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Peripheral Synthesis of an Atypical Protein Kinase C Mediates the Enhancement of Excitability and the Development of Mechanical Hyperalgesia Produced by Nerve Growth Factor

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Abstract—Nerve growth factor (NGF) plays a key role in the initiation as well as the prolonged heightened pain sensitivity of the inflammatory response. Previously, we showed that NGF rapidly augmented both the excitability of isolated rat sensory neurons and the mechanical sensitivity of the rat's hind paw. The increase in excitability and sensitivity was blocked by the myristoylated pseudosubstrate inhibitor of atypical PKCs (mPSI), suggesting that an atypical PKC may play a key regulatory role in generating this heightened sensitivity. Our findings raised the question as to whether NGF directs changes in translational control, as suggested for long-lasting long-term potentiation (LTP), or whether NGF leads to the activation of an atypical PKC by other mechanisms. The current studies demonstrate that enhanced action potential (AP) firing produced by NGF was blocked by inhibitors of translation, but not transcription. In parallel, *in vitro* studies showed that NGF elevated the protein levels of PKMζ, which was also prevented by inhibitors of translation. Intraplantar injection of NGF in the rat hind paw produced a rapid and maintained increase in mechanical sensitivity whose onset was delayed by translation inhibitors. Established NGF-induced hypersensitivity could be transiently reversed by injection of rapamycin or mPSI. These results suggest that NGF produces a rapid increase in the synthesis of PKMζ protein in the paw that augments neuronal sensitivity and that the ongoing translational expression of PKMζ plays a critical role in generating as well as maintaining the heightened sensitivity produced by NGF.

Key words: sensory neuron, sensitization, neurotrophin, protein synthesis, excitability, hyperalgesia.

INTRODUCTION

Neurotrophins, such as nerve growth factor (NGF), play key roles in the initiation of the inflammatory response by their ability to activate or traffic a variety of immune cells to a site of injury (Levi-Montalcini et al., 1996; Skaper, 2001; Villoslada and Genain, 2004; Nockher and Renz, 2006; Linker et al., 2009; Seidel et al., 2010). An early study demonstrated that the levels of NGF were elevated in blister exudates obtained from the hindpaw skin of rats (Weskamp and Otten, 1987). Additionally, application of NGF was shown to lead to the release of histamine (Bruni et al., 1982; Mazurek et al., 1986) and serotonin (Horigome et al., 1993) from rat peritoneal mast cells. NGF was proven to be chemotactic for human (Gee et al., 1983) and mouse (Boyle et al., 1985) polymorphonuclear leukocytes. Human B cells express the TrkA receptor for NGF (Otten et al., 1989; Brodie and Gelfand, 1992) and their proliferation was augmented upon exposure to NGF (Thorpe and Perez-Polo, 1987; Otten et al., 1989; Brodie and Gelfand, 1992). Finally, immune-competent cells, such as mouse CD4+ and CD8+ T lymphocytes (Ehrhard et al., 1993; Santambrogio et al., 1994) and rat peritoneal mast cells (Leon et al., 1994), express the mRNA for NGF as well release biologically active NGF upon activation.

In this capacity, NGF can also enhance the sensitivity of nociceptive sensory neurons to different modalities of stimulation and thereby lead to heightened pain states (reviewed by McMahon, 1996; Woolf, 1996). Intraperitoneal injection of NGF was reported to greatly enhance the sensitivity to both mechanical and thermal stimulation of the hindpaw of a rat (Lewin et al., 1993). Intraplantar

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Abbreviations: APs, action potentials; CFA, complete Freund's adjuvant; DRG, dorsal root ganglion; HPRT, hypoxanthineguanine phosphoribosyltransferase; LTP, long-term potentiation; MPE, maximal possible effect; mPSI, myristoylated pseudosubstrate inhibitor of atypical PKCs; NGF, nerve growth factor; RE, response efficiency; VFH, von Frey hair; ZIP, zeta inhibitory peptide.

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injection of complete Freund's adjuvant (CFA) also augments the hindpaw sensitivity to mechanical or thermal stimulation; this CFA-induced hypersensitivity was blocked by injection of an antibody to NGF, indicating that elevated NGF directly mediates this inflammatory pain (Woolf et al., 1994; Lewin et al., 1994; Nicol and Vasko, 2007).

The hypersensitivity of nociceptive sensory neurons after exposure to inflammatory mediators has, in some ways, been likened to the effects of agonist or high-frequency stimulation of nerve fibers in the hippocampus that result in long-term potentiation (LTP). The long-lasting or maintenance phase of LTP depends on the synthesis of new proteins (Stanton and Sarvey, 1984; Kelleher et al., 2004; Costa-Mattioli et al., 2009) wherein one key protein associated with LTP is the atypical PKC known as PKMζ (Sacktor et al., 1993; Sacktor, 2011). PKMζ can be expressed from an internal promoter within the full-length PKCζ gene, resulting in a truncated product that lacks the regulatory domain, rendering this product constitutively active (Hernandez et al., 2003). Several different studies suggested that this variant plays a key role in the maintenance of long-term synaptic strength. For example, treatment with the myristoylated pseudosubstrate inhibitor (mPSI) of atypical PKCs reversed established LTP (Ling et al., 2002). In an *in vivo* study of conditioned taste aversion (CTA) as an animal model of memory, infusion of mPSI into the insular cortex suppressed the CTA memory (Shema et al., 2007). Finally, lenti-viral over-expression of PKMζ enhanced CTA memory, whereas introduction of a dominant-negative PKMζ led to suppression (Shema et al., 2011).

Our previous studies demonstrated that treatment with NGF acutely enhanced the excitability of isolated rat sensory neurons (Zhang et al., 2002, 2012) and that intraplantar injection of NGF produced a significant hypersensitivity to mechanical and thermal stimulation of the rat's hindpaw (Khodorova et al., 2013, 2017). The NGF-induced augmentation of excitability, mechanical, and thermal sensitivity was blocked by pretreatment with mPSI. In addition, siRNA targeted to PKCζ significantly reduced the expression of PKMζ, but not that of either PKCζ or PKCλ/ι, and blocked the NGF-mediated increases in the excitability of sensory neurons (Zhang et al., 2012). These observations indicate that PKMζ plays a key regulatory role in generating the heightened sensitivity resulting from exposure to NGF. Our findings then suggest two possible explanations: NGF engages the translational control pathway, as has been suggested for long-lasting LTP in the central nervous system, or by some other mechanisms NGF leads to the activation of an atypical PKC in the peripheral nervous system.

EXPERIMENTAL PROCEDURES

Isolation and maintenance of sensory neurons

Sensory neurons were harvested from young adult Sprague–Dawley rats (80–150 g) (Harlan Laboratories, Indianapolis, IN, USA). Briefly, male rats were killed by placing them in a chamber that was then filled with CO_2. Dorsal root ganglia (DRG) were isolated and collected in a conical tube with sterilized Puck's solution. The tube was centrifuged for 1 min at approximately 2000×*g* and the pellet was resuspended in 1 ml Puck's solution containing 10 U of papain (Worthington, Lakewood, NJ, USA). After 15-min incubation at 37 °C, the tube was centrifuged at 2000×*g* for 1 min and the supernatant was replaced by 1 ml F-12 medium containing 1 mg collagenase IA and 2.5 mg dispase II (Roche Diagnostics, Indianapolis, IN, USA). The DRGs were resuspended and incubated at 37 °C for 20 min. The suspension was centrifuged for 1 min at 2000×*g* and the supernatant was removed. The pellet was resuspended in F-12 medium supplemented with 10% heat-inactivated horse serum and 30 ng/ml NGF (Harlan Bioproducts, Indianapolis, IN, USA) and mechanically dissociated with fire-polished glass pipette until all visible chunks of tissue disappeared. Isolated cells were plated onto either plastic coverslips (electrophysiology experiments) or 6-well tissue culture plates (Western blotting experiments); both surfaces were previously coated with 100 μg/ml poly-D-lysine and 5 μg/ml laminin. Cells were then maintained in culture in an F-12 medium supplemented with 30 ng/ml NGF at 37 °C and 3% CO_2 for either 18–24 h before electrophysiological recording or for 48 h before administering treatments and collecting cell lysates for Western blotting experiments. All procedures were approved by the Animal Use and Care Committee of the Indiana University School of Medicine.

Electrophysiology

Recordings were made using the whole-cell patch-clamp technique as previously described (Zhang et al., 2012). Briefly, a coverslip with sensory neurons was placed into a culture dish containing normal Ringer's solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, with pH adjusted to 7.4 using NaOH; after approximately 15 min, the cover slip was transferred to the recording chamber filled with Ringer's solution. Recording pipettes were pulled from borosilicate glass tubing (Model G85165T-4, Warner Instruments, Hamden, CT, USA). Recording pipettes had resistances of 2–5 MΩ when filled with the following solution (in mM): 140 KCl, 5 $MgCl_2$, 4 ATP, 0.3 GTP, 0.25 $CaCl_2$, 0.5 EGTA, (calculated free Ca^{2+} concentration of 100 nM, MaxChelator), and 10 HEPES, at pH 7.2 adjusted with KOH. Whole-cell voltages were recorded with an Axopatch 200 or Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were acquired and analyzed with pCLAMP 10 (Molecular Devices). All drugs were applied with a VC-8 bath perfusion system (Warner Instruments). NGF was used at a concentration of 100 ng/ml, which was based on the observation that this concentration produced a significant sensitization of the capsaicin-gated current in small-diameter rat sensory neurons (Shu and Mendell, 1999). In the current-clamp experiments, the neurons were held at their resting potentials (between −45 and −65 mV) and a depolarizing current ramp (1000 ms in duration) was applied. The amplitude of a ramp was adjusted to produce between 2 and 4 action potentials (APs) under control conditions

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