Published in final edited form as:

Pain. 2008 September 15; 138(3): 604–616. doi:10.1016/j.pain.2008.02.022.

A-Kinase Anchoring Protein Mediates TRPV1 Thermal Hyperalgesia through PKA Phosphorylation of TRPV1

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Abstract

Certain phosphorylation events are tightly controlled by scaffolding proteins such as A-Kinase Anchoring Protein (AKAP). On nociceptive terminals, phosphorylation of transient receptor potential channel type 1 (TRPV1) results in the sensitization to many different stimuli, contributing to the development of hyperalgesia. In this study, we investigated the functional involvement of AKAP150 in mediating sensitization of TRPV1, and found that AKAP150 is co-expressed in trigeminal ganglia (TG) neurons from rat and associates with TRPV1. Furthermore, siRNA-mediated knock-down of AKAP150 expression led to a significant reduction in PKA phosphorylation of TRPV1 in cultured TG neurons. In CHO cells, the PKA RII binding site on AKAP was necessary for PKA enhancement of TRPV1-mediated Ca^{+2} -accumulation. In addition, AKAP150 knock-down in cultured TG neurons attenuated PKA sensitization of TRPV1 activity and *in vivo* administration of an AKAP antagonist significantly reduced prostaglandin E2 sensitization to thermal stimuli. These data suggest that AKAP150 functionally regulates PKA-mediated phosphorylation/ sensitization of the TRPV1 receptor.

Keywords

TRPV1; AKAP; Trigeminal; Pain; PKA; Hyperalgesia

Introduction

Transient receptor potential channel 1 (TRPV1) is a calcium permeable ion channel responsible for the transduction of certain chemical (capsaicin, acid, anandamide) and thermal ($> 42^{\circ}C$)

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stimuli into nociceptive signaling [7,8,45]. Reports have identified several serine and threonine residues on the receptor that are modified through phosphorylation to sensitize receptor activity [3,4,26,33]. In particular, protein kinase A (PKA,[3]) and protein kinase C (PKC, ([8,9]) and demonstrate a significant role in TRPV1 phosphorylation.

Several inflammatory mediators such as prostaglandin E₂ (PGE₂, [30]), sensitize TRPV1 activity through PKA activation. The phosphorylation of TRPV1 by PKA significantly increase TRPV1 activity upon stimulation with CAP or heat [30,31,37]. However, given that TRPV1 lacks known PKA binding domains, the mechanism by which PKA is targeted to TRPV1 is unclear.

Phosphorylation events are controlled via interactions with a variety of scaffolding proteins. The scaffolding protein A-kinase anchoring protein (AKAP) serves to localize PKA to membrane sites of action [44], including the NMDA [12] and glutamate [21,41] receptor complexes. A number of AKAP isoforms are expressed amongst differing species and cell types, including AKAP 15, AKAP220, and AKAP150/79 [28]. Despite apparent differences in sequence homology, the AKAP isomers function similarly to localize PKA to distinct sites of action, organizing signal transduction events by the kinase.

Previous reports suggest a potential association between AKAP and TRPV1. Rathee et al reported a reduction in forskolin-induced potentiation of I_{heat} in cultured DRG neurons following pre-treatment with the PKA/AKAP associative inhibitor, St-Ht31P [37]. Indeed, forskolin-induced potentiation of I_{heat} in HEK293 cells expressing TRPV1 was blocked following treatment with St-Ht31P [37]. Furthermore, St-Ht31P blocked the calcium-induced potentiation of I_{heat} in cultured DRG, further suggesting a role for AKAP [15]. These data suggest a role for AKAP in regulating PKA-phosphorylation and sensitization of heat-transducing channels, including TRPV1, TRPV3, and/or TRPV4. However, a direct functional association between the scaffolding protein and these channels has not been established, nor is it known whether this interaction contributes to the development of behavioral hyperalgesia.

In the present set of studies, we employed biochemical and molecular approaches to precisely determine that AKAP150 co-localizes and associates with TRPV1 in trigeminal ganglia neurons. Calcium imaging and electrophysiology results indicate that AKAP150, and not AKAP220, is a functional regulator of PKA sensitization of TRPV1 activity, and this finding is substantiated by monitoring the impact of an AKAP inhibitor on the development of PGE₂-evoked peripheral thermal hyperalgesia in rats. Results from this study demonstrate that the specific association of AKAP150 with TRPV1 in peripheral neurons is essential and necessary for PKA phosphorylation and sensitization of the receptor.

Materials & Methods

Tissue Culture

All procedures utilizing animals were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health. Trigeminal ganglia (TG) were cultured as previously described [24]. Cultures were maintained at 37°C, 5% CO₂, and grown in 10 cm plates for 5 – 7 days for phosphorylation experiments, 16-36 hr for electrophysiology experiments. Chinese hamster ovary (CHO) cells were utilized for heterologous expression of cDNA constructs. They were maintained at 37°C, 5% CO₂ and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions.

siRNA Design and Transfection

Specific siRNA duplexes to knock down AKAP150 or AKAP220 were designed and ordered from Dharmacon (<http://www.dharmacon.com>, Lafayette, CO). The sequences for the sense strands of AKAP150 siRNA are as follows: AKAP150-1, GCAUGUGAUUGGCAUAGAA-dTdT; AKAP150-2, GCAUAGGACUGGAAUU AGA-dTdT. The sequence for the sense strands of the AKAP220 siRNA is as follows: AKAP220-1, GGAUCUAGCUGCAGUUUCA-dTdT; AKAP220-2, ACAGAUGCAGC UCAUAUGA-dTdT. siRNA duplexes were transfected into cultured TG neurons using HiPerFect (Qiagen, Valencia, CA), following manufacturer's directions (125 ng siRNA/cover slip; 625 ng siRNA/10 cm plate). In order to identify transfected neurons in electrophysiology experiments, siRNA with the same sequence as AKAP150-1 with 3'-Alexa Fluor 488 tagged ands were acquired from Qiagen. Alexa Fluor 488-tagged AKAP150-1 siRNA yielded a transfection efficiency of approximately 60-70% following Qiagen manufacturer's instructions for HiPerFect use (data not shown). Silencer negative control #2 siRNA (Ambion, Austin, TX) was used as a negative control to determine specificity and efficiency of AKAP siRNAs.

Western Blot and ³²P Autoradiography

The lysis of TG cells, quantification of protein concentration, immunoprecipitation, and Western blotting were conducted as previously described [24]. Antibodies used for Western blotting were AKAP150 (1:1000, Upstate, Lake Placid, NY), AKAP220 (1:1000) and PKA RII subunit (1:500, BD Biosciences, Palo Alto, CA), TRPV1 (1:1000, Calbiochem, La Jolla, CA), PSD-95 (1:1000, Chemicon, Temecula, CA), SAP-97 (1:500, Stressgen, Victoria, BC). Densitometry data analyzed by one-way ANOVA, *p<0.05, **p<0.01, NS=not significant, results are representative of 3-5 independent trials.

Electrophysiology

All recordings were made in a whole-cell voltage clamp configuration at a holding potential (V_h) of -60 mV. Recordings and following analysis were carried out at 22-24°C from small-to-medium sized (20-35 pF) cultured TG neurons (24 h post-transfection) using an Axopatch 200B amplifier and pCLAMP9.0 software (Axon Instruments, Union City, CA). Data were filtered at 0.5 kHz and samples at 2 kHz. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 3-5 MΩ in perforated patch pipette solution. If necessary, access resistance (R_s) was compensated by 40-80% to 8-10 MΩ.

All recordings are made in the presence of 2 mM Ca^{+2} in external solution. Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 HEPES, pH 7.4. The pipette solution consisted of (in mM): 110 K-methanesulfonate, 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES pH 7.3, 0.2 GTP-Na and 2.5 ATP- Mg_2 (Sigma, St. Louis, MO). Drugs were applied using a computer controlled pressure-driven 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA). FITC-labeled AKAP150-1 siRNA (Qiagen) used to positively identify transfected neurons. Specificity of siRNA confirmed by Western blot analysis (Supplemental Figure 1E). Data were analyzed by one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, NS=not significant.

Calcium Imaging

CHO cells were transfected as outlined above (*Tissue Culture*) with pEGFP-N1 (Clontech, Mountain View, CA, to identify transfected cells) and cDNA vectors containing inserts corresponding to rat TRPV1 (generously provided by David Julius, UCSF, San Francisco, CA), rat AKAP150 wt and AKAP150ΔPKA (generously provided by John D. Scott, Vollum Institute, OHSU, Portland OR). To measure intracellular $[Ca^{+2}]$ levels, the dye Fura-2 AM (2 μM; Molecular Probes, Carlsbad, CA) was loaded for 30 min at 37°C into cells in the presence

of 0.05% Pluronic (Calbiochem). Fluorescence was detected with a Nikon Eclipse TE 2000-U microscope fitted with a 20x/0.8 NA Fluor objective. Fluorescence images from 340 nm and 380 nm excitation wavelengths were collected and analyzed with the MetaFluor Software (MetaMorph, Web Universal Imaging Corporation, Downingtown, PA). The net change in Ca^{+2} ($\Delta\text{F}_{340/380}$) was calculated by subtracting the basal $\text{F}_{340/380}$ Ca^{+2} level (mean value collected for 60 s prior to agonist addition) from the peak $\text{F}_{340/380}$ Ca^{+2} level achieved after exposure to the agonists. For each transfection/treatment group, 31-57 cells were imaged, statistical significance determined by one-way ANOVA analysis.

Immunohistochemistry

Trigeminal ganglia were dissected from 3 rats, sectioned (30 sections/TG) and fixed with 4% paraformaldehyde for 30 min at 25°C. Following fixation, 3 representative tissue sections from each TG were rinsed three times in PBS, incubated with 2% NGS, 0.3% Triton X-100, and 20mg/ml bovine- γ -globulin in PBS (blocking solution) for 90 min at 25°C. Tissue sections were then incubated with antisera directed specifically towards AKAP150 (1:250, Upstate) or AKAP220 (1:250, BD Transduction), TRPV1 (1:1,000, Guinea pig, Chemicon/Millipore, Bedford, MA), and PKA RII (1:250, BD Transduction) in blocking solution overnight at 4°C. The antisera directed against the C-terminus of rat TRPV1 has been used reproducibly in several species, demonstrating specificity [1,16,23,35,36,46]. Tissue sections were then rinsed three times and incubated with appropriate secondary antibodies in blocking solution (1:500) for 90 min at 25°C. Following rinsing three times with PBS, tissue sections were dried for 30 min at 25°C, and prepared for visualization by confocal microscopy.

Cultured rat TG cells were grown on poly-D lysine coated coverslips for 5-7 days in normal media. Coverslips were rinsed with PBS, and fixed with 4% paraformaldehyde for 10 min at 25°C. Following fixation, coverslips were rinsed twice with PBS, and incubated with 5% normal goat serum, 0.5% Triton X-100 in PBS for 30 min at 25°C. Coverslips were then incubated with antisera directed specifically towards AKAP150 or AKAP220, TRPV1, and PKA RII overnight at 4°C. Coverslips were then rinsed three times and incubated with appropriate secondary antibodies one hour at 25°C. Following rinsing three times with PBS, coverslips were mounted to microscope slides and dried overnight. For triple-label immunofluorescence, cover-slipped images were acquired using a 40X objective lens mated to a Nikon E600 microscope (Melville, NY, USA) equipped with a Photometrics SenSys digital CCD camera (Roper Scientific, Tucson, AZ, USA) connected to a computer equipped with Metamorph V4.1 image analysis software (Universal Image Corporation, Downingtown, PA, USA). Results are representative of 2-3 individual animals.

PKA Activity Assay

PKA activity from TG neurons transfected with AKAP150-specific siRNA, (–) Silencer control siRNA (Ambion, Austin, TX) or mock-transfected were lysed in Co-IP Buffer (20 mM HEPES, 120 mM NaCl, 20 mM NaF, 20 mM 2-glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM orthovanadate, 0.1% Triton X-100, 1 mM benzamidine, pH to 7.45), quantified by Bradford analysis [5]. Aliquots (250 μg) of cell lysates from transfected cells were assayed for PKA activity following manufacturer's instructions (Upstate). Results are representative of 6 independent trials.

RNA Isolation and Real-time PCR

Total RNA was isolated from TG cultures treated with siRNA and the appropriate controls using the guanidinium thiocyanate method [10]. The isolated RNA was then treated with DNA-free reagent (DNase I; Ambion, Austin, TX, USA). Approximately 1 μg of RNA was used for first-strand cDNA synthesis (SuperScript III kit, Invitrogen, Valencia, CA, USA), and cDNA samples equivalent to 4 ng (18S rRNA) and 45 ng (AKAP) of RNA were used as template for

the reactions. Real-time amplification of target sequences was detected by a sequence detector, ABI 7700 (Applied Biosystems, Foster City, CA, USA), utilizing TaqMan Gene Expression Assays on Demand (Applied Biosystems), with specific primers and probes for A kinase anchor protein 150 (PRKA, assay #Rn01786021-m1) and eukaryotic 18S rRNA genes (assay #Hs99999901-s1). The reactions were run in triplicates of 25 μ L as described previously [14]. The comparative $\Delta\Delta C_t$ was used to normalize the data based on 18S rRNA expression and to determine the relative fold change after the exclusion criteria were verified by comparing primer efficiencies of AKAP150 and 18S rRNA [27].

Behavioral Test for Inflammatory Hyperalgesia

PGE₂-evoked thermal paw withdrawal latencies (PWL) were measured as described [20,38]. Animals were acclimated on the Hargreaves' apparatus for 30 min following which, baseline readings were collected. After collection of basal PWL, rats were injected intraplantarly (50 μ L) with Tris/saline vehicle, St-HT31P (1mM) or St-HT31P-C (1mM), and then with 50 μ L 0.01% ethanol/saline or 0.3 μ g PGE₂ 15 min later. PWL was measured 20 min later by blinded observers. Data were analyzed with n=6-10 animals/group, employing 2-way ANOVA with Bonferroni correction, **p<0.01, NS=not significant.

Results

Expression of AKAP150 in TRPV1-positive sensory neurons

Several recent studies have characterized the expression of AKAP isoforms in central and peripheral nervous tissues [13,18,37,40,41]. To investigate AKAP expression in TG neurons, confocal microscopy was utilized to identify cells that co-express the transient receptor potential channel V1 (TRPV1), A-kinase anchoring protein 150 (AKAP150), and protein kinase A subunit RII (PKA RII) in both intact and cultured TG (Figure 1A-H). These data provide the first reported evidence for co-expression of AKAP150 and PKA in TRPV1-positive TG neurons.

Interaction of AKAP150 and TRPV1 in sensory neurons

We next co-immunoprecipitated TRPV1 and AKAP150 to establish whether the proteins are physically associated in culture. Using antibodies previously verified for specificity (TRPV1 [24], AKAP150 [21], Supplemental Figure 1), we found TRPV1 and AKAP150 to co-immunoprecipitate from cultured TG. In addition, PKA RII also co-immunoprecipitated with AKAP150 in these pull-down experiments, but not with TRPV1 (Figure 1I-K). These results can be interpreted in the context of this method, as the use of 1% Triton X-100 in the general lysis buffer was specifically used to reduce non-specific association, which would account for the lack of PKA immunoreactivity in TRPV1 immunoprecipitation. Membrane-associated guanylate kinase homologs (MAGUK proteins) PSD-95 and SAP-97, reported as bridging proteins between AKAP and other receptors [12], displayed little expression in cultured TG neurons relative to rat brain lysate (Figure 1L-M). In addition, neither PSD-95 nor SAP-97 co-immunoprecipitated with the TRPV1/AKAP150 complex. These results suggest either a direct association between AKAP150 and TRPV1 or the involvement of an unknown MAGUK bridging protein.

AKAP150 is involved in PKA-mediated phosphorylation of TRPV1 in sensory neurons

We employed siRNA-mediated knockdown of AKAP150 expression in cultured TG neurons to study the role of AKAP150 in PKA phosphorylation of TRPV1. To demonstrate the specificity of this molecular approach in TG neurons, we characterized the time course for AKAP150 knockdown, relative to TRPV1 and β -actin expression, and found a 95% reduction in normalized AKAP150 protein expression 1 day post-transfection (Figure 2A-B, β -actin

expression was equal across transfection samples, data not shown). Furthermore, AKAP150 expression recovered after 2 days to levels observed in non-transfected cells, suggesting the structural protein has a rapid turnover rate. To confirm the specificity of the siRNA, we tested 2 different AKAP150-specific siRNA sequences, finding that both effectively knocked down expression (Figure 2A). Furthermore, the collection of mRNA one day post-transfection (Figure 2B) from cultured TG neurons transfected with the AKAP150-1 siRNA, was used for real time PCR to demonstrate knockdown of the AKAP150 transcript (Figure 2C). In this regard, the siRNA labeled as “AKAP-1” in Figure 2 was used for the remainder of experiments requiring AKAP150 knock-down.

AKAP is typically described as a scaffolding protein for PKA, among other enzymes, that brings the kinase into close spatial orientation with substrate proteins. To confirm whether this relationship exists for AKAP150/PKA and TRPV1, we suppressed AKAP expression with specific siRNA and measured ^{32}P -orthophosphate incorporation by TRPV1 (receptor phosphorylation) in TG cultures stimulated with 8-Br-cAMP (a membrane-permeable activator of PKA; 10 μM , 5 min). Results illustrated in Figure 2D demonstrated that PKA-stimulated phosphorylation of TRPV1 was reduced to basal levels following siRNA-mediated knock-down of AKAP150 expression, with no measurable difference in PKA RII expression (data not shown). Control experiments demonstrated no change in biotinylated, plasma membrane expression of TRPV1 in TG neurons transfected with AKAP150 siRNA, indicating that the results could not be attributed to a difference in TRPV1 localization to the plasma membrane in response to siRNA-mediated AKAP150 knock-down (Figure 2E). Taken together, these findings suggest that AKAP150 may be necessary for PKA phosphorylation of the TRPV1 receptor, thereby controlling PKA sensitization of TRPV1 channel activity.

Role of AKAP150 in PKA-mediated sensitization of TRPV1 activities

To confirm the functional importance of AKAP150 association with TRPV1, we transiently transfected CHO cells with rat cDNAs corresponding to TRPV1 and AKAP150 wild type or AKAP150 ΔPKA (PKA binding site deletion mutant). In co-transfected CHO cells, both wild type and mutant AKAP proteins co-immunoprecipitated with TRPV1, as shown above in cultured TG neurons (Figure 3A-B). Importantly, only AKAP150 wild type was able to demonstrate association with PKA in transfected CHO cells, whereas AKAP150 ΔPKA was unable to co-immunoprecipitate the kinase. Furthermore, as previously shown for cultured TG neurons, TRPV1 immunoprecipitation failed to produce PKA co-precipitation. We next employed Ca^{+2} imaging of co-transfected CHO cells to determine whether AKAP150 functionally regulates PKA-mediated sensitization of TRPV1 activity. As shown in Figure 3C, co-transfected cells were only capsaicin (CAP)-sensitive with TRPV1 transiently expressed, and these cells displayed equal amounts of CAP (100 nM)-induced Ca^{+2} influx when expressing either AKAP150 wild type or mutant. However, cells co-expressing TRPV1 and AKAP wild type demonstrated a significant increase in CAP-induced Ca^{+2} influx following 8-Br-cAMP (10 μM , 5 min) treatment, a sensitization that was not seen in cells co-expressing TRPV1 and AKAP150 ΔPKA . As shown in Figure 3D, this difference in Ca^{+2} influx was not attributable to changes in PKA activity, suggesting that AKAP150-mediated sensitization of TRPV1 activity by PKA is based upon the known ability of AKAP150 to bind the kinase.

We next employed whole-cell recordings in siRNA-transfected TG neurons to further investigate the regulation of TRPV1 activities by AKAP150. Both pharmacological desensitization and sensitization of TRPV1 by PKA stimulation were characterized. Thus, I_{CAP} (100 nM) tachyphylaxis was not statistically different between mock-, scrambled-, and AKAP150 siRNA-transfected cells (Figure 4A-E). However, PKA-mediated sensitization of I_{CAP} was modulated by AKAP150 expression. Transfection of sensory neurons with AKAP150-specific siRNA, in contrast to mock- or scrambled-siRNA transfection, led to a

significant reduction in 8-Br-cAMP (10 μ M, 5 min)-induced sensitization of I_{CAP} (Figure 5A, C-E). Importantly, transfection with AKAP150-specific siRNA did not alter PKA activity itself, since mock- and AKAP150 siRNA-transfected cells revealed similar kinase activities that were equally stimulated with the addition of 8-Br-cAMP (Figure 5B). These experiments agree with previously published studies examining the importance of PKA phosphorylation to TRPV1 activation by CAP [30], and serve to demonstrate that AKAP150 expression is necessary in TG to sensitize TRPV1 I_{CAP} via PKA.

To evaluate the specificity of the functional effects observed following AKAP150 knock-down, we sought to evaluate TRPV1 modulation by another AKAP isoform expressed in peripheral neurons. In intact as well as cultured TG, we discovered strong co-expression of TRPV1 and AKAP220, a scaffolding protein related to AKAP150 that also serves to mediate PKA signaling [43] (Figure 6A-D). In cultured TG neurons, siRNA-mediated knock-down of AKAP220 expression had no effect on AKAP150 expression, and vice versa, suggesting specific and independent siRNA knock-down (Supplemental Figure 1E). Importantly, and unlike treatment with siRNA to AKAP150, the knock-down of AKAP220 expression in cultured TG neurons failed to alter 8-Br-cAMP-stimulated I_{CAP} sensitization (Figure 6F and Figure 5). These control experiments implicate a specific role for the functional association of AKAP150 and TRPV1.

AKAP150 modulates PKA-mediated peripheral thermal hyperalgesia

To investigate the potential pharmacological modulation of AKAP150 and TRPV1 association, we utilized a commercially available peptide inhibitor of AKAP150/PKA association that has been previously characterized [11,32] and is cell-permeable. Cultured TG neurons were treated with the inhibitor St-Ht31P and the inactive control peptide St-Ht31P-C to determine the potential effects on TRPV1 phosphorylation, following a similar paradigm as described for Figure 2. TRPV1 receptors in neurons that were pre-treated with St-Ht31P (50 μ M, 15 min) incorporated less 32 P-orthophosphate incorporation following 8-Br-cAMP (10 μ M, 5 min), in comparison to cells pre-treated with 10 mM Tris vehicle or the control peptide St-Ht31P-C (50 μ M, 15 min) followed by 8-Br-cAMP (Figure 7A). These results indicate that TRPV1 phosphorylation by PKA can be suppressed by peptidergic inhibition of AKAP150/PKA association.

To translate our biochemical findings into an animal model, we employed the Hargreaves test for thermal hyperalgesia in rats treated with PGE₂, an inflammatory mediator shown to stimulate PKA activity via prostaglandin receptor activation [2]. Rathee et al. previously demonstrated an integral role for AKAP150/PKA RII interaction in TRPV1-expressing HEK293 cells, using St-Ht31P to block forskolin-induced potentiation of heat-activated currents [37]. However, the *in vivo* importance of a functional interaction between AKAP150 and PKA RII in terms of thermal hyperalgesia has yet to be determined. Rats were first analyzed for potential algescic responses to 50 μ l injections of the St-HT31P inhibitor at 500, 50 and 5 μ mol dosages (data not shown). This inhibitor did not elicit any significant differences in basal withdrawal latencies relative to Tris/saline vehicle. Rats injected with St-HT31P (50 μ mol /50 μ l injection) produced a near complete blockade of PGE₂ (0.3 μ g/50 μ l injection)-induced thermal hypersensitivity, with paw withdrawal levels indistinguishable from rats treated with vehicle/vehicle (Figure 7B). Rats treated with the control peptide (St-HT31P-C, 50 μ mol)/PGE₂ demonstrated no significant difference in withdrawal latency from rats treated with vehicle/PGE₂. Similarly, rats treated with St-HT31P/vehicle failed to display antinociceptive properties, demonstrating that St-HT31P serves to attenuate PGE₂-stimulated thermal hyperalgesia in a behavioral model. These results encompass the first known behavioral test of the St-Ht31P associative inhibitor, and are expected to spur further studies with this agent.

Discussion

The post-translational modification of proteins and enzymes throughout the cell are tightly controlled by various scaffolding and regulatory proteins. In this study, we examined the role of AKAP150 in the sensitization of TRPV1 receptor activity by PKA phosphorylation. In both cultured and intact TG, confocal immunohistochemistry revealed co-localization of AKAP150, TRPV1 and PKA RII. Upon further biochemical characterization, AKAP150 was found to co-immunoprecipitate with TRPV1, suggesting an intracellular association between the protein and the receptor. Results from radioactive orthophosphate labeling experiments coupled with siRNA-mediated knockdown of AKAP150 in cultured TG demonstrated that AKAP150 scaffolding significantly regulates 8-Br-cAMP-stimulated phosphorylation of TRPV1. To further characterize this finding, we transfected CHO cells with TRPV1 and either AKAP150wt or AKAP150 Δ PKA, and discovered that the PKA-binding site on AKAP150 was necessary for PKA-mediated sensitization of CAP-induced Ca^{+2} -influx by 8-Br-cAMP pre-treatment. Utilizing whole cell recordings, siRNA-mediated knock-down of AKAP150 in cultured TG attenuated PKA-stimulated re-sensitization of TRPV1 following repeated CAP treatments, further implicating the scaffolding protein as necessary for PKA modulation of TRPV1 activity. Finally, results from behavioral analyses of paw withdrawal latencies suggest that AKAP150 association with PKA is necessary to sensitize TRPV1 responses following PGE₂ treatment. Taken together, the results presented here support the hypothesis that AKAP150 regulates TRPV1 phosphorylation and subsequent sensitization by PKA.

The findings presented in this manuscript provide a complete and thorough examination of the functional role that AKAP150 demonstrates in PKA-directed phosphorylation and sensitization of the TRPV1 receptor-channel in TG neurons. Previous reports have provided a background for the study of this phenomenon in primary afferent nociceptive systems. In 2002, Rathee et al. demonstrated that administration of the “AKAP inhibitor” St-Ht31P attenuated forskolin-stimulated translocation of the PKA catalytic subunit to the plasma membrane in DRG sensory neurons [37]. Additional work by Distler et al. in 2003 demonstrated that St-Ht31P attenuates Ca^{+2} -induced potentiation of I_{heat} in cultured DRG sensory neurons [15]. These previous works highlight the importance of AKAP in mediating PKA-translocation and heat-evoked currents in sensory neurons. In the current manuscript, we use CAP as a specific activator of TRPV1 current and activity, demonstrating that PKA phosphorylation and sensitization of the receptor-channel requires functional AKAP150 participation.

AKAP has been reported in literature to associate with various plasma membrane receptor channels, including the NMDA and AMPA receptors [12], KCNQ2 channel [22], L-type voltage-gated Ca^{+2} channel [17], and aquaporin water channel [25]. The association of AKAP with these channels was shown to modulate post-translational modifications, such as phosphorylation, thereby regulating their subsequent activities. The association of AKAP with these channels is governed, in part, by one of several conjugate proteins, also known as MAGUK proteins [12]. Interestingly, the association between AKAP150 and TRPV1, as revealed by the present co-immunoprecipitation experiments, was found to lack some of the more notable MAGUK proteins such as PSD-95 or SAP-97, suggesting that either the association is direct, involves an uncharacterized MAGUK-like protein, or possibly requires a non-MAGUK bridging protein.

Electrophysiological results from cultured TG neurons transfected with AKAP150-specific siRNA in Figures 4 and 5 yielded significant results. On a primary level, the data suggests that basal PKA phosphorylation of TRPV1, that is reduced following AKAP150 knock-down, is a controlling factor in TRPV1 current response to CAP stimulation. Indeed, several studies support this notion. Bhawe et al demonstrated in 2002 that co-incubation of CHO-K1 cells with 8-Br-cAMP and the PKA inhibitor H-89 attenuated the normalized second CAP-response [3].

In 2003, Mohapatra and Nau reported a rightward shift in the CAP concentration response curve in CHO cells transfected with the PKA-phosphorylation site mutant TRPV1 S774A compared to cells expressing wild-type TRPV1 (S774A EC_{50} 837 ± 13 nM, Hill coefficient 1.73 ± 0.04 ; wild-type EC_{50} 245 ± 11 nM, Hill coefficient 1.29 ± 0.06) [30]. Furthermore, St-Ht31 peptide use has been shown to attenuate Ca^{+2} -induced potentiation of I_{heat} in comparison to vehicle treated cells [15]. In conjunction with these reports, our data would suggest that PKA phosphorylation is important to basal TRPV1 activity.

In our behavioral analyses, the veh-PGE₂ group produced a significant thermal hyperalgesia that was blocked by pre-treatment with St-Ht31P. These data are consistent with the hypothesis that *in vivo* development of PGE₂-evoked thermal hyperalgesia requires a functional AKAP-PKA complex. In addition, the data showed a minor trend for the control peptide, St-Ht31P-C, to also inhibit PGE₂-evoked hyperalgesia at the 20 min time-point, although this effect was not significant. It is unclear whether this small effect is due to non-AKAP actions of the control peptide or whether it is due to previously unrecognized interactions with PGE₂. In either case, the major finding from the *in vivo* study is that interventions known to disrupt the AKAP-PKA complex significantly and completely disrupt PGE₂-evoked thermal hyperalgesia.

Results from the behavioral investigation studying St-HT31P-inhibition of PGE₂-induced sensitization of rats to a thermal stimulus does not directly indicate the functional link between AKAP150 and TRPV1 that the *in vitro* results in this study identify. Indeed, there are other thermo-sensitive TRP channels that are expressed in the periphery and have been shown to respond to heat, including TRPV2 [6], TRPV3 [34,39,48], TRPV4 [19]. Of these, TRPV4 is the only channel other than TRPV1 to undergo regulation following phosphorylation by a kinase (Src-family kinase, [47]), whereas PKA has been shown to complex with TRPV2 in mast cells [42]. Mice lacking the type 1 regulatory subunit of PKA display a significantly reduced capacity for reacting to inflammatory pain [29]. This finding suggests that the permanent, peptidergic inhibition of AKAP150/PKA association may also result in a diminished capacity for sensing inflammatory pain, such as that following PGE₂ injection. However, AKAP150 complex formation with TRPV2 and/or TRPV4 has yet to be investigated. Therefore, our conclusive *in vitro* results support our *in vivo* studies, despite our inability to accurately conclude that AKAP150 modulates PKA-regulated sensitization of TRPV1 to heat in the rat hindpaw.

In our search to identify another AKAP expressed in cultured TG, we discovered AKAP220, a scaffolding protein implicated in the mediation of PKA-induced inhibition of GSK3 β [43]. In agreement with previously published work in DRG neurons [37], we demonstrated the expression of AKAP220 in cultured TG neurons. However, the siRNA-mediated knock-down of AKAP220 in cultured TG had no effect on PKA-stimulated sensitization of TRPV1 activity. These results not only suggest that AKAP150 regulation of TRPV1 activity is specific, but also suggest that TRPV1 specifically interacts with AKAP150. This novel finding allows for a greater understanding of the control mechanisms governing phosphorylation/ sensitization of TRPV1, and provides a potential point for therapeutic manipulation to relieve inflammatory hyperalgesia.

Acknowledgements

We would like to acknowledge Dharshini Amarneethi and Griffin Perry for their excellent technical assistance, David Julius for TRPV1 cDNA, and John D. Scott for AKAP150 cDNAs. These studies were supported by NIH NIDCR DE016500 (NAJ), NIH NIDCR DE13942 (MH), and NIH NIDA DA19585 (KMH).

References

1. Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA, Bunnett NW. Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J Neurosci* 2004;24(18):4300–12. [PubMed: 15128844]
2. Bauman GP, Bartik MM, Brooks WH, Roszman TL. Induction of cAMP-dependent protein kinase (PKA) activity in T cells after stimulation of the prostaglandin E2 or the beta-adrenergic receptors: relationship between PKA activity and inhibition of anti-CD3 monoclonal antibody-induced T cell proliferation. *Cell Immunol* 1994;158(1):182–94. [PubMed: 8087864]
3. Bhawe G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* 2002;35(4):721–31. [PubMed: 12194871]
4. Bhawe G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci U S A* 2003;100(21):12480–5. [PubMed: 14523239]
5. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54. [PubMed: 942051]
6. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 1999;398(6726):436–41. [PubMed: 10201375]
7. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389(6653):816–24. [PubMed: 9349813]
8. Cesare P, Moriondo A, Vellani V, McNaughton PA. Ion channels gated by heat. *Proc Natl Acad Sci U S A* 1999;96(14):7658–63. [PubMed: 10393876]
9. Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA. Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. *Neuron* 1999;23(3):617–24. [PubMed: 10433272]
10. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162(1):156–9. [PubMed: 2440339]
11. Colledge M, Scott JD. AKAPs: from structure to function. *Trends Cell Biol* 1999;9(6):216–21. [PubMed: 10354567]
12. Colledge M, Dean RA, Scott GK, Langeberg LK, Hagan RL, Scott JD. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. *Neuron* 2000;27(1):107–19. [PubMed: 10939335]
13. Dell'Acqua ML, Smith KE, Gorski JA, Horne EA, Gibson ES, Gomez LL. Regulation of neuronal PKA signaling through AKAP targeting dynamics. *Eur J Cell Biol* 2006;85(7):627–33. [PubMed: 16504338]
14. Diogenes A, Patwardhan AM, Jeske NA, Ruparel NB, Goffin V, Akopian AN, Hargreaves KM. Prolactin modulates TRPV1 in female rat trigeminal sensory neurons. *J Neurosci* 2006;26(31):8126–36. [PubMed: 16885226]
15. Distler C, Rathee PK, Lips KS, Obreja O, Neuhuber W, Kress M. Fast Ca²⁺-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. *J Neurophysiol* 2003;89(5):2499–505. [PubMed: 12740405]
16. Galoyan SM, Petruska JC, Mendell LM. Mechanisms of sensitization of the response of single dorsal root ganglion cells from adult rat to noxious heat. *Eur J Neurosci* 2003;18(3):535–41. [PubMed: 12911749]
17. Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD, Hosey MM. cAMP-dependent regulation of cardiac L-type Ca²⁺ channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* 1997;19(1):185–96. [PubMed: 9247274]
18. Gorski JA, Gomez LL, Scott JD, Dell'Acqua ML. Association of an A-kinase-anchoring protein signaling scaffold with cadherin adhesion molecules in neurons and epithelial cells. *Mol Biol Cell* 2005;16(8):3574–90. [PubMed: 15930126]
19. Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M. Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* 2002;22(15):6408–14. [PubMed: 12151520]

20. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32(1):77–88. [PubMed: 3340425]
21. Hoshi N, Langeberg LK, Scott JD. Distinct enzyme combinations in AKAP signalling complexes permit functional diversity. *Nat Cell Biol* 2005;7(11):1066–73. [PubMed: 16228013]
22. Hoshi N, Zhang JS, Omaki M, Takeuchi T, Yokoyama S, Wanaverbecq N, Langeberg LK, Yoneda Y, Scott JD, Brown DA, Higashida H. AKAP150 signaling complex promotes suppression of the M-current by muscarinic agonists. *Nat Neurosci* 2003;6(6):564–71. [PubMed: 12754513]
23. Hwang SJ, Burette A, Rustioni A, Valtchanoff JG. Vanilloid receptor VR1-positive primary afferents are glutamatergic and contact spinal neurons that co-express neurokinin receptor NK1 and glutamate receptors. *J Neurocytol* 2004;33(3):321–9. [PubMed: 15475687]
24. Jeske NA, Patwardhan AM, Gamper N, Price TJ, Akopian AN, Hargreaves KM. Cannabinoid WIN 55,212-2 Regulates TRPV1 Phosphorylation in Sensory Neurons. *J Biol Chem* 2006;281(43):32879–90. [PubMed: 16954222]
25. Jo I, Ward DT, Baum MA, Scott JD, Coghlan VM, Hammond TG, Harris HW. AQP2 is a substrate for endogenous PP2B activity within an inner medullary AKAP-signaling complex. *Am J Physiol Renal Physiol* 2001;281(5):F958–65. [PubMed: 11592953]
26. Jordt SE, Julius D. Molecular basis for species-specific sensitivity to “hot” chili peppers. *Cell* 2002;108(3):421–30. [PubMed: 11853675]
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25(4):402–8. [PubMed: 11846609]
28. Lohmann SM, DeCamilli P, Einig I, Walter U. High-affinity binding of the regulatory subunit (RII) of cAMP-dependent protein kinase to microtubule-associated and other cellular proteins. *Proc Natl Acad Sci U S A* 1984;81(21):6723–7. [PubMed: 6093118]
29. Malmberg AB, Brandon EP, Idzerda RL, Liu H, McKnight GS, Basbaum AI. Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase. *J Neurosci* 1997;17(19):7462–70. [PubMed: 9295392]
30. Mohapatra DP, Nau C. Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem* 2003;278(50):50080–90. [PubMed: 14506258]
31. Mohapatra DP, Nau C. Regulation of Ca²⁺-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. *J Biol Chem* 2005;280(14):13424–32. [PubMed: 15691846]
32. Moita MA, Lamprecht R, Nader K, LeDoux JE. A-kinase anchoring proteins in amygdala are involved in auditory fear memory. *Nat Neurosci* 2002;5(9):837–8. [PubMed: 12172550]
33. Numazaki M, Tominaga T, Takeuchi K, Murayama N, Toyooka H, Tominaga M. Structural determinant of TRPV1 desensitization interacts with calmodulin. *Proc Natl Acad Sci U S A* 2003;100(13):8002–6. [PubMed: 12808128]
34. Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P, Bevan S, Patapoutian A. A heat-sensitive TRP channel expressed in keratinocytes. *Science* 2002;296(5575):2046–9. [PubMed: 12016205]
35. Plant TD, Zollner C, Mousa SA, Oksche A. Endothelin-1 potentiates capsaicin-induced TRPV1 currents via the endothelin A receptor. *Exp Biol Med (Maywood)* 2006;231(6):1161–4. [PubMed: 16741069]
36. Price TJ, Flores CM. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *J Pain* 2007;8(3):263–72. [PubMed: 17113352]
37. Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M. PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci* 2002;22(11):4740–5. [PubMed: 12040081]
38. Schuligoi R, Donnerer J, Amann R. Bradykinin-induced sensitization of afferent neurons in the rat paw. *Neuroscience* 1994;59(1):211–5. [PubMed: 8190269]

39. Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L, Egerton J, Charles KJ, Smart D, Randall AD, Anand P, Davis JB. TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* 2002;418(6894):186–90. [PubMed: 12077606]
40. Smith KE, Gibson ES, Dell'Acqua ML. cAMP-dependent protein kinase postsynaptic localization regulated by NMDA receptor activation through translocation of an A-kinase anchoring protein scaffold protein. *J Neurosci* 2006;26(9):2391–402. [PubMed: 16510716]
41. Snyder EM, Colledge M, Crozier RA, Chen WS, Scott JD, Bear MF. Role for A kinase-anchoring proteins (AKAPS) in glutamate receptor trafficking and long term synaptic depression. *J Biol Chem* 2005;280(17):16962–8. [PubMed: 15718245]
42. Stokes AJ, Shimoda LM, Koblan-Huberson M, Adra CN, Turner H. A TRPV2-PKA signaling module for transduction of physical stimuli in mast cells. *J Exp Med* 2004;200(2):137–47. [PubMed: 15249591]
43. Tanji C, Yamamoto H, Yorioka N, Kohno N, Kikuchi K, Kikuchi A. A-kinase anchoring protein AKAP220 binds to glycogen synthase kinase-3 β (GSK-3 β) and mediates protein kinase A-dependent inhibition of GSK-3 β . *J Biol Chem* 2002;277(40):36955–61. [PubMed: 12147701]
44. Theurkauf WE, Vallee RB. Molecular characterization of the cAMP-dependent protein kinase bound to microtubule-associated protein 2. *J Biol Chem* 1982;257(6):3284–90. [PubMed: 6277931]
45. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 1998;21(3):531–43. [PubMed: 9768840]
46. Wisnoskey BJ, Sinkins WG, Schilling WP. Activation of vanilloid receptor type I in the endoplasmic reticulum fails to activate store-operated Ca²⁺ entry. *Biochem J* 2003;372(Pt 2):517–28. [PubMed: 12608892]
47. Xu H, Zhao H, Tian W, Yoshida K, Roulet JB, Cohen DM. Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. *J Biol Chem* 2003;278(13):11520–7. [PubMed: 12538589]
48. Xu H, Ramsey IS, Kotecha SA, Moran MM, Chong JA, Lawson D, Ge P, Lilly J, Silos-Santiago I, Xie Y, DiStefano PS, Curtis R, Clapham DE. TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature* 2002;418(6894):181–6. [PubMed: 12077604]

Figure 1. TRPV1, AKAP150 and PKA RII co-express and co-immunoprecipitate in TG Neurons
A-D.) Immunohistochemical co-expression of TRPV1 (blue), AKAP150 (red) and PKA RII (green) in intact TG neurons excised from rat, scale bar in yellow represents 50 μ m. **E-H.)** Immunocytochemical co-expression of TRPV1 (blue), AKAP150 (red) and PKA RII (green) in cultured TG neurons from rat, scale bar in yellow represents 50 μ m. **I-M.)** Co-immunoprecipitation of proteins from cultured TG neurons. Lane 1: AKAP150 immunoprecipitation, lane 2: TRPV1 immunoprecipitation, lane 3: 50 μ g TG neuron cell lysate, lane 4: 50 μ g brain homogenate. Blots were probed for (left to right) AKAP150 (**I**), TRPV1 (**J**), PKA RII (**K**), PSD-95 (**L**), SAP-97 (**M**).

Figure 2. siRNA directed knock-down of AKAP150 expression in TG neurons

A.) Cultured TG neurons transfected with nothing (-), mock transfected (Mock), scrambled/control negative silencer #2 siRNA (Scram), AKAP150-1 siRNA (AKAP-1), or AKAP150-2 siRNA (AKAP-2). Western blot expression of AKAP150 (above) following transfection quantified and normalized to expression of TRPV1.

B.) Cultured TG neurons transfected with AKAP150-1 harvested day 0, 1, 2, 3 or 4 following transfection. Western blot expression of AKAP150 (above) following transfection quantified and normalized to expression of TRPV1.

C.) Real-time RT-PCR experiments were performed with RNA samples from cultured TG neurons left alone (control), mock transfected (Mock), transfected with scrambled/control negative silencer #2 control siRNA (Scram), or with AKAP150-1 (AKAP-1 siRNA) following 1 day post-transfection. Reactions were performed using primers specific for AKAP150 (PRKA) gene and the internal control (18S). Data were normalized to the relative amount of control AKAP150 mRNA/18S. Data are presented as mean $\pm$ SEM ($n = 6$ per group).

D.) Cultured TG neurons mock transfected (Mock), with scrambled/control negative silencer #2 control siRNA (Scram), or with AKAP150-1 (AKAP-1) were analyzed for ³²P-incorporation following vehicle (H₂O) or 8-Br-cAMP (10 μM, 5 min). Autoradiographic results of labeled TRPV1 quantified and normalized to total TRPV1 expression.

E.) Cultured TG neurons mock transfected or with AKAP150-1 siRNA were surface biotinylated and analyzed for TRPV1 plasma membrane expression relative to total cell expression of AKAP150.

All plotted data are expressed as mean ± SE, **p<0.01, ***p<0.005, NS = not significant.

Figure 3. PKA association with AKAP150 in CHO is necessary for PKA sensitization of TRPV1 activity

A.) Co-immunoprecipitation of AKAP150, with Western blot analysis of association with TRPV1 and PKA RII in co-transfected CHO cells.

B.) Co-immunoprecipitation of TRPV1, with Western blot analysis of association with AKAP150 and PKA RII in co-transfected CHO cells.

C.) Calcium imaging of CHO cells transfected with AKAP150, TRPV1, and/or AKAP150ΔPKA followed by pretreatment with either vehicle or 8-Br-cAMP (10 μM, 5 min), followed by stimulation of all groups with capsaicin (100 nM).

D.) Basal *in vitro* activity of immunoprecipitated PKA from co-transfected CHO cells, with Western blot analysis of AKAP150, TRPV1 and PKA RII from cell lysates to demonstrate equal protein expression.

All plotted data are expressed as mean ± SE, ***p<0.005.

Figure 4. Pharmacological desensitization of TRPV1 in sensory neurons is independent of AKAP150 expression

A.) Cultured TG neurons mock transfected (Mock), transfected with scrambled/control negative silencer #2 control siRNA (Scram) or AKAP150-1 (AKAP-1) were analyzed for capsaicin (CAP; 50 nm, 30 sec)-activated current (I_{CAP}) tachyphylaxis, n shown on graph. Veh represents first response to CAP application.

B.) I_{CAP} values after second application of CAP (50 nm) normalized to first application of CAP (i.e. control). Recordings taken from cultured TG neurons as in panel A, n shown on graph.

C.) Representative trace of recordings taken from cultured TG neurons mock transfected, treatments indicated.

D.) Representative trace of recordings taken from cultured TG neurons transfected with scrambled/control negative silencer #2 siRNA (negative control), treatments indicated.

E.) Representative trace of recordings taken from cultured TG neurons transfected with AKAP150-1 specific siRNA, treatments indicated.

All plotted data are expressed as mean $\pm$ SE, * p <0.05, ** p <0.01, *** p <0.005, NS = not significant.

Figure 5. PKA sensitization of TRPV1 activity is dependent on AKAP150 expression

A.) Cultured TG neurons transfected as in A, analyzed for I_{CAP} (CAP: 50nm, 30 sec) sensitization following vehicle (H_2O) or 8-Br-cAMP (10 μM , 5 min) pre-treatment. Illustrated data normalized to vehicle treatment, n shown on graph.

B.) *in vitro* activity of immunoprecipitated PKA from mock (Mock) or AKAP150-1 (AKAP)-transfected TG neurons following H_2O vehicle or 8-Br-cAMP (10 μM , 5 sec) treatment, with Western blot analysis of AKAP150 and PKA RII from cell lysates to demonstrate equal protein expression.

C.) Representative trace of recordings taken from cultured TG neurons mock transfected, treatments indicated.

D.) Representative trace of recordings taken from cultured TG neurons transfected with scrambled/control negative silencer #2 siRNA (negative control), treatments indicated.

E.) Representative trace of recordings taken from cultured TG neurons transfected with AKAP150-1 specific siRNA, treatments indicated.

All plotted data are expressed as mean $\pm$ SE, ** $p < 0.01$, *** $p < 0.005$, NS = not significant.

Figure 6. AKAP220 expression in cultured TG neurons

A-D.) Immunohistochemical co-localization of TRPV1 (blue), AKAP220 (red) and PKA RII (green) in cultured TG neurons from rat, scale bar in yellow represents 50 μm .

E.) Western blot analysis of AKAP220 and AKAP150 expression from 50 μg TG neuronal lysate following siRNA knock-down: lane 1, no transfection; lane 2, mock; lane 3, AKAP150-1 FITC siRNA; lane 4, AKAP220-1 siRNA; lane 5, AKAP220-2 siRNA.

F.) Cultured TG neurons mock transfected (Mock) or transfected with AKAP220-1 siRNA (AKAP220 siRNA) and analyzed for I_{CAP} (50 nm) following vehicle (H_2O) or 8-Br-cAMP (10 μM) treatment. $n=7-12/\text{group}$.

G-H.) Representative trace of recordings taken from cultured TG neurons transfected with mock (**G**) or AKAP220 (**H**) siRNA, treatments indicated.

All plotted data are expressed as mean $\pm$ SE, ** $p<0.01$, *** $p<0.005$.

Figure 7. AKAP150/PKA association is necessary for TRPV1 phosphorylation and sensitization
A.) Cultured TG neurons pre-treated with tris/H₂O veh, St-HT31P (50 μ mol), or StHT31P-C (50 μ mol) 15 min, followed by 8-Br-cAMP (10 mM, 5 min), and analyzed for TRPV1 phosphorylation by autoradiographic densitometry normalized to Western blot immunoreactivity. * p <0.05, NS = not significant.

B.) Rats were injected with 50 μ l Tris/saline veh, St-HT31P (5 μ mol), or St-HT31P-C (5 μ mol) in the right hindpaw. The same paws were injected with 0.01% ethanol/saline veh or 0.3 μ g PGE₂ 15 min later, upon which thermal paw withdrawal latencies were measured by blinded observers. Data are depicted as change in paw withdrawal latency (sec) from baseline valuations taken before injections, such that PGE₂ produces shorter paw withdrawal latency values vs. baseline). All plotted data expressed as mean $\pm$ SEM, n = 6–10 animals per group, two-way ANOVA with Bonferroni post hoc test, ** p <0.01, NS=not significant.