP2 System, Leica) for 12-well culture plates. After 48 h of transfection, the EGFP-positive plasmids using the Effectene Transfection Reagent (QIAGEN) protocol for 12-well culture plates. After 48 h of transfection, the EGFP-positive neurons were visualized by a Confocal Laser Scanning Microscope (TCS SP2 System, Leica) fitted with PL APO 100x/ 1.40-0.7 OIL objective. Sequential scans of EGFP fluorescence were collected and merged and quantified using Leica TCS SP2 Software ver. 3.0.

**BRIEF OVERVIEW OF PAST LITERATURE ON PKC IN NEURONAL AND PAIN SIGNALING**

**General role of PKC in neuronal signaling**

Nerve cells can transmit signals over long distances up to 1 m by means of bioelectrical impulses. Opening of voltage-dependent Ca²⁺ channels (VDCCs) following depolarization of the presynaptic membrane by an action potential transduces the biochemical signal into several chemical messages. The influx of Ca²⁺ triggers an exocytotic release of a variety of neurotransmitters from synaptic vesicles within nerve terminals. The chemical messages are then reverted to a bioelectrical form by channel-linked receptors such as nicotinic, metabotropic glutamate (mGluR), and GABA_A receptors located on postsynaptic membranes. Many proteins related to these processes of synaptic transmission may be the prime targets of PKC action. PKC is present in high concentrations in neuronal tissues and has been implicated in a broad spectrum of neuronal functions. Activation of PKC in nerve cells is frequently associated with the modulation of ion channels, desensitization of receptors and enhancement of neurotransmitter release. The PKC pathway may modulate the efficacy of synaptic transmission.
On the other hand, non-channel-linked receptors respond to agonists by initiating a cascade of second messengers [Figure 4]. The first step in this cascade is activation of G proteins, which may either interact directly with ion channels or control the production of intracellular second messengers. When phospholipases are activated via G protein-linked receptors, PKC is activated by increased amounts of DAG in membranes, as a result of agonist-induced hydrolysis of inositol phospholipids by phospholipase C (PLC) [62,83] [Figure 4]. Upon cell stimulation, DAG is detected in various intracellular compartments at different times during the cellular responses. The early peak of DAG is transient and reverts back to basal levels within seconds, or maybe several minutes, temporally corresponding to the formation of inositol 1,4,5-triphosphate (IP3) and the rise in intracellular Ca2+ concentration. At a relatively later phase of cellular responses, formation of DAG has a slow onset but is more sustained. It is most likely derived from the hydrolysis of major constituents of the phospholipid bilayer such as phosphatidylcholine (PC) by phospholipase D (PLD) to yield phosphatidic acid, which is then dephosphorylated to DAG [84]. Activation of PLC and PLD is often accompanied by a signal-dependent release of arachidonic acid through phospholipase A2 (PLA2)-catalyzed hydrolysis [Figure 4]. The reaction products of PC hydrolysis by PLA2, cis-unsaturated fatty acid (FFA), and lysophosphatidylcholine (lysoPC), are both enhancer molecules of PKC activation [62,85] [Figure 4]. It is plausible that the agonist-induced cascade of degeneration of various membrane phospholipids is necessary for transducing full information from extracellular signals across the membrane.

**PKC regulates sensitization of peripheral and central pain signaling**

Activation of peripheral terminals of C and Aδ nociceptors by high-intensity peripheral stimuli lasts for tens of milliseconds and encodes information on the onset, intensity, quality, location, and duration of the noxious stimulus [20]. This activation is transferred from peripheral terminals via the thalamus to the cortex, leading to translation of an acute pain sensation (physiological

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**Figure 4:** Schematic showing the signaling events between thermo-TRP channels and PKC. Activation and translocation of cytosolic cPKC βI, βII, and nPKCε, δ isoforms to plasma membrane may mediate phosphorylation, and hence modulation of plasma membrane-associated thermo-TRP channels. Diacylglycerol (DAG), Phospholipase C (PLC), Inositol 1,4,5-triphosphate (IP3), intracellular calcium (Ca2+), Phosphatidylcholine (PC), Phospholipase D (PLD), Arachidonic acid (AA), Phospholipase A2 (PLA2), cis-unsaturated fatty acid (FFA), and lysophosphatidylcholine (lysoPC), Inositol phospholipids (IP).
pain).[23] Activation of nociceptors by noxious stimuli that result in an activity- or use-dependent neuronal plasticity in the spinal cord modifies the subsequent performance of the nociceptive pathway by exaggerating or prolonging the response to noxious inputs (hyperalgesia) and enabling normally innocuous inputs to activate it (allodynia).[86]

Such neuronal plasticity in the spinal cord is regulated in part by PKC, some aspects of which have been covered in an excellent review elsewhere.[87] Briefly, PKC is activated by (i) calcium entry through ionotropic receptors, (ii) voltage-gated ion channels, (iii) activation of G-protein-coupled receptors, and (iv) tyrosine kinase receptors in response to excitatory amino acid, peptide, or protein neurotransmitter/neuromodulators released by C fiber central terminals following noxious peripheral stimuli. This activation leads to changes in gene expression of sensory neurons and phosphorylation of membrane receptors and ion channels that can alter the excitability of nociceptive neurons as well as non-nociceptive neurons. Such increases in the excitability of sensory neurons result in the recruitment of responses of neurons to normally subthreshold inputs that may have low intensity or even be innocuous.

In the CNS, post-translational changes are responsible for the production of central sensitization, which manifests as an increase in responsiveness to noxious stimuli, spread of pain sensitivity to tissue other than the injured one and development of pain in response to low-intensity stimuli.[88] In summary, nociceptive input can activate transcription factors and evoke a change in gene expression in peripheral nociceptors, resulting in central sensitization and chronic pain conditions.[86,89-91]

There is substantial evidence supporting a role for PKC expressed in dorsal horn neurons in regulating pain hypersensitivity in a number of different pain models.[92-95] As mentioned earlier, an important action of [Ca2+]i, in conjunction with DAG is the induction of PKC.[87] The following observations are a few of the many that support the concept that PKC plays a pivotal role in the processes underlying central sensitization and enhancement of nociception.

1. Stimulation and inhibition of spinal pools of PKC, respectively enhance and reduce the inflammatory hyperalgesia provoked by intraplantar injection of formalin.[92,93,97]
2. PKC inhibitors attenuate the capsaicin-induced sensitization of spinothalamic tract neurons to mechanical stimuli.[98-101] In addition, the PKC inhibitor, staurosporine, blocks the facilitatory influence of NK1 receptors on N-methyl-D-aspartate (NMDA) receptor-mediated activity in the dorsal horn, whereas phorbol esters, which enhance PKC activity, mimic the facilitatory influence of PKC on NMDA receptor-mediated activity.[102-105]
3. Activation of PKC inhibits opioidergic mechanisms of antinociception.[106]
4. Levels of membrane-bound (translocated) PKC increase in the dorsal horn during inflammatory hyperalgesia following both peripheral nerve injury and noxious stimulation.[84,107]
5. There is reduced neuropathic and inflammatory pain, but preserved acute nociceptive pain, in mice lacking the γ isoform of PKC.[109] This isoform is expressed only in a subset of neurons in the inner lamina II of the dorsal horn.[108] PKCγ in the dorsal horn appears to be a trigger for activation of silent synapses between some nociceptors and the dorsal horn.[109]

To summarize, the modulation of peripheral or central terminals of nociceptive neurons is effected via PKC transduction mechanisms, which are facilitated largely by DAG/[Ca2+]i. A related and crucial intracellular event of interest in this review is the underlying changes in the excitability of nociceptive neurons following phosphorylation of the polymodal thermo-TRPs by PKC.

MODULATION OF THERMO-TRPS BY PKC AND ITS IMPLICATIONS IN PAIN SIGNALING

TRPV1-4 modulation by PKC

Functional studies have revealed a consistent picture of TRPV1 as an ion channel with polymodal gating that is subject to modulation by kinases and lipid mediators.[110] Capsaicin-sensitive sensory DRG neuron cell bodies have provided the most popular model to investigate TRPV1-associated nociceptive signaling. Since phosphorylation catalyzed by PKC exerts modulation of nociception,[22] the distribution and activity of specific isoforms of PKC in DRG neurons is suggested to be important in pain signaling.[78] Of the 16 putative serine (S) and threonine (T) PKC phosphorylation sites found on TRPV1, only S502 and S800 were reported to be regulating the increased sensitivity of TRPV1 to capsaicin or heat[111] [Table 2]. Furthermore, S800 was reported to be critical for increasing the sensitivity of TRPV1 desensitized by capsaicin[112,113] [Table 2]. These studies have clearly shown that among several isoforms of PKC expressed in the nociceptive DRG neurons, PKCζ is the predominant isoform that targets S502 and S800 sites on TRPV1 for functional regulation [Table 2]. Such specific information may have implications towards micro-managing chronic pain symptoms like thermal hyperalgesia. With this information, pharmacotherapeutics blocking PKC sites of phosphorylation on TRPV1 or the PKCζ itself could be designed. Another isoform, PKCκ...
has been shown to be necessary for TRPV1 activation\cite{114} [Table 2]. This mechanism was, however, shown to occur in nociceptive DRG neurons obtained from embryonic rats,\cite{114} which could limit the implications of these findings to the embryonic stages of development. In addition, the embryonic PKCα isoform did not have a role in the activation of postnatal TRPV1.\cite{114} An expression of PKCz and its role in modulating TRPV1 was not observed in neonatal DRG neurons.\cite{73} These studies indicate that there may be a developmental expression pattern for the PKCz isoform. Also, TRPV1 phosphorylation sites for the PKCα isoform are unknown. Further studies on the effect of other PKC isoforms and their phosphorylation sites on TRPV1 may have implications for a complete understanding of the mechanisms of chronic pain mediated via phosphorylation of specific sites of TRPV1 by specific PKC isoforms. It is possible that individual isoforms phosphorylate-specific sites on TRPV1 and may thus mediate differential modulation of the receptor. Differential modulation may also be governed by differences in expression patterns of different isoforms during development (embryonic to adult). Studies have suggested that some PKC activators can directly open (gate) the TRPV1 channels.\cite{118} Although there is some evidence for such a claim, this could merely be an artifact of TRPV1 activation at lowered temperature threshold (room temperature) in the presence of PKC activators.\cite{111} Moreover, non-phosphorylating sites that may be involved in the direct gating of TRPV1 by PKC activators have not yet been identified.

There are no reports on the direct modulation of the thermo-TRPs TRPV2 and TRPV3 by PKC [Table 2]. These two thermo-TRPs have been implicated in pain mechanisms, thus making necessary further investigations on their modulation by PKC and identification of putative serine/threonine phosphorylation sites. Such information may be useful in designing pharmacotherapeutics targeting modalities of chronic pain mediated by TRPV2 or TRPV3 modulation by PKC. Sensitization to repeated noxious heat stimuli was seen in nociceptors expressing TRPV2.\cite{116} A role for PKC in such sensitization has not been investigated. On the other hand, sensitization of TRPV3 by repeated application of non-noxious heat or camphor\cite{38} was shown to be independent of PKC.\cite{117} However, sensitization of TRPV2 or TRPV3 to single stimulus of heat following activation of PKC is not known. Hence, investigations need to be undertaken to identify putative serine/threonine residues on TRPV2 and TRPV3 receptors. Once identified, further studies must be undertaken to elucidate the effect of phosphorylation of these serine/threonine residues by PKC on the threshold of TRPV3 activation by heat or camphor, as this could have implications underlying pathological conditions of inflammation or injury.

| Table 2: Isoforms of PKC and PKC phosphorylation sites that are involved in modulating thermo-TRPs (TRPV1-4, TRPM8, and TRPA1) |
|-----------------------------------------------|
| Thermo-TRP | Modulator PKC isoforms (direct or indirect) | PKC phosphorylation sites critical for modulation |
| TRPV1 | PKCα and PKCε | S502 and S800 for PKCα |
| TRPV2 | Not known | Not known |
| TRPV3 | Independent of PKC modulation | Not directly via known sites, which include S9, S12, S20, S221, S268, S312, S319, S541, and T556 |
| TRPV4 | Not known | S162, S189, and T175 |
| TRPM8 | PKCα and/or PKCβ | Not known |
| TRPA1 | Not known | Not known |

The fourth thermo-TRPV member, TRPV4, has been characterized to have putative PKC phosphorylation sites\cite{118,119} [Table 2]. These include S162, S189 and T175,\cite{118} and S824.\cite{119} However, further investigations are required to identify specific isoforms of PKC that can phosphorylate these sites on TRPV4. Modulation of TRPV4 function by PKC has also been established.\cite{93} This modulation has been postulated to induce mechanical hyperalgesia.\cite{120} It remains to identify TRPV4 sensitization by specific isoforms of PKC mediating mechanical hyperalgesia in nociceptive and TRPV4-expressing subset of DRG neurons. Moreover, similar to TRPV1, there is evidence that PKC activators can also directly open (gate) TRPV4 channels.\cite{121,122} Evidence for gating but not direct interaction of TRPV4 by a PKC activator 4alpha-PDD was shown at non-phosphorylating site pairs, namely, Leu584 and Trp586 or Tyr591 and Arg594.\cite{123} As in the case of TRPV1, this gating mechanism of TRPV4 channels by PKC activators can well be an artifact or additive effect of the lowered threshold of sensitized TRPV4 channels, which may then open at room temperatures. Investigations undertaken to clarify such issues will have significant implications for micro-management of chronic pain conditions where PKC-sensitized TRPV4 channels mediate mechanical hyperalgesia.

**TRPM8 modulation by PKC**

Unlike the TRPV subfamily of thermo-sensitive TRP’s, TRPM8, the receptor for cool temperatures and menthol is desensitized by PKC.\cite{125} Such negative regulation of TRPM8 by PKC has been shown to occur indirectly via PKC-mediated activation of phosphatase calcineurin\cite{126} [Table 2]. Calcineurin, in turn, inhibits TRPM8 via dephosphorylation.\cite{126} This inhibition involves pushing the threshold of TRPM8 activation by cool temperatures to very cold temperatures.\cite{127} Since the desensitization
of TRPM8 by PKC was calcium-dependent, the putative isoforms of PKC that may be involved could be among the conventional PKCs (cPKCs) (α, βI, βII, and/or γ), which are calcium-dependent. One study showed evidence for indirect modulation of TRPM8 by PKCα and/or PKCβI isoforms.[125] [Table 2]. The same study showed that none of the nine putative serine/threonine PKC phosphorylation sites (S9, S12, S20, S221, S268, S312, S319, S541, and T556) were involved in desensitizing TRPM8, indicating indirect modulation of the receptor by PKC.[125] [Table 2]. However, calcium-independent isoforms cannot be ruled out. Activation of the PLC signaling cascade that mediates hydrolysis of phosphatidyl inositol 4,5-phosphate 2 (PIP2), a membrane-associated phospholipid, was shown to maintain open probability of TRPM8.[9] Here, PLC signaling results in the phospholipid, was shown to maintain open probability of TRPM8 channels.[128,129] Moreover, PLC-mediated hydrolysis of PIP2 generates DAG,[62,130] a potent activator of calcium-dependent conventional PKCs (cPKCs) (α, βI, βII, and/or γ) as well as calcium-independent novel PKCs (nPKCs) (δ, ε, η, and θ). Inhibition of TRPM8 by PKC has interesting implications for pain signaling in the subsets of DRG neurons that express TRPM8. In the subset of DRG neurons that are nociceptive and express pain peptides and TRPV1, PKC activation would inhibit TRPM8 and sensitize TRPV1. This would result in increased pain signaling. Hence, blocking PKC in this subset would be a useful strategy. In addition, for the subset of DRG neurons that expresses TRPM8 without the pain peptides or TRPV1 and is considered non-nociceptive, blocking PKC would again be a useful strategy to leave TRPM8-mediated analgesia open as a therapy for chronic pain. However, it remains to be determined whether the PKC isoforms that mediate inhibition of TRPM8 and sensitization of TRPV1 are the same or different in the subsets of DRG neurons that express TRPM8 alone or co-express TRPM8 and TRPV1.[41,44] Also, further investigations are necessary to determine PKC isoforms that inhibit TRPM8 via de-phosphorylation[125] or the PLC-signaling cascade.[128,131] It would be interesting to see whether PKC isoforms that mediate the de-phosphorylation of TRPM8 or modulate TRPM8 downstream of the PLC-signaling cascade are the same. Current knowledge of PKC mechanisms regulating TRPM8 leads us towards a paradox. On one hand, blocking PKC may help target TRPM8-mediated analgesia. On the other hand, blocking PKC would prove counterproductive in cases of cold allodynia or cold hyperalgesia mediated by TRPM8.[48] This necessitates further studies to identify specific PKC isoforms, revisit putative sites of PKC phosphorylation, and elucidate their mechanisms in specific subsets of nociceptive and non-nociceptive DRG neurons expressing TRPM8.

TRPA1 modulation by PKC

There have been no reports yet on direct phosphorylation of TRPA1 by PKC. An early study showed clear activation of PKC in the spinal cord dorsal horn and enhancement of mustard oil-induced pain.[93] Since the cloning of TRPA1, studies involving TRPA1 knockout mice have implicated mustard oil as a specific agonist of TRPA1.[47] These results suggest a putative role of PKC in enhancing TRPA1-mediated pain. Studies in TRPA1 knockout mice have also shown inhibition of bradykinin-mediated hypersensitivity,[47] suggesting a possible role for PKC as in case of TRPV1.[7] Also, like TRPV1, the PLC- or PLA2-mediated pathway sensitization of TRPA1 has been shown.[132] [Table 2]. TRPA1 was sensitized via PLC disinhibition of PIP2 mediated by the activation of proteinase activated receptor (PAR)-2 in models of cold hyperalgesia and inflammatory pain.[52,133,134] These studies provide further insights into PKC-TRPA1 signaling as a downstream effect of PLC or PLA2 cascades. However, further studies are necessary to delineate the PKC isoforms and putative PKC phosphorylation sites on TRPA1. Since most of the TRPA1 is co-expressed within the TRPV1 subsets of nociceptive DRG neurons, it is important to identify whether the PKC isoforms modulating each of these channels are the same or different.

CONCLUSION

This review highlights that the current understanding of PKC modulation of thermo-sensitive TRP channels expressed in nociceptive DRG neurons is incomplete. A complete knowledge of such interactions could have significant implications for micro-management of symptoms arising from pain signaling between PKC and thermo-sensitive TRPs. We have shown some of our own data to highlight differential expression, localization, and translocation patterns of specific PKC isoforms in nociceptive DRG neurons expressing TRPV1. The purpose of showing some of our data is to bring forward tools that can be used for future investigations into delineating specific PKC isoforms involved in modulating specific subsets of thermo-TRPs in addition to TRPV1. Real-time translocation in DRG neurons or heterologous expression systems of specific PKC isoforms and thermo-TRP channels in response to pain stimuli and PKC activators can serve as a powerful in vitro assay for such studies. Finally, with this review, we would like to stimulate a further comprehensive research into mechanisms that can potently alter somatosensation, resulting in chronic pain.

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