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Activation of NMDA receptors leads to phosphorylation of TRPV1 S800 by protein kinase C and A-Kinase anchoring protein 150 in rat trigeminal ganglia

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ABSTRACT

A-Kinase anchoring protein 150 (AKAP150) is required for the phosphorylation of transient receptor potential cation channel subfamily V member 1 (TRPV1) by PKA or PKC in sensory neurons and, hence, affects TRPV1-dependent hyperalgesia under pathological conditions. Recently, we showed that the activation of *N*-methyl-D-aspartate (NMDA) receptors sensitizes TRPV1 by enhancing serine phosphorylation through PKC in trigeminal nociceptors. In this study, we extended this observation by investigating whether AKAP150 mediates NMDA-induced phosphorylation of TRPV1 via PKC in native sensory neurons in the rat. By adopting a phospho-specific antibody combined with a surface biotinylation assay, we first assessed NMDA-induced changes in the phosphorylation level of serine 800 residues (S800) in TRPV1 delimited to cell surface membrane in cultured trigeminal ganglia (TG). The biotinylation assay yielded that the application of NMDA significantly increased the phosphorylation of S800 (p-S800) of TRPV1 at time points correlating with the development of NMDA-induced mechanical hyperalgesia [10]. We then obtained a siRNA sequence against AKAP150 that dose-dependently down-regulated the AKAP150 protein. Pretreatment of TG culture with the siRNA, but not mismatch sequences, prevented the NMDA-induced phosphorylation of serine residues of total TRPV1 as well as S800 of membrane bound TRPV1. We confirmed that AKAP150 co-immunoprecipitated with TRPV1 and demonstrated that it also co-immunoprecipitated with NMDA receptor subunits (NR1 and NR2B) in TG. These data offer novel information that the activation of NMDA-induced TRPV1 sensitization involves p-S800 of TRPV1 in cell surface membrane in native sensory neurons and that AKAP150 is required for NMDA-and PKC-mediated phosphorylation of TRPV1 S800. Therefore, we propose that the NMDA receptor, AKAP150, and TRPV1 forms a signaling complex that underlies the sensitization of trigeminal nociceptors by modulating phosphorylation of specific TRPV1 residues.

1. Introduction

Transient receptor potential cation channel subfamily V member I (TRPV1) is a ligand-gated, non-selective cation channel with a high permeability for Ca^{2+} and is activated by capsaicin, noxious heat, acid and various lipids [1–4]. The role of TRPV1 in pain sensation has been rigorously studied [5,6]. The function of TRPV1 is regulated by multiple G-protein coupled receptors (GPCR) found in sensory neurons. For example, the augmentation of TRPV1 function by a type I metabotropic glutamate receptor (mGluR5) in dorsal root ganglia (DRG) has been proposed to serve as the underlying mechanism for thermal hyperalgesia [7]. Other GPCR such as protease activated receptor 2 and neurokinin receptor sensitize TRPV1 through PKC activation and enhance pain behaviors [8,9]. We have recently reported that the activation of NMDA receptors leads to PKC-dependent phosphorylation of serine residues of TRPV1 in TG neurons, which provides an intracellular mechanism for TRPV1 sensitization and the development of orofacial mechanical hyperalgesia [10]. Thus, TRPV1 serves as a downstream integrator for signals arising from not only GPCR, but also from another ligand-gated ion channel in sensory neurons.

A-Kinase anchoring protein (AKAP) families are comprised of scaffolding proteins that anchor receptors and signaling molecules to physiological substrates [11]. AKAP79/150 is a member of AKAP family that binds to the regulatory subunit of PKA and PKC [12–14]. AKAP150, the rodent homolog of human AKAP79, is ubiquitously expressed in the brain and modulates several ion channels such as voltage-gated M-type K^+ channels, L-type Ca^{2+} channels and acid-sensing ion channels expressed in neurons [15–17]. AKAP150 is also expressed in sensory neurons and mediates phosphorylation of TRPV1, an event that underlies nociceptor

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sensitization [18,19]. Knocking down AKAP150 in trigeminal sensory neurons significantly attenuates PKA sensitization of TRPV1 activity and the administration of an AKAP150 antagonist reduces thermal hyperalgesia in rats [20]. Interestingly, the activation of GPCR results in the formation of the PKC-AKAP150 signaling complex, which modulates TRPV1 phosphorylation/sensitization in DRG neurons [19]. These findings suggest that AKAP150 is a key molecule required for TRPV1 modulation by intracellular signals arising from upstream receptors or channels.

In this study, we examined whether AKAP150 is also involved in NMDA-induced phosphorylation of serine residues of TRPV1 in TG neurons. Of the two serine residues in TRPV1that are phosphorylated by PKC, i.e. S502 and S800, the phosphorylation of S502 requires AKAP150 [19]. However, it is not known whether AKAP150 also targets S800. By taking advantage of the specific antibody that detects phosphorylation at S800 (p-S800) combined with a biotinylation assay, we examined whether the activation of NMDA receptors results in changes in p-S800 of TRPV1 at the surface membrane in an AKAP150 dependent manner in cultured TG neurons.

2. Material and methods

2.1. Primary TG culture

Male Sprague Dawley rats (200–250 g) were used for primary TG culture as described previously [10]. TG were minced in cold Hanks's Balanced Salt Solution (HBSS) and incubated in 5 ml of Dulbecco's Modified Eagle Medium (DMEM)/F-12 containing collagenase and trypsin in a shaking incubator at 37 °C for 30 min. TG extracts were mechanically dissociated and resuspended in the culture medium before plating on a 12 well plate coated with laminin. Dissociated TG neurons were maintained with DMEM/F-12 media containing 10% FBS, 1% penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Cultures were used in immunoprecipitation experiments and biotinylation assays three to four days after plating.

2.2. Biotinylation of cell surface proteins

To examine changes in proteins localized to the plasma membrane, we performed biotinylation as described previously [21,22]. Briefly, dissociated TG cells were washed three times in cold PBS. For membrane protein biotinylation, TG cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS at 4 °C for 30 min. To quench the reaction, cells were washed three times with cold PBS containing 100 mM glycine. Next, TG cells were lysed in lysis buffer containing protease inhibitor cocktail followed by centrifugation at 1000*g* for 5 min. The 100–150 μg of collected lysate was incubated with streptavidin cross linked to agarose beads (Pierce) for 2 h at 4 °C. The beads were then washed twice with lysis buffer, and eluted with LDS loading buffer by heating at 100 °C for 5 min. The membranes were incubated with antibody against p-S800 TRPV1antibody (1:500, polyclonal, anti-rabbit, Cosmo) for three days at 4 °C. The specificity of this antibody is previously verified [23]. In order to normalize the amount of protein loaded and to examine contamination of cytosolic components in the biotinylation assay, the stripped membranes were incubated with GAPDH antibody (1:5000, monoclonal, anti mouse, Sigma). For the relative quantification of p-S800 TRPV1, GAPDH level of the corresponding sample was used as the normalization control.

2.3. siRNA preparation and transfection

The siRNA construct of AKAP150 was made by Thermo Scientific (Dharmacon). The sequence of the sense strand of AKAP150 siRNA was GCAUGUGAUUGGCAUAGAA-dTdT. The efficiency of this sequence in knocking down AKAP150 has been demonstrated in TG [20]. Isolated TG neurons were transfected with either AKAP150 siRNA or mismatch (MM, silencer-1, Ambion) using RNAi reagent (Invitrogen) as instructed by the manufacturer. TG neurons were transfected with two doses of the siRNA sequence (0.05, 0.1 nM) or MM as a negative control for 24 h.

2.4. Immunoprecipitation and co-immunoprecipitation

TG cultures were treated with lysis buffer containing protease inhibitor cocktail. To extract protein, the lysate was centrifuged at 12,000 rpm at 4 °C for 20 min. The protein concentration of the cell lysate was measured using a Bio-Rad protein assay reagent kit. The sample proteins were immuonoprecipitated with TRPV1 antibody (1 μg, polyclonal, anti-rabbit, Calbiochem) overnight at 4 °C, and then with protein A/G-Sepharose beads (Santa Cruz) for 2hr. LDS loading dye including SDS was added and boiled at 100 °C for 5 min to elute proteins from the bead complex. The denatured protein was then fractionated on a 4–12% gradient Nu-PAGE electrophoresis gel and blotted onto a PVDF or Nitrocellulose membrane. The membrane was blocked and incubated overnight at 4 °C with a monoclonal phosphor-serine antibody (1:500, monoclonal, anti-mouse, Santa Cruz). The bound primary antibody was detected with a horseradish peroxidase conjugated anti-mouse IgG secondary antibody. The membranes were re-probed with anti-TRPV1 (1:1000, polyclonal, anti-rabbit, Calbiochem) following a stripping process to examine the same amount of running proteins. The immunocomplex was visualized using ECL reagent (Amersham) and recorded on X-ray film. The band signals on the film were scanned and quantified with Image J software. When normalized to p-Ser, the re-probed TRPV1 on the same membrane was used as a loading control.

For co-immunoprecipitation (Co-IP) experiments involving AKAP 150 and NMDA receptors, lysates were incubated with anti-AKAP150 (2 μg, polyclonal, anti-rabbit, Upstate) for 4 h at 4 °C and then followed the same protocol described above for immunoprecipitation. The following antibodies were used: NR1 (1:500, monoclonal, anti-mouse, Millipore), NR2B (1:500, monoclonal, monoclonal anti-mouse, Millipore) and TRPV1 (1:500, polyclonal, anti-goat, Santa Cruz). The specificities of these antibodies have previously been established [24–27].

2.5. Data analysis

For immuonoprecipitation studies, serine phosphorylation levels of each sample were normalized to TRPV1 in the same sample. In biotinylation experiments, p-S800 TRPV1 expression level of each sample was normalized to GAPDH in the same lysate. The Kruskal–Wallis one-way analysis of variance on ranks was used to detect statistical differences between treatments. Dunnett's comparison test was used for post hoc analysis. Mann–Whitney Rank Sum test was performed for two group comparisons. The significance level was set at $p < 0.05$.

3. Results

3.1. NMDA induced PKC-mediated phosphorylation of S800 of TRPV1 in TG culture

In our previous study, we demonstrated that the activation of NMDA receptors leads to PKC-dependent phosphorylation of serine residues of TRPV1 in rat TG neurons [10]. In this study, we further investigated whether the application of NMDA in TG culture increases the phosphorylation of a specific serine residue, S800 of