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研究課題名（和文）Roles of spinal dorsal horn TRP channels in neuropathic pain

研究課題名（英文）Roles of spinal dorsal horn TRP channels in neuropathic pain

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研究成果の概要（和文）：Sパルミトイル化は可逆的な翻訳後修飾の一つで、蛋白質の形質膜発現、空間的分布、相互作用や機能的活性を調節する。そこで、様々な細胞機能に関与するTRPチャネルのSパルミトイル化を検討した。パルミトイル化酵素阻害剤2BPはHEK293T細胞に発現させたTRPM8，TRPA1，TRPV2のチャネル活性を変化させた。また、パルミトイル化酵素DHHC7を共発現させると、TRPM8，TRPA1のチャネル活性が増大した。一方、脱パルミトイル化剤NtBHAと2BP処置でマウス感覚神経のTRPM8，TRPA1活性は減弱した。Sパルミトイル化は複数のTRPチャネル発現を制御することが分かった。

研究成果の概要（英文）：S-palmitoylation is a reversible post-translational modification that can control the surface expression, spatial organization, interactions, and functional activities of proteins. However, little is known about whether and how TRP channels are regulated by S-palmitoylation. We investigated the role of S-palmitoylation in the in vitro and in vivo activity of several TRP channels. Treatment with a palmitoyl acyltransferase (PAT) enzyme inhibitor 2-bromopalmitate (2BP) altered the agonist-induced responses of TRPM8, TRPA1, and TRPV2 channels in HEK293T cells. Meanwhile, co-expression of TRP channels with the global PAT enzyme DHHC7, but not DHHC3, significantly increased the responses of TRPM8 and TRPA1. Moreover, treatment with 2BP and N-tert-butyl-hydroxylamine (NtBHA), a chemically cleaving reagent of thioester linkage, decreased the responses and percentages in a population of endogenous TRPM8 and TRPA1. Thus, expression of some TRP channels are regulated by S-palmitoylation.

研究分野：分子細胞生理学

キーワード：生理学

１．研究開始当初の背景

S-palmitoylation is a reversible post-translational modification of proteins wherein palmitoyl acyltransferases (PAT) catalyze the attachment of a 16-carbon saturated fatty acid (palmitic acid) to intracellular cysteine residues through a thioester linkage. This lipid modification increases the hydrophobicity of proteins that facilitates their translocation and insertion into intracellular and plasma membranes. In response to specific signaling pathways, palmitoylation and depalmitoylation cycles can act to shuttle and relocalize proteins dynamically between intracellular and plasma membranes. Recent studies confirmed that many ion channels are regulated by *S*-palmitoylation through two aspects: plasma membrane expression and channel activities and kinetics. For example, the plasma expression of voltage-gated sodium (Nav1.2) and potassium (Kv1.5) channels was shown to be regulated by *S*-palmitoylation in the early biosynthetic pathway. On the other hand, *S*-palmitoylation of the β2α subunit of N-type calcium channel controls voltage-dependent inactivation and *S*-palmitoylation of the β subunit of epithelial sodium channel affects its gating. To date, more than 40 different ion channels have been shown to be regulated by *S*-palmitoylation. However, the regulation of transient receptor potential (TRP) channels by *S*-palmitoylation is little known. Examination and elucidation of *S*-palmitoylation can provide a better understanding of the mechanism by which this post-translational modification regulates TRP channels in physiological and pathological conditions.

TRP channels belong to a large family of non-selective cation channels that are broadly expressed in both neuronal and non-neuronal cells. TRP channels share a similar topology to Kv channels, with cytoplasmic N- and C- termini, six transmembrane segments and a pore region that lies between the fifth and sixth segments. Numerous reports showed that TRP channels play important roles in many physiological events, including sensory transduction, neuronal axon growth, and insulin secretion. Similar to Kv channels, TRP channels undergo post-translational modifications in both the ER and Golgi apparatus. Appropriately folded and assembled proteins are subsequently sorted into vesicles for trafficking and delivery to the plasma membrane by a constitutive or regulated pathway of vesicle exocytosis. The constitutive exocytosis pathway is primarily used for turnover of both membrane proteins and lipids for the removal of aged or damaged membrane components. In contrast, the regulated pathway is used by specialized cells, such as endocrine cells, neurons and pancreatic acinar cells for cell communication. Increasing evidence suggested that translocation of TRP channels to the plasma membrane can be mediated by various stimuli through the regulated pathway. While there have been intense studies on TRP channel translocation governed by the regulated pathway, the detailed mechanisms occurring in the constitutive pathway that controls plasma membrane expression and protein translocation remain poorly understood. Recently, several studies indicated that some TRP channels could be regulated by *S*-palmitoylation. Although these studies suggested that *S*-palmitoylation is an important post-translation modification for TRP channels, a systematic screening and evaluation of *S*-palmitoylation effects on TRP channels has not been studied, and as such whether and how TRP channels are regulated by *S*-palmitoylation and how this post-translational modification is involved in physiological functions is unclear.

２．研究の目的

We explored the effects of *S*-palmitoylation on several TRP channels, with a main focus on the TRPV, TRPM and TRPA subfamilies.

３．研究の方法

Male C57BL/6NCr mice (4-5 weeks old) were maintained in a controlled environment with food and water *ad lib*. All procedures involving the care and use of animals were approved by the Institute Animal Care and Use Committee of the National Institute for Physiological Sciences. All animal experiments were carried out following the National Institutes of Health *Guide for the care and use of laboratory animals.*

HEK293T cells were maintained in DMEM containing FBS, L-glutamine, penicillin and streptomycin. Cells were transfected with plasmids using Lipofectamine reagent. After transfection, the cells were reseeded onto cover slips and incubated overnight with DMSO, 2BP (100 μM), NtBHA (500 μM) or 2BP with NtBHA. The treated cells were used the following day for biochemical and electrophysiological studies.

Transfected HEK293T cells were washed with PBS and incubated with EZ-link NHS-SS-Biotin for 30 min. The cells were then washed three times with quenching buffer and twice with PBS. Biotinylated plasma membrane proteins were precipitated overnight with Dynabeads® MyOne™ Streptavidin T1 with rotation at 4 °C. Beads were collected with a magnet and denatured at 95 °C for 5 min with SDS and DTT. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The primary antibodies used were anti-TRPM8, anti-TRPA1 (1:1000 dilution), anti-TRPV1 (1:50,000 dilution) and anti-β-actin

(1:1000 dilution). Secondary antibodies were anti-rabbit or anti-mouse IgG HRP-linked antibodies (1:5000 dilution). Band density quantification was performed using Image J software.

Mice were anesthetized with pentobarbital sodium (50 mg/kg, ip) and sacrificed by cervical dislocation. DRG neurons were rapidly isolated and digested by collagenase type XI for 25 min at 37 °C. After digestion, cells were gently dissociated using a fire-polished Pasteur pipettes and seeded onto poly-D-lysine pre-coated cover slips. DRG neurons were maintained in DMEM/F12 containing 10% FBS, L-glutamine, penicillin, and streptomycin at 37 °C in a 5% CO_2 incubator.

Calcium imaging experiments were carried out after DRG neurons were incubated overnight with DMSO, 2BP (100 μM), NtBHA (500 μM) or 2BP with NtBHA. Calcium influx was observed after fura-2 loading. A positive response was determined when the ratio increased to more than 0.2. The standard bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 D-glucose (pH 7.4, adjusted with NaOH). In whole-cell patch clamp experiments, the standard bath solution was the same as that used for the calcium imaging experiments. The pipette solution contained (in mM) 140 KCl, 5 EGTA and 10 HEPES (pH 7.4, adjusted with KOH). Data were sampled at 10 kHz and filtered at 5 kHz. The membrane potential was clamped at -60 mV and a 50 ms voltage ramp (-100 mV to +100 mV) was applied in a 5 s duration.

4．研究成果

We first screened the effect of *S*-palmitoylation on TRP channel functions using a heterologous expression system in HEK293T cells. Overnight incubation with 2BP decreased the agonist-induced responses of TRPM8 and TRPA1 (Figs. 1 and 2).

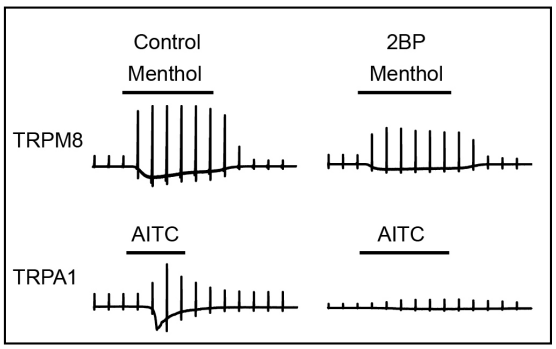

Figure 1

If the reduced current densities of TRPM8 and TRPA1 were solely due to the inhibition of *S*-palmitoylation by 2BP, then co-expression of PAT enzymes should increase current densities for these channels. To test this possibility, we co-expressed TRPM8 and TRPA1 with a subfamily of PAT enzyme, DHHC7. As expected, the agonist-induced responses of TRPM8 and TRPA1 were significantly increased at both +60 and -60 mV by co-expression with DHHC7.

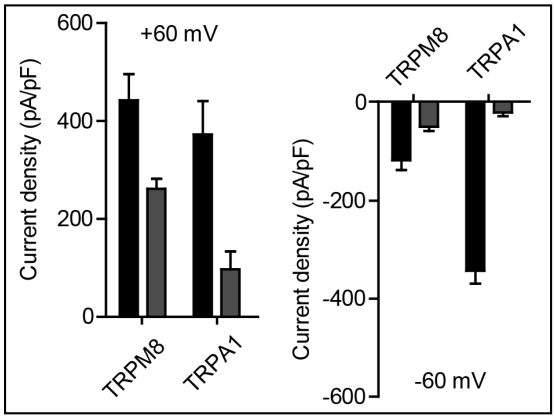

Figure 2

Although we demonstrated that *S*-palmitoylation regulates TRPM8 and TRPA1 activity in HEK293T cells, whether this modification regulates these channels in native cells is unclear. Since TRPM8 and TRPA1 were previously reported to be highly expressed in dorsal root ganglia (DRG) neurons, we examined the effect of *S*-palmitoylation on these channels in freshly isolated DRG neurons treated with 2BP and NtBHA, which cleaves thioester linkages with high potency and specificity. Upon overnight incubation with 2BP and/or NtBHA, menthol- and ATIC-induced Ca^{2+} influx responses were significantly decreased (Fig. 3).

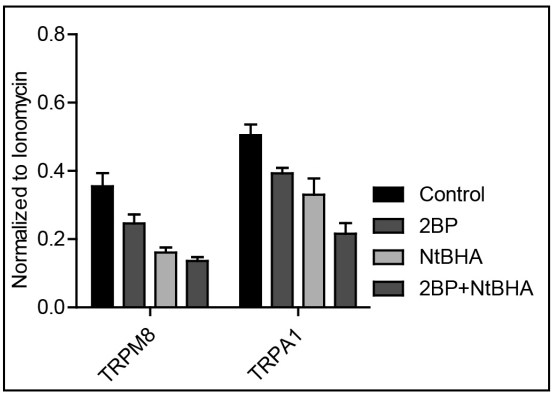

Figure 3

To understand the detailed mechanism of how TRPM8 and TRPA1 are regulated by *S*-palmitoylation, we examined their plasma membrane expression levels in HEK293T cells using a surface biotinylation assay following overnight incubation with 2BP and/or NtBHA. Both 2BP and NtBHA significantly decreased the plasma membrane expression of TRPM8 and TRPA1, which indicates that *S*-palmitoylation regulates TRPM8 and TRPA1 by affecting the translocation of these channels to the plasma membrane.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計１件）

① Zhou Y, Suzuki Y, Uchida K and Tominaga M. Identification of a splice variant of mouse TRPA1 that regulates TRPA1 activity. Nat. Commun. 4: 2408, 2013. doi: 10.1038/ncomms3399**（査読有り）**

〔学会発表〕（計３件）

① **周一鳴、奥慎一郎、深田優子、深田正紀、富永真琴**（2014.3.17）**パルミトイル化の** TRP **チャネル機能への影響の研究**. **第** 91 **回日本生理学会大会**, **鹿児島大学(鹿児島県・鹿児島市)**

② Zhou Y, Suzuki Y, Uchida K and Tominaga M (2013.12.18) Identification of a splice variant of mouse TRPA1 that regulates TRPA1 activity. The 44th NIPS International Symposium & the 5th Asian Pain Symposium, **岡崎カンファレンスセンター（愛知県・岡崎市）**

③ Zhou Y, Suzuki Y, Uchida K and Tominaga M (2013.10.21) A novel mouse TRPA1 alternative splicing variant regulates its channel activity. NIPS-Chulalongkorn University Joint Symposium, Bangkok （Thailand）

〔その他〕
ホームページ等
http://www.nips.ac.jp/cs/

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