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RESEARCH PAPER

Signaling Pathways Relevant to Nerve Growth Factor-induced Upregulation of Transient Receptor Potential M8 Expression

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Abstract—Transient receptor potential melastatin 8 (TRPM8) is a nonselective cation channel that primarily detects the innocuous cold. In pathological conditions, TRPM8 plays a role in the development of cold hyperalgesia/allodynia. Nerve growth factor (NGF) is an important mediator involved in various pain disorders. In the present study, the NGF-TrkA pathway increased TRPM8 expression by stabilizing *TRPM8* mRNA through the actions of phosphatidylinositol 3-kinase and p38 MAP kinase. Moreover, c-Jun N-terminal kinase and Src tyrosine kinase were identified as a positive and negative regulator of TRPM8 expression, respectively, via post-transcriptional mechanisms independent of mRNA stabilization. PTEN activity was found to increase protein TRPM8 expression. Calcium imaging confirmed that NGF induced TRPM8 functional upregulation. Time-lapse fluorescence microscopic analysis and a cell fractionation assay revealed that NGF promoted the trafficking of TRPM8 to the plasma membrane. In the presence of NGF, lysosome-associated membrane protein-2 (LAMP-2) was localized to TRPM8-positive dot-like and linear structures, the latter of which were observed in the periphery of the cytoplasm. It was inferred that LAMP-2 was involved in the vesicular transport of TRPM8. Pharmacological blockade of the proteasome with MG132 led to a further increase in NGF-induced TRPM8 expression, indicating that the proteasome system played a pivotal role in the degradation of TRPM8. Our findings provide novel insight into the signaling pathways involved in NGF-mediated TRPM8 upregulation and its reversion to the normal state.

Key words: transient receptor potential M8 (TRPM8), nerve growth factor (NGF), receptor trafficking, neuropathic pain, inflammatory pain, proteasome.

INTRODUCTION

For all organisms, it is extremely important to perceive noxious stimuli for self-defense. Primary sensory neurons with the ability to detect noxious and usually injurious stimuli exist in the dorsal root ganglia (DRG) and trigeminal ganglia (TG) (Basbaum et al., 2009). Some of these neurons possess unmyelinated C fibers or thin myelinated Aδ fibers and are known to express molecular receptors required for nociception, which include the transient receptor potential (TRP) family (Laing and Dhaka, 2016). There are several distinct TRP family members; for instance, TRP vanilloid 1 (TRPV1) is activated by noxious heat (> 42 °C), protons and pungent compounds, such as capsaicin (Caterina et al., 1997). TRPV1 is a nonselective cation channel that transduces these noxious stimuli into a pain signal within nociceptors. Subsequently, the signal is transmitted to the central nervous system and perceived as actual pain. The cell-surface expression level of TRPV1 is known to be upregulated

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Abbreviations: 4-OHT, 4-hydroxytamoxifen; ANOVA, analysis of variance; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; EmGFP, Emerald green fluorescent protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; KO, knockout; LAMP-2, lysosome-associated membrane protein-2; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-activated kinase kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PTEN, phosphatase and Tensin homolog deleted from Chromosome 10; Src, sarcoma-family kinase; TG, trigeminal ganglion; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid 1; VAMP7, vesicle-associated membrane protein 7.

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by inflammatory mediators, such as nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1) and ATP (Camprubi-Robles et al., 2009). These facilitating actions for TRPV1 plasma membrane expression are accomplished by transcriptional upregulation and/or increased trafficking to the plasma membrane. The insertion of TRPV1 into the plasma membrane is controlled by post-translational modifications, such as phosphorylation of specific amino acid residues of TRPV1. For instance, NGF is known to drive the TrkA-phosphatidylinositol 3-kinase (PI3K)-Src kinase pathway, which culminates in TRPV1 phosphorylation of the tyrosine residue at position 200 (Y200) (Zhang et al., 2005). This phosphorylation facilitates the translocation of TRPV1 to the plasma membrane. The upregulation of cell-surface TRPV1 expression by inflammatory mediators is relevant to the development of inflammatory hyperalgesia and allodynia. Hyperalgesia is defined as an increased response to painful stimuli (Basbaum et al., 2009).

The cold sensing mechanism of the peripheral nerve terminals appears to be complex (Babes et al., 2004; Madrid et al., 2006). TRP melastatin 8 (TRPM8) is activated by innocuous cold ($\leq$26 °C), menthol, and other cooling substances, such as eucalyptol and icilin (McKemy et al., 2002; Dhaka et al., 2007). It is likely that intricate modulatory actions determine the threshold temperature at which TRPM8 is activated (de la Peña et al., 2005). TRPM8 activation does not cause pain under normal circumstances; rather, a soothing sensation arises from TRPM8 activation. Cooling (TRPM8 activation) can relieve heat pain, a consequence of TRPV1 activation (Kayama et al., 2017). Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is a cardinal cellular lipid component, and it is hydrolyzed into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC). Evidence shows that TRPM8 channel gating is enhanced by PtdIns(4,5)P_2 (Liu and Qin, 2005; Rohacs et al., 2005). After hydrolysis of PtdIns(4,5)P_2 by PLC, IP_3 elevates the intracellular Ca^{2+} concentration by mobilizing Ca^{2+} stores from the endoplasmic reticulum, which is followed by activation of Ca^{2+}/calmodulin kinase (CaMK). On the other hand, DAG activates protein kinase C (PKC). Both kinases are known to downregulate TRPM8 channel activity (Premkumar et al., 2005; Abe et al., 2006; Sarria et al., 2011). In contrast, PtdIns(4,5)P_2 attenuates TRPV1 channel activity when it is inserted into the extracellular leaflet of the plasma membrane (Senning et al., 2014). In this regard, an inhibitory modification on TRPV1 concomitant with TRPM8 activation by PtdIns(4,5)P_2 may contribute to the specialization in cold sensing of primary sensory neurons positive for both TRPM8 and TRPV1 (Takashima et al., 2007; Dhaka et al., 2008; Kayama et al., 2017). In pathological conditions, TRPM8 activation contributes to the development of cold hyperalgesia/allodynia. Colburn et al. (2007) showed that either subcutaneous complete Freund's adjuvant (CFA) administration or nerve injury led to heightened sensitivity to a cooling effect with acetone, which was consistent with the development of cold hyperalgesia/allodynia. In *TRPM8* knockout (KO) mice, such acetone hypersensitivity was significantly attenuated in inflammatory and neuropathic pain models, indicating that TRPM8 is required for the development of cold hyperalgesia/allodynia in both pain models. Cold hyperalgesia/allodynia is an extremely bothersome medical problem not only in inflammatory settings but also in diabetic and chemotherapy-induced neuropathic conditions (Descoeur et al., 2011; Brix Finnerup et al., 2013; Ewertz et al., 2015). Currently, no efficacious therapy exists for cold hyperalgesia/allodynia. The neurotrophic factors, NGF and artemin, are known to evoke cold sensitization *in vivo* (Lippoldt et al., 2013). In particular, there is ample evidence showing that NGF is implicated in the pathophysiology of a myriad of inflammatory and neuropathic pain states (Mizumura and Murase, 2015). Although experimental data indicate that NGF upregulates TRPM8 expression (Babes et al., 2004; Luo et al., 2007), little is known about the precise mechanism underlying this phenomenon. In addition, it remains elusive how such an elevated TRPM8 expression state is rectified thereafter. In the present study, we primarily aimed to clarify the molecular mechanisms whereby NGF upregulates TRPM8 expression. We also explored what molecules were involved in TRPM8 degradation.

We established a PC12 cell line that stably expressed an Emerald GFP (EmGFP)-rat full-length TRPM8-V5 fusion protein. We demonstrate that NGF-TrkA pathway activation enhances both whole-cell and cell-surface expression of TRPM8 with functional upregulation. Moreover, we clarify the intracellular signaling that mediates NGF-induced TRPM8 upregulation by pharmacological analysis. Lastly, we show that the proteasome system plays a pivotal role in the intracellular degradation of TRPM8.

EXPERIMENTAL PROCEDURES

Stable transformants expressing an EmGFP-rat full-length TRPM8-V5 epitope fusion protein

Total RNA was prepared from the TG of an adult male Sprague–Dawley rat using TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). Full-length TRPM8 cDNA was amplified by PCR using a set of sequence-specific primers (forward: 5′-caccatggccttcgagggagccagg-3′, reverse: 5′-tttgactttat tagcaatctctttcag-3′). The amplified DNA fragment was subcloned into pcDNA™3.2-DEST (Invitrogen). The integrity of the plasmid was verified by DNA sequencing. The EmGFP-rat full-length TRPM8-V5 expression vector was transfected into PC12 cells using Lipofectamine 2000 (Invitrogen). Stably transfected cells were isolated using 10 μg/ml Blasticidin (Invitrogen) and are referred to as EmGFP-TRPM8-V5 cells hereafter. All experimental procedures were approved by the Keio University School of Medicine Safety Committee on Genetically Modified Organisms (Authorization No. 20-017-5). Rat NGF (N2513, Sigma-Aldrich, St. Louis, MO, USA) dissolved in distilled water at 100 mg/ml was stored at −20 °C as frozen aliquots until use. NGF was added to the culture media at concentrations of 10–200 ng/ml.

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