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Multiple PKC ϵ -dependent Mechanisms Mediating Mechanical Hyperalgesia

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Abstract

We have recently implicated mitochondrial mechanisms in models of neuropathic and inflammatory pain, in some of which a role of protein kinase C ϵ (PKC ϵ) has also been implicated. Since mitochondria contain several proteins that are targets of PKC ϵ , we evaluated the role of mitochondrial mechanisms in mechanical hyperalgesia induced by proinflammatory cytokines that induce PKC ϵ -dependent nociceptor sensitization, and by a direct activator of PKC ϵ (ψ εRACK), in the rat. Prostaglandin E₂ (PGE₂)-induced hyperalgesia is short lived in naïve rats, while it is prolonged in ψ εRACK pre-treated rats, a phenomenon referred to as priming. Inhibitors of two closely-related mitochondrial functions, electron transport (complexes I-V) and oxidative stress (reactive oxygen species), attenuated mechanical hyperalgesia induced by intradermal injection of ψ εRACK. In marked contrast, in a PKC ϵ -dependent form of mechanical hyperalgesia induced by prostaglandin E₂ (PGE₂), inhibitors of mitochondrial function failed to attenuate hyperalgesia. These studies support the suggestion that at least two downstream signaling pathways can mediate the hyperalgesia induced by activating PKC ϵ .

Keywords

Hyperalgesia; Mitochondria; Priming; Protein kinase C ϵ ; Pain

Introduction

While PKCepsilon (PKC ϵ) a novel protein kinase C (PKC) isoform, plays an important role in primary afferent nociceptor sensitization and mechanical hyperalgesia [28,3,43,40,10,42] mechanisms downstream of PKC ϵ remain to be elucidated. The mitochondrion, an intracellular organelle to which PKC ϵ can translocate [15,11,36] which contains proteins phosphorylated by PKC ϵ such as the mitochondrial respiratory chain protein, cytochrome oxidase subunit IV (COX IV) [32,8,12], mitochondrial ATP-sensitive K⁺ channel [38,11], and glycogen synthetase kinase-3beta [7,29], has recently been implicated in mechanical hyperalgesia [24,25]. In the present study we evaluated the hypothesis that the

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Conflict of interest: Jon Levine is a consultant to KAI Pharmaceutical Inc., which is performing clinical trials of a PKC ϵ -inhibitor for pain.

mitochondrion is a downstream target of PKC ϵ mediating the induction of mechanical hyperalgesia. In previous studies [26] we have shown that administration of carrageenan or ψ ERACK can trigger long-lasting hypersensitivity of nociceptors to inflammatory cytokines. This phenomenon “hyperalgesic priming” was demonstrated to be PKC ϵ dependent [35,21]. We studied the role of four key mitochondrial mechanisms, electron transport (complexes I-V), oxidative stress (reactive oxygen species, ROS), caspase signaling, and intracellular calcium mobilization [30,14,6], in mechanical hyperalgesia induced by inflammatory mediator G-protein coupled receptors that produce PKC ϵ -dependent hyperalgesia [19,31] and by a more generalized activator of PKC ϵ by pseudo-receptor for activated C kinase (ψ ERACK), a direct activator of PKC ϵ [13].

Experimental procedures

Animals

Male (220–300 g) Sprague–Dawley rats (Charles River, Hollister, CA) used in these experiments were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12-h light/dark cycle. All experimental protocols were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee, and conformed to NIH guidelines for the care and use of experimental animals. Effort was made to limit the number of animals used and their discomfort.

Drugs: Preparation and administration

The drugs used in these experiments were: rotenone (3 acetylphenyl N-(P-Tolyl) carbamate) a mitochondrial electron transport chain complex I (mETC-I) inhibitor; 3-NP (3 nitropropionic acid), a mETC-II inhibitor; antimycin, a mETC-III inhibitor; sodium cyanide, a mETC-IV inhibitor; oligomycin, a mETC-V inhibitor; α -lipoic acid, an antioxidant, TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester), an intracellular calcium mobilization blocker, prostaglandin E₂ (PGE₂), and epinephrine (Sigma, St. Louis), ZVAD-FMK, a nonspecific caspase inhibitor (EMD Bioscience, San Diego), and ψ ERACK, a selective PKC ϵ activator [13] prepared by SynPep (Dublin, CA). Rotenone, 3-NP, antimycin, oligomycin, α -lipoic acid and ZVAD-FMK were dissolved in 10% DMSO, sodium cyanide, TMB-8, ψ ERACK, and epinephrine were dissolved in physiological saline. PGE₂ stock solution was prepared in 10% ethanol and diluted with physiological saline so that the final concentration of alcohol was < 1%. All compounds were administered intradermally (i.d.) on the dorsum of the hind paw in a volume of 5 μ l, using a 30-gauge hypodermic needle connected to a 10- μ l Hamilton syringe. When studying the effect of co-administration of the above inhibitors with other agents, the solutions were drawn up into the syringe, separated by a small air bubble, such that the inhibitor reached the paw prior to the other test agent. Paw withdrawal thresholds were measured at different time points (30 min and 4 hrs) except for time course determination, when it was measured up to 96 hrs after test agent administrations. Doses of test agents were selected based on the results of our previous studies [23,24].

Mechanical nociceptive testing

Mechanical nociceptive threshold was quantified by the Randall–Selitto paw pressure test [37], using an Analgesymeter (Stoelting, Chicago, IL) in which a force that increases linearly over time is applied to the dorsum of the rat's hind paw. For testing, rats were lightly restrained in transparent cylinders with slits on the side that allowed access to the hind leg. All rats were acclimated to the testing procedure. Nociceptive threshold was defined as the force (in grams) at which the rat withdrew its paw. Paw-withdrawal threshold was defined as the mean of three readings. Each paw was treated as an independent measure

and each experiment performed on a separate group of rats. All behavioral testing was done between 10:00 and 16:00 h.

Hyperalgesic priming was induced, as described previously [26,34,21]. In brief, the PKC ϵ activator, ψ εRACK (1 μ g) was injected intradermally on the dorsum of a rat's hind paw. ψ εRACK-induced mechanical hyperalgesia was measured every hour for 6 hours on the first day and continued every day for four days. Five days later, when nociceptive threshold had returned to pre- ψ εRACK treatment baseline, the nociceptive effect of PGE₂ was evaluated. In this primed state PGE₂ hyperalgesia develops PKC ϵ dependence not present in the naïve control rat [4].

Statistics

Data are expressed as percentage decrease in nociceptive threshold, group data are presented as mean $\pm$ SEM, and analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test. The accepted level for statistical significance was set at a p-value of 0.05. Separate groups of animals were used for each experiment.

Results

Time course of ψ εRACK-, PGE₂- and epinephrine-induced mechanical hyperalgesia

As previously demonstrated [5,26] intradermal injection of ψ εRACK (1 μ g), PGE₂ (100 ng) and epinephrine (epi, 100 ng) induced a decrease in mechanical nociceptive threshold in control rats (Fig. 1A). The hyperalgesia induced by PGE₂- and epinephrine- was short lived (< 4hr, Fig. 1A, both n = 6), while that induced by ψ εRACK persisted for more than 24 hours (Fig. 1A, n = 6), returning to baseline by the 3rd to 4th day. In primed rats, which were previously treated with ψ εRACK, administration of PGE₂ (100 ng) now induced hyperalgesia that lasted for >4 hrs (Fig. 1B, n = 6). PGE₂ and epinephrine were tested similarly and even when co-administered they failed to produce priming (data not shown).

Mitochondrial electron transport chain (mETC) complex inhibitors differentially affect ψ εRACK-, PGE₂- and epinephrine-induced hyperalgesia

To determine whether mitochondria play a role in mechanical hyperalgesia, we tested the effect of electron transport chain complex (mETC I-V) inhibitors on ψ εRACK-PGE₂- and epinephrine-induced hyperalgesia. Inhibitors of all five mETCs significantly attenuated ψ εRACK-induced acute hyperalgesia (Fig. 2A, all, p < 0.001, n = 8/group) while all failed to inhibit PGE₂- or epinephrine-induced hyperalgesia (Fig. 2B&C, all p = ns, n = 8/group). All mETC (I-V) inhibitors have been tested for their *per se* effect previously [24]. These compounds did not change the mechanical paw withdrawal thresholds of naïve rats at the doses employed.

Antioxidant differentially affect ψ εRACK-, PGE₂- and epinephrine-induced hyperalgesia

To evaluate the role of oxidative stress in mechanical hyperalgesia, we tested the effect of α -lipoic acid, an antioxidant, on ψ εRACK-, PGE₂- and epinephrine-induced mechanical hyperalgesia. α -lipoic acid markedly attenuate ψ εRACK and slightly but significantly epinephrine-induced hyperalgesia (Fig. 2A&C, p < 0.001, for both ψ εRACK and epi, n = 8/ for both groups), but failed to have any effect on PGE₂-induced hyperalgesia (Fig. 2B, all p = ns; n = 8/group).

Caspase inhibitor does not inhibit ψ εRACK-, PGE₂- or epinephrine-induced hyperalgesia

To evaluate the role of apoptotic signaling pathways we tested the effect of a nonspecific caspase inhibitor Z-VAD-FMK on ψ εRACK-, PGE₂- and epinephrine-induced mechanical

hyperalgesia. Z-VAD-FMK had no effect on mechanical hyperalgesia induced by ψ εRACK-, PGE₂- or epinephrine (Fig. 2 A,B&C, all $p = ns$; $n = 8/\text{group}$).

Calcium mobilization blocker does not inhibit ψ εRACK-, PGE₂- or epinephrine-induced mechanical hyperalgesia

To evaluate the role of calcium mobilization, we tested the effect of TMB-8, an intracellular calcium mobilization blocker, on ψ εRACK-, PGE₂- and epinephrine-induced mechanical hyperalgesia. TMB-8 did not inhibit the mechanical hyperalgesia induced by ψ εRACK, PGE₂ or epinephrine (Fig. 2A,B&C, all $p = ns$; $n = 8/\text{group}$).

Role of mitochondria in PGE₂ hyperalgesia after priming

After recovery from ψ εRACK-induced mechanical hyperalgesia, PGE₂ produces an enhanced (30 min) and PKCε-dependent prolonged (>4 hrs) hyperalgesia, a phenomenon referred to as hyperalgesic priming [4], in naïve control rats intradermal PGE₂-induced hyperalgesia is mediated by protein kinase A (PKA) [4]. The mitochondrial electron transport chain complex (mETC I-V) inhibitors, α-lipoic acid, Z-VAD-FMK and TMB-8 were tested against PGE₂-induced hyperalgesia in the primed state. In primed rats, none of these agents produced a significant reduction in the PGE₂-induced hyperalgesia (Fig. 3A&B, all $p = ns$; $n = 6/\text{group}$).

Discussion

The mechanical hyperalgesia induced in several models of inflammatory [4,5,24,25], neuropathic [2,19,25] and generalized widespread pain [27] syndromes are, at least in part, PKCε dependent. Several molecules have been demonstrated to be upstream of PKCε in nociceptor function including G-protein coupled and death family cell surface receptors [3,20,24] and the second messengers G_i-protein [27] phospholipase Cβ3 [39,35,23,21], ceramide [23] and the guanine exchange factor, Epac [20]. However, the mechanisms downstream of PKCε in primary afferent nociceptors that mediate these pain syndromes, are largely unknown. The possibility that mitochondria play a role in transduction mechanisms in sensory neurons has been suggested by the finding that they are at especially high concentration in the peripheral terminals of presumed nociceptors in synovial [16] and corneal [17] afferents. Functional studies suggest that they may play a role in shaping nociceptor function [18,33]. We have recently demonstrated a role of mitochondrial mechanisms in a model of cancer chemotherapy (vincristine)-induced painful neuropathy in which mechanical hyperalgesia was PKCε – dependent [22,24]. Since it has been shown in other cells that PKCε can translocate to mitochondria [15,36], which contain proteins that are phosphorylated by PKCε including, mitochondrial respiratory chain proteins [32,8,12], in the present experiments we tested the hypothesis that mitochondrial mechanisms are downstream targets of PKCε mediating mechanical hyperalgesia.

While of the four major mitochondrial functions (i.e., electron transport and ATP synthesis, reactive oxygen species generation, caspase pathway signaling and intracellular calcium mobilization) [30,14,6], all have been implicated in mechanical hyperalgesia in one or more animal models, in the present studies we found that the hyperalgesia induced by direct activation of PKCε is mediated by only two of these mitochondrial mechanisms, the closely linked mitochondrial electron transport chain, and ROS generation. We did not detect a significant contribution from the other two mechanisms, caspase signaling and intracellular calcium mobilization, at doses found effective in other pain models [1,23].

While ψ εRACK is likely able to activate PKCε in most if not all subcellular locations, it is known that activation of PKCε by different upstream signaling pathways causes it to

translocate and therefore be activated at different sites in the cell (e.g., mitochondria, plasma membrane, Golgi, and juxta-nuclear region) [41,9,20,36]. Therefore, we also evaluated whether mitochondria are downstream of PKC ϵ in receptor-activated signaling pathways involved in nociceptor sensitization. We chose to evaluate a model of chronic inflammatory pain, hyperalgesic priming, in which PKC ϵ has been implicated in nociceptor sensitization and mechanical hyperalgesia [26,34,21]. In contrast to the effect of the more generalized activator of PKC ϵ , hyperalgesia induced by PGE₂ in primed paws was not dependent on any of the four major mitochondrial functions. While these data support a role for PKC ϵ at a site in the cell other than that associated with the mitochondrial compartment in the cell, the location of the relevant pool of PKC ϵ involved remains to be established. Of note, however, we have previously shown that a mediator that produces hyperalgesia by action at a G-protein coupled receptor (i.e., the β_2 -adrenergic receptor), which causes PKC ϵ to translocate from the cytoplasm to plasma membrane [20], in the present study produced mitochondrial-independent hyperalgesia. Future studies will need to address both interactions between the mitochondrial mechanisms contributing to PKC ϵ -induced hyperalgesia, as well as downstream targets of these mitochondrial mechanisms contributing to pain.

Acknowledgments

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Figure 1. Time Course of $\psi\epsilon$ RACK, PGE₂ and epinephrine hyperalgesia

A. Time course of the mechanical hyperalgesia induced by intradermal injection of prostaglandin E₂ (PGE₂, 100 ng), epinephrine (epi, 100 ng) and a PKC ϵ activator ($\psi\epsilon$ RACK, 1 μ g) on the dorsum of the hind paw. All three agents tested, produced a decrease in mechanical nociceptive threshold measured at 30 min (all $p < 0.001$, $n = 6$ /group), the mechanical hyperalgesia was short lived (< 4 hr) in PGE₂-and epinephrine- treated rats, but prolonged (> 72 hr) in $\psi\epsilon$ RACK-treated rats.

B. Time course of the mechanical hyperalgesia induced by intradermal injection of prostaglandin E₂ (PGE₂, 100 ng) on the dorsum of the hind paw of rats treated 5 days previously with $\psi\epsilon$ RACK, 1 μ g (i.e., primed). In $\psi\epsilon$ RACK primed rats, PGE₂ (100 ng)-

induced peripheral mechanical hyperalgesia was significantly prolonged (> 24 hr, $p < 0.001$, $n = 6$).

Figure. 2. Effect of Mitochondrial inhibitors on ψ εRACK, PGE_2 and epinephrine-induced hyperalgesia

A. Intradermal injection of inhibitors of mitochondrial electron complexes (I-V, each 5 μ g) or ROS inhibitor α -lipoic acid (5 μ g) together with ψ εRACK (1 μ g), significantly attenuated ψ εRACK-induced hyperalgesia (all $p < 0.001$, $n = 8$ /group). Non-selective caspase inhibitor, ZVAD-FMK and intracellular calcium mobilization blocker TMB-8 (both 5 μ g, both $p = ns$; $n = 8$ /group) did not affect ψ εRACK-induced hyperalgesia.

B. Intradermal injection of inhibitors of mitochondrial electron complexes (I-V), ROS inhibitor α -lipoic acid, non-selective caspase inhibitor, ZVAD-FMK and intracellular

calcium mobilization blocker TMB-8 did not attenuate PGE₂ (100 ng)-induced hyperalgesia (all $p = \text{ns}$; $n = 8/\text{group}$).

C. Intradermal injection of inhibitors of mitochondrial electron complexes (I-V), non-selective caspase inhibitor, ZVAD-FMK and intracellular calcium mobilization blocker TMB-8 did not attenuate epinephrine (100 ng)-induced hyperalgesia (all $p = \text{ns}$; $n = 8/\text{group}$). However, ROS inhibitor α -lipoic acid significantly attenuated epinephrine-induced hyperalgesia ($p < 0.001$, $n = 6$).

Figure. 3. Effect of Mitochondrial inhibitors on PGE₂-induced hyperalgesia in primed (previously treated with ψ εRACK) rats

A. Intradermal injection of mitochondrial electron complex inhibitors (I-V, each, 5 μ g), ROS inhibitor α -lipoic acid, non-selective caspase inhibitor, ZVAD-FMK and intracellular calcium mobilization blocker TMB-8 (all 5 μ g), did not attenuate PGE₂ (100 ng)-induced hyperalgesia in ψ εRACK-primed rats, when determined 30 min after the administration of PGE₂ (all p = ns; n = 6/group).

B. Intradermal injection of inhibitors of mitochondrial electron complexes (I-V), ROS inhibitor α -lipoic acid, non-selective caspase inhibitor, ZVAD-FMK and intracellular calcium mobilization blocker TMB-8 (all n = 6/group) did not attenuate PGE₂ (100 ng)-

induced hyperalgesia in ψ εRACK-primed rats, when determined 4 hr after the administration of PGE₂ (all p = ns; n = 6/group).