BJ www.biochemj.org

BJ Signal

Biochem. J. (2010) **432**, 549–556 (Printed in Great Britain) doi:10.1042/BJ20100936

PP2B/calcineurin-mediated desensitization of TRPV1 does not require AKAP150

Elaine D. POR*, Bret K. SAMELSON†, Sergei BELUGIN‡, Armen N. AKOPIAN‡, John D. SCOTT† and Nathaniel A. JESKE*§[1]

*Department of Pharmacology, Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, U.S.A., †Howard Hughes Medical Institute, Department of Pharmacology, Box 357750, University of Washington School of Medicine, Seattle, WA 98195-7280, U.S.A., ‡Department of Endodontics, Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, U.S.A., and §Department of Oral and Maxillofacial Surgery, Center for Biomedical Neuroscience, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, U.S.A.

Activation of protein kinases and phosphatases at the plasma membrane often initiates agonist-dependent signalling events. In sensory neurons, AKAP150 (A-kinase-anchoring protein 150) orientates PKA (protein kinase A), PKC (protein kinase C) and the Ca^{2+}/calmodulin-dependent PP2B (protein phosphatase 2B, also known as calcineurin) towards membrane-associated substrates. Recent evidence indicates that AKAP150-anchored PKA and PKC phosphorylate and sensitize the TRPV1 (transient receptor potential subfamily V type 1 channel, also known as the capsaicin receptor). In the present study, we explore the hypothesis that an AKAP150-associated pool of PP2B catalyses the dephosphorylation and desensitization of TRPV1. Biochemical, electrophysiological and cell-based experiments indicate that PP2B associates with AKAP150 and TRPV1 in cultured TG (trigeminal ganglia) neurons. Gene silencing of AKAP150 reduces basal phosphorylation of TRPV1. However, functional studies in neurons isolated from AKAP150$^{-/-}$ mice indicate that the anchoring protein is not required for pharmacological desensitization of TRPV1. Behavioural analysis of AKAP150$^{-/-}$ mice further support this notion, demonstrating that agonist-stimulated desensitization of TRPV1 is sensitive to PP2B inhibition and does not rely on AKAP150. These findings allow us to conclude that pharmacological desensitization of TRPV1 by PP2B may involve additional regulatory components.

Key words: A-kinase-anchoring protein 150 (AKAP150), calcineurin, pain, phosphorylation, transient receptor potential subfamily V type 1 channel (TRPV1), trigeminal ganglion.

INTRODUCTION

Anchoring and scaffolding proteins function to target proteins to distinct subcellular environments or within distinct signalling pathways, controlling the activity of neighbouring substrates [1]. AKAP150 (A-kinase-anchoring protein 150) is a scaffolding protein that organizes the phosphorylation and/or dephosphorylation of various plasma membrane targets [2]. The human AKAP orthologue AKAP79 and the murine AKAP150 interact with multiple signalling enzymes, including PKA (protein kinase A), PKC (protein kinase C) and PP2B (protein phosphatase 2B) [3]. Specifically, anchoring of PKC and PKA via AKAP150 is necessary for the phosphorylation and sensitization of TRPV1 (transient receptor potential subfamily V type 1 channel) [4–6]. Phosphorylation of TRPV1 results in sensitization of the channel to activation by multiple pain-evoking stimuli, including noxious heat (>42 °C), acidic pH and CAP (capsaicin), the active ingredient in chilli peppers [7,8]. Previous studies have demonstrated that truncation or mutation of the PKA-binding site of AKAP150 interferes with the phosphorylation and subsequent sensitization of TRPV1 [9]. As a consequence, the functional involvement of AKAP150 in TRPV1 phosphorylation and sensitization demonstrates a critical role for this scaffolding complex in peripheral nociception [4].

Several investigators have demonstrated that sustained stimulation of TRPV1 leads to pharmacological desensitization of the receptor [10–12]. Dephosphorylation of TRPV1 by PP2B is a critical mechanism that leads to desensitization of the channel [13–16].

The Ca^{2+}/calmodulin-dependent serine/threonine phosphatase PP2B is a heterodimeric protein composed of a 60 kDa catalytic A subunit and a 19 kDa regulatory B subunit [17]. AKAP150 contains a principle PP2B-binding site within its C-terminus (amino acids 605–647), and mutation of this site demonstrates that anchoring of PP2B to AKAP150 is necessary for the dephosphorylation of various protein targets [18,19].

Specificity in cell signalling can be influenced by the targeting of different enzyme combinations to substrates [18]. In the present study, we examine whether dephosphorylation and desensitization of TRPV1 in primary TG (trigeminal ganglia) neurons relies on AKAP150-mediated targeting of PP2B. This detailed investigation into AKAP150-mediated regulation of TRPV1 is necessary to understand the underlying molecular mechanisms involved in pain and nociception.

EXPERIMENTAL

Tissue culture

All procedures using animals were approved by the Institutional Animal Care and Use Committee of UTHSCSA (University of Texas Health Centre at San Antonio), and were conducted in accordance with policies for the ethical treatment of animals established by the NIH (National Institutes of Health). TG neurons

Abbreviations used: AKAP150, A-kinase-anchoring protein 150; CAIP, calcineurin autoinhibitory peptide; CAP, capsaicin; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; NMP, N-methylpyrrolidinone; PKA, protein kinase A; PKC, protein kinase C; PP2B, protein phosphatase 2B; SES, standard external solution; siRNA, small interfering RNA; TG, trigeminal ganglia; TRPV1, transient receptor potential subfamily V type 1 channel.

[1] To whom correspondence should be addressed (email Jeske@uthscsa.edu).

were dissected bilaterally from male Sprague–Dawley rats (200–250 g; Charles River Laboratories) and disassociated by treatment with collagenase (catalogue number S5K8219, Worthington) for 30 min, following by treatment with trypsin (Sigma) for 15 min. Cells were centrifuged (2000 ***g*** for 2 min at 25 °C), aspirated and re-suspended in DMEM (Dulbecco's modified Eagle's medium, Invitrogen) supplemented with 10 % FBS (fetal bovine serum, Invitrogen), 100 ng/ml nerve growth factor (Harlan Laboratories), 1 % penicillin/streptomycin (Invitrogen) and 1 % glutamine (Sigma), and then placed on poly-D-lysine-coated plates. Cultures were maintained at 37 °C and 5 % CO_2 and grown for 5–7 days. CHO (Chinese-hamster ovary) cells were utilized for heterologous expression of cDNA constructs. CHO cells were maintained at 37 °C and 5 % CO_2 and transfected using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions.

siRNA (small interfering RNA) transfection

siRNA duplexes designed to specifically knockdown AKAP150 were constructed by Thermo Scientific (Dharmacon). The sequence for the sense strand of AKAP150 siRNA used was GCAUGUGAUUGGCAUAGAA-dTd [4]. TG neurons were transfected with scrambled siRNA (Silencer-1, Ambion) or AKAP150 siRNA (750 ng of siRNA/10 cm plate) using HiPerFect (Qiagen), following the manufacturer's protocol. TG neurons were utilized for experimentation at 1 day post-transfection, as validated previously [4].

Immunoprecipitation and Western blot analysis

For each experimental condition, cells were treated with the appropriate compounds and harvested as described previously [14]. Protein quantification of cell lysates was completed using the Bradford assay [20] (Sigma) according to the manufacturer's protocol. Following protein quantification, cleared lysates (500 μg) were immunoprecipitated with 1 μg of anti-AKAP150 (R-300, Santa Cruz Biotechnology), anti-TRPV1 (R-130, Santa Cruz Biotechnology) or anti-PP2B-B (Abcam) antiserum. Next, immunoprecipitates were resolved via SDS/PAGE (12.5 % gels), and transferred on to PVDF membrane (Millipore). Western blots were blocked in 5 % non-fat dried skimmed milk in TBS-Tw (Tris-buffered saline/Tween 20:15.35 mM Tris/HCl, 136.9 mM NaCl, pH 7.6, with 0.1 % Tween 20) and visualized using anti-AKAP150, anti-TRPV1 or anti-PP2B-B antibodies, followed by the appropriate horseradish-peroxidase-conjugated secondary antisera (GE Healthcare) and ECL (enhanced chemiluminescence) detection following the manufacturer's protocol (GE Healthcare). To study TRPV1 phosphorylation, TG neurons were incubated with 1 mCi of [^{32}P]P_i (PerkinElmer) for 4 h at 37 °C in phosphate-free DMEM (Invitrogen) on the day of experimentation. Crude plasma membranes were prepared for immunoprecipitation, SDS/PAGE separation and transfer following UTHSCSA radiation safety protocols. Autoradiographs were developed after 18 h exposure to blots at − 80 °C. Densitometry measurements were determined using NIH Image 1.62, with the reported pixel density = (band density) − (lane background density).

Crude plasma membrane preparation

Following homogenization by 20 strokes in a Potter–Elvehjem homogenizer in a hypotonic homogenization buffer [25 mM Hepes, 25 mM sucrose, 1.5 mM $MgCl_2$ and 50 mM NaCl (pH 7.2)], the cell extract was incubated on ice for 15 min and then centrifuged at 1000 ***g*** for 1 min at 4 °C to remove nuclei and non-lysed cells from the homogenate. The resulting supernatant was centrifuged at 16000 ***g*** for 30 min at 4 °C, separating cytosolic proteins from cell membrane proteins. The pellet (crude membrane fraction) was then resuspended in 500 μl of homogenization buffer containing 1 % Triton X-100.

Electrophysiology

All recordings were made in a perforated patch 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 using a MultiClamp 700B amplifier and pCLAMP 10.0 software (Axon Instruments). Data were filtered at 0.5 kHz and sampled at 2 kHz. Borosilicate pipettes (Sutter) were polished to resistances of 8–10 MΩ in perforated patch pipette solution. If necessary, access resistance (R_s) was compensated by 40–80 % to <25 MΩ.

All recordings were made in the presence of 2 mM Ca^{2+} in external solution. SES (standard external solution) comprised 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 10 mM Hepes (pH 7.4). The pipette solution comprised 140 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$ and 10 mM Hepes (pH 7.3), and contained 250 mg/ml amphotericin (Sigma). Drugs were applied using a computer-controlled pressure-driven eight-channel system (ValveLink8; AutoMate Scientific). Data were analysed by one-way ANOVA, and significance is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$.

Ca^{2+} imaging

To measure intracellular [Ca^{2+}] levels, the dye fura 2 AM (fura 2 acetoxymethyl ester) (2 μM; Molecular Probes) was incubated with cells for 30 min at 37 °C in the presence of 0.05 % Pluronic® (Calbiochem/EMD Biosciences). Fluorescence was detected with a Leica DM IRB microscope fitted with a ×20/0.8 NA (numerical aperture) fluorescence objective. Fluorescence images from 340 nm and 380 nm excitation wavelengths were collected and analysed with MetaFluor Software (MetaMorph, Web Universal Imaging). Transfected cells were identified by their GFP (green fluorescent protein) fluorescence. To assess for Ca^{2+} accumulation following TRPV1 activation, CAP (capsaicin, 50 nM, Tocris Bioscience) was administered for 30 s, followed by a 3 min washout with SES buffer. For analysis of TRPV1 desensitization, repeated CAP (50 nM) applications were interrupted by SES washout for 3 min. The net change in Ca^{2+} (ΔF340/380) was calculated by subtracting the basal F340/380 Ca^{2+} amounts of accumulation from the peak F340/380 Ca^{2+} accumulation achieved. Cell-permeant CAIP (calcineurin autoinhibitory peptide; EMD Biosciences) (sequence Ac-RRRRRRRRRRGGGRMAPPRRDAMPSDA-NH_2) was used to treat cells at 100 mM for 30 min at 37 °C prior to experimentation.

Behavioural assay

All procedures using animals were approved by the Institutional Animal Care and Use Committee of UTHSCSA, and were conducted in accordance with policies for the ethical treatment of animals established by the NIH. On the day of experimentation, CAP was dissolved in a 20 % NMP (N-methylpyrrolidinone) solution. AKAP-knockout mice were generated as described previously [21]. Prior to the experiment, wild-type and AKAP150$^{-/-}$ male C57/Bl6 mice (littermates, 5–10-weeks-old) were acclimated to both the room and testing equipment for 30 min prior to testing. For experiments evaluating CAP-induced desensitization, the right hindpaw of animals was injected with either vehicle or CAP (0.5 μg/10 μl) followed 15 min later

with CAP (0.5 μg/10 μl). For experiments evaluating the effect of FK-506 on CAP-induced desensitization, the plantar surface of the right hindpaw of animals was first injected with either vehicle or FK-506 (20 μg/10 μl) followed 15 min later by the CAP administration schedule described above. NMP (20 %) was used as a control for all individual experiments. The observer assessing nociceptive behaviour was blinded to the genotype of animals and injection contents used in all experiments. CAP-induced nocifensive behaviour was assessed by recording the time spent by the animal grooming and flinching of the injected hindpaw over a 5 min period [22]. In total, $n = 6$–10 animals per genotype/treatment were used. All reagents used were obtained from Sigma, unless otherwise indicated.

PKA RII subunit overlay analysis

Solid-phase-binding overlays were performed following previously published protocols [23], from freshly frozen, whole TG neurons, using 50 μg of total protein for each assay.

RESULTS AND DISCUSSION

Multiple studies have determined that pharmacological desensitization of TRPV1 is sensitive to PP2B inhibition [13–16]. Furthermore, recent studies have demonstrated that AKAP150 and TRPV1 associate in peripheral afferent neurons [4–6,9]. Since biochemical and cell-based studies have demonstrated that AKAP150 also contains a PP2B-binding site [19,24], we sought to determine whether the anchoring protein, the phosphatase and TRPV1 form a ternary complex in neurons cultured from TG. Western blot detection revealed that PP2B was detected in immune complexes isolated from crude plasma membrane fractions of TG neurons with antibodies against AKAP150 or TRPV1 (Figure 1). Similar amounts of PP2B immunoreactivity were detected in both AKAP150 and TRPV1 immunoprecipitates. There are two possible explanations for these findings: (i) the phosphatase, anchoring protein and channel form a ternary complex, or (ii) that AKAP150 and TRPV1 both have the capacity to independently interact with PP2B. In either case, the net effect would be to maintain PP2B in close proximity to TRPV1.

The kinase-anchoring function of AKAP150 directs PKA- and PKC-mediated phosphorylation of TRPV1 and the concomitant sensitization of the ion channel's activity [4–6]. Therefore we established whether the AKAP150 signalling complex influences the phosphorylation state of TRPV1. The incorporation of [^{32}P]P_i into TRPV1 was measured by autoradiography. Primary cultures of TG neurons were transfected with AKAP150-specific siRNA. Additional control experiments monitored the incorporation of [^{32}P]P_i into TRPV1 in neurons transfected with scrambled oligonucleotide control. Western blot analysis with an AKAP150-specific antibody confirmed gene silencing of the anchoring protein (Figure 2A). Previous studies have established the specificity of this and alternate AKAP150 siRNA oligonucleotide duplexes as reagents that suppress the expression of AKAP150 in TG neurons [4]. A significant decrease in basal phosphorylation of TRPV1 as indicated by ^{32}P incorporation was observed following gene silencing of AKAP150 in TG neurons (Figures 2B and 2D). In contrast, there were no significant differences in basal TRPV1 phosphorylation levels in control samples prepared from mock-transfected neurons or those transfected with scrambled siRNA (Figure 2D). TG neurons were treated with the TRPV1 agonist CAP (100 nM) for 30 s to determine whether agonist-dependent dephosphorylation of TRPV1 [25] requires AKAP150 expression. A significant reduction in phosphorylation was

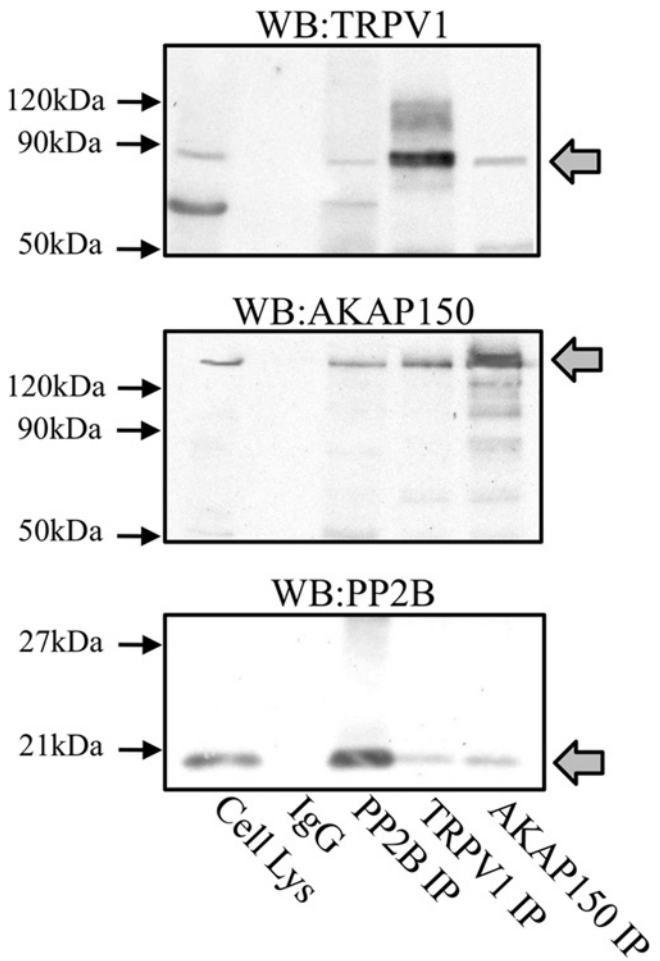

Figure 1 AKAP150 and TRPV1 associate with the PP2B-B subunit in rat primary sensory neurons

TG neurons were lysed and incubated with antibodies specific against TRPV1, AKAP150 and PP2B-B. Western blotting (WB) was performed to detect co-immunoprecipitates (IP) and expression levels in plasma membrane fractions. IgG and cell lysates (Cell Lys) were assessed for specificity of the TRPV1 antibody and expression profiles of all proteins. Molecular mass markers (in kDa) are shown on the left-hand side. Arrows on the right-hand side denote immunoreactive bands of interest. Results are representative of four independent experiments.

observed following CAP treatment of AKAP150 siRNA-treated neurons as compared with untreated neurons (Figures 2C and 2E). Normalization of the basal phosphorylation values revealed no significant difference in ^{32}P incorporation by TRPV1 between the transfection conditions (Figure 2F). TG neurons transfected with AKAP150 siRNA demonstrated similar levels of PP2B activity as compared with mock- and scrambled-transfected cells (results not shown). Taken together, these results not only indicate that AKAP150 may play a role in the basal phosphorylation of TRPV1, but importantly suggest that AKAP150 is not required for CAP-stimulated dephosphorylation of TRPV1.

Following the characterization of the AKAP150-specific siRNA, we sought to determine whether PP2B associates with TRPV1 directly or through AKAP150-assisted anchorage. TG neurons were cultured and transfected in a mock setting or with AKAP150-specific siRNA to knockdown expression of the scaffolding protein. Following knockdown, cultures were homogenized and crude plasma membrane fractions were subjected to co-immunoprecipitation. Results shown in Figure 3 indicate that PP2B associates with TRPV1 following CAP

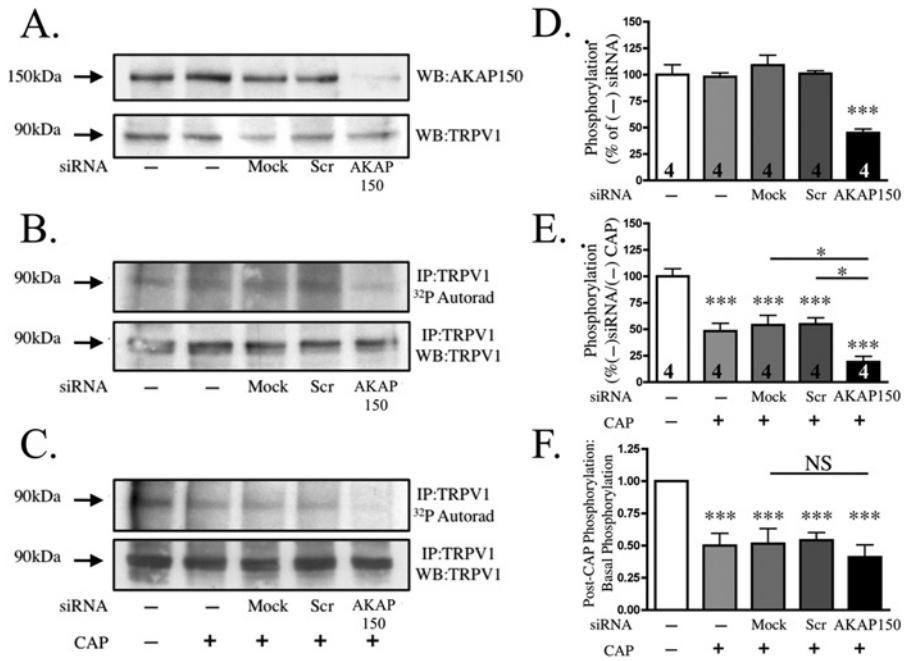

Figure 2 AKAP150 does not mediate CAP-induced dephosphorylation of TRPV1 in primary sensory neurons

TG neurons were transfected with siRNA directed against AKAP150, silencer negative siRNA (Scr) or no siRNA (mock) and assessed for [^{32}P]P_i incorporation (TRPV1 phosphorylation) by immunoprecipitation (IP) and Western blotting (WB). (**A**) AKAP150 expression levels in untransfected (–), mock, scrambled- and AKAP150-siRNA transfected cells. (**B**) Basal phosphorylation levels of TRPV1 following siRNA transfection. (**C**) Phosphorylation of TRPV1 following application of CAP (100 nM; 30 s). For (**A**–**C**) the molecular mass in kDa is indicated on the left-hand side. (**D**) Quantification of TRPV1 basal phosphorylation levels following untransfected (–), mock, scrambled- and AKAP150-siRNA transfection (significant compared with mock-transfected cells). Phosphorylation levels of TG-transfected neurons following activation (**E**) were normalized with basal levels of TRPV1 phosphorylation (**F**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$, as determined using two-way ANOVA with Bonferroni correction. Results are representative of four independent experiments.

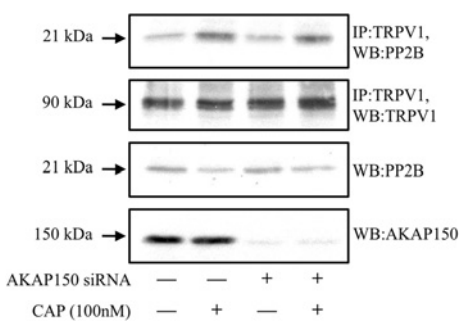

Figure 3 PP2B associates with TRPV1 in the absence of AKAP150 in primary sensory neurons

TG neurons were either mock-transfected or transfected with siRNA directed against AKAP150, and treated with vehicle or CAP (100 nM, 30 s). Following treatment, crude plasma membrane homogenates were collected for each treatment condition and immunoprecipitated (IP) with antibodies specific for TRPV1. Arrows on the left-hand side denote the predicted molecular mass of the protein of interest. Results are representative of three independent experiments. WB, Western blot.

treatment, in both the presence and absence of AKAP150 expression. These findings suggest that TRPV1 does not require AKAP150 to dynamically associate with PP2B for potential dephosphorylation events.

Next, we sought to determine whether the anchoring of PP2B to AKAP150 is critical to the pharmacological desensitization of TRPV1. CHO cells were transiently transfected with rat TRPV1 and rat AKAP150. In parallel, cells were transfected with TRPV1 and an internally deleted form of the anchoring protein AKAP150ΔPP2B, which is unable to anchor the phosphatase [18]. Since TRPV1 is a non-selective cation channel with a preference for Ca^{2+} that is directly activated by CAP [26], changes in TRPV1 activity were determined indirectly by fluorescent measurement of CAP-induced Ca^{2+} accumulation. Cells transfected with TRPV1 demonstrated normal desensitization/tachyphylaxis upon repeated applications of CAP (50 nM), in the presence of low endogenous levels of AKAP150 expression (Figure 4A). CHO cells co-transfected with TRPV1 and AKAP150 (Figures 4A and 4B) demonstrated a similar response when compared with controls (Figures 4A and 4B). Importantly, the introduction of AKAP150ΔPP2B failed to affect the typical CAP-mediated desensitization pattern (Figures 4A–4C) of TRPV1 following repeated stimulation with CAP. In Figure 4(D), CHO cells were pre-treated with a cell-permeant CAIP to demonstrate the significant role of calcineurin across all three sets of transfected cells. These results indicate that neither AKAP150 overexpression nor AKAP150-mediated anchorage of PP2B contribute to the desensitization of TRPV1 in the heterologous CHO expression system.

More sophisticated experiments were performed in cultured neurons from wild-type and AKAP150$^{-/-}$ mice. Ablation of the AKAP150 gene was demonstrated with a RII overlay assay (Figure 5A). Biochemical characterization confirmed that the anchoring protein was not expressed in the cell lysates of TG neurons from AKAP150$^{-/-}$ mice, although surface biotinylation of TRPV1 was unaffected (Figure 5). Gene silencing of AKAP150 in TG neuronal cultures supported this point, demonstrating similar levels of TRPV1 expression in plasma membrane fractions generated from mock- and AKAP150 siRNA-transfected cells (Figure 5C). β1-Integrin was used as a positive control for plasma membrane proteins.

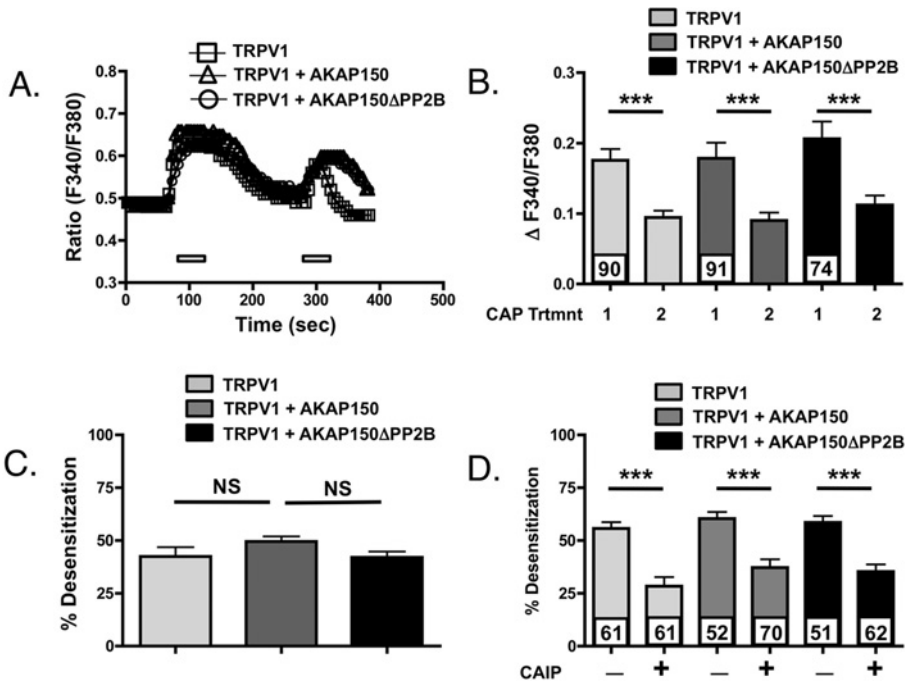

Figure 4 AKAP150 association with PP2B does not mediate CAP-induced desensitization of TRPV1

CHO cells transiently transfected with TRPV1, TRPV1 + AKAP150 or TRPV1 + AKAPΔPP2B were assessed for CAP-induced Ca^{2+} accumulation. (**A**) Representative traces. Following acquisition of baseline, CAP (100 nM) was administered for 30 s (shaded bar), and the cells were rinsed with SES buffer for 3 min. To assess desensitization, a second application of CAP was administered for 30 s following the 3 min rinse. Accumulation of Ca^{2+} was calculated from the 340/380 ratio. (**B**) The net change in Ca^{2+} (ΔF340/380) was calculated by subtracting the basal F340/380 Ca^{2+} level from the peak F340/380 Ca^{2+} level achieved, $n = 74$–90 neurons per transfection group. (**C**) Ca^{2+} accumulation from CAP/CAP treatments were normalized to those for vehicle/CAP treatments, and are displayed as normalized desensitization. (**D**) Ca^{2+} accumulation from CAP/CAP treatments were normalized to those for vehicle/CAP treatments following pre-treatment with vehicle (H_2O) or cell-permeant CAIP (100 mM, 30 min), and are displayed as normalized desensitization. $^{***}P < 0.005$ and NS = no significance, as determined by one-way ANOVA, with Bonferroni post-hoc analysis. *n* values are indicated in the bars of each histogram.

In order to further investigate this finding, we took a behavioural approach. Age-matched wild-type and AKAP150$^{-/-}$ littermates were monitored for changes in nocifensive behaviour following the repeated administration of CAP. Injection of vehicle (20 % NMP) followed by a CAP (0.5 μg) injection in the right hindpaw of wild-type and AKAP150$^{-/-}$ mice resulted in nocifensive behaviour in both groups, as determined by the time spent licking and flinching the injection site. Interestingly, AKAP150$^{-/-}$ mice demonstrated a decreased response to the single CAP injection as compared with wild-type mice (Figure 6). To test for CAP-specific desensitization of TRPV1, an initial injection of CAP (0.5 μg) was administered, followed by a second dose of CAP (0.5 μg) 15 min later. This resulted in decreased nocifensive behaviour in both groups (Figure 6). Importantly, pre-injection of the animals with the PP2B-specific inhibitor FK-506 (20 mg/10 ml, [27]) at the injection site reversed CAP/CAP pharmacological desensitization in both wild-type and AKAP150$^{-/-}$ populations. Taken together, these behavioural results indicate that TRP agonist-mediated PP2B activation through TRPV1 drives pharmacological desensitization of TRPV1 in both wild-type and AKAP150$^{-/-}$ animals.

Next, we conducted electrophysiological experiments in a perforated patch-clamp configuration to detect differences in CAP/CAP pharmacological desensitization of TRPV1 in TG neurons cultured from wild-type and AKAP150$^{-/-}$ animals. Normalized desensitization of TRPV1 to repeated CAP applications in the presence/absence of FK-506 or the calmodulin inhibitor W-7 were measured in multiple neurons, as illustrated in Figure 7. Results from these studies indicate no significant differences between wild-type and AKAP150$^{-/-}$ animals in normalized CAP desensitization of TRPV1, although both W-7 and FK-506 treatment reversed a significant portion and all of the desensitization in both genotypes respectively. Taken together, these results suggest that PP2B is capable of regulating the pharmacological desensitization of TRPV1 in the absence of AKAP150-assisted targeting, and that the process involves calmodulin, as other research groups have demonstrated [15,28,29].

Stimulation of TRPV1 by certain agonists including CAP and resiniferatoxin leads to receptor desensitization following dephosphorylation by PP2B [15,30]. The results of the present paper elucidate the role of AKAP150 associated with PP2B in localized catalysis of CAP-induced TRPV1 desensitization. In the present study, we have demonstrated that both AKAP150 and TRPV1 associate with PP2B at the plasma membrane. Moreover, the loss of functional AKAP150 expression through siRNA-mediated gene silencing and ablation of the AKAP150 gene in mice indicated that tachyphylactic (i.e. pharmacological) desensitization of TRPV1 occurs in the absence of the scaffolding protein. This observation is supported by behavioural tests that monitor CAP-induced nociception [31]. Taken together, these results suggest that, although AKAP150 has been shown to direct the activities of PP2B with other proteins, the scaffolding protein is not required for the dynamic pharmacological desensitization of TRPV1 by PP2B.

Previous findings have implicated AKAP150 in TRPV1 desensitization [6], although studies presented herein offer different data and an alternative explanation. In agreement with Zhang et al. [6], we demonstrate that knockdown of AKAP150 or deletion of the PP2B-binding site in the C-terminus of

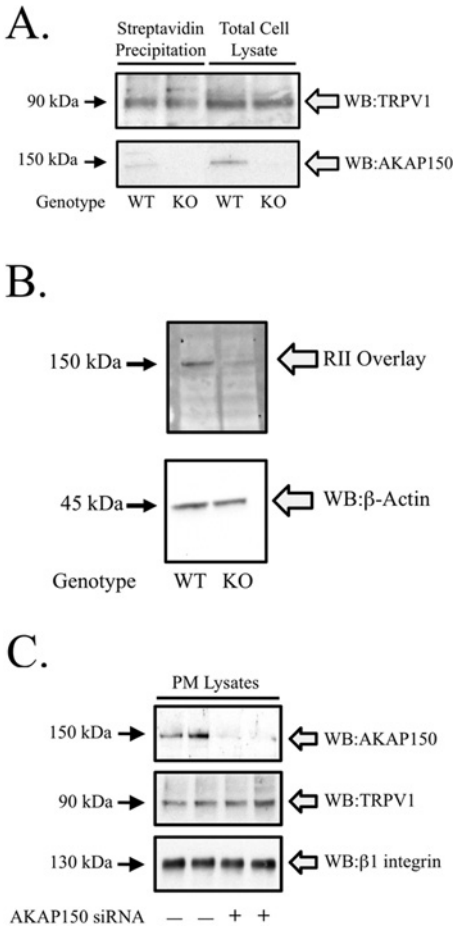

Figure 5 AKAP150 knockdown or knockout does not effect TRPV1 localization to the plasma membrane

(**A**) TG neurons isolated from wild-type (WT) and AKAP150$^{-/-}$ (KO) mice were cultured and surface biotinylated to identify plasma membrane proteins exposed to the extracellular space. (**B**) PKA RII subunit-binding proteins were detected in lysates generated from homogenized TGs dissected and frozen from wild-type (WT, AKAP150$^{+/+}$) and AKAP150$^{-/-}$ (KO) mice, using ^{32}P-radiolabelled RIIα as a probe. Control blots indicate correct expression profiles for AKAP150 and β-actin. (**C**) TG neurons from rats were transfected in a mock fashion or with AKAP150-specific siRNA, and homogenates were differentially centrifuged to isolate the crude plasma membrane fraction. Equal aliquots (30 μg) of plasma membrane (PM) lysates were resolved and analysed for AKAP150, TRPV1 and β1-integrin expression. The molecular mass in kDa is indicated on the left-hand side. Arrows on the right-hand side denote immunoreactive bands of interest. Results are representative of four independent experiments.

AKAP150 does not completely abolish TRPV1 desensitization. However, the utilization of AKAP150$^{-/-}$ animals in behaviour and electrophysiological experiments designed to test the functional and pharmacological desensitization of TRPV1 respectively, would indicate that AKAP150 has no role in mediating PP2B-driven desensitization of the channel. Indeed, if calmodulin assists in PP2B-mediated pharmacological desensitization, as suggested previously, the N-terminal and C-terminal calmodulin-binding sites could become important tools to study the desensitization of TRPV1 [28,32,33]. It is also interesting that a reported 14-amino-acid AKAP150-binding site on TRPV1 [6] is located 19 residues upstream of the C-terminal calmodulin-binding site [28]. This relatively small C-terminal region that governs important intermolecular interactions with TRPV1 would certainly be subject to tight biochemical control to account for the numerous protein–protein interactions and subsequent chemical reactions that affect TRPV1 activity.

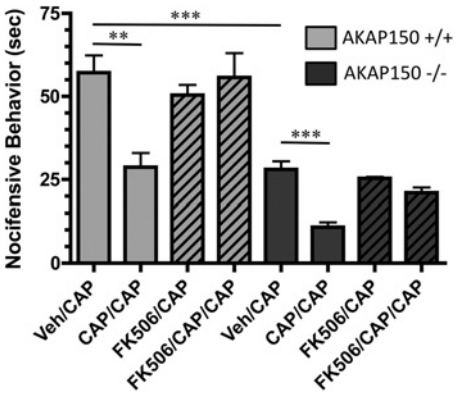

Figure 6 AKAP150 is not required for behavioural TRPV1 desensitization

Wild-type (AKAP150$^{+/+}$) and AKAP150$^{-/-}$ mice were assessed for nocifensive behaviour as determined by time spent licking and flinching the injected hind-paw. For control experiments, both groups were injected with vehicle (20 % NMP) followed 15 min later with CAP (0.5 μg) in a 10 μl injection volume. For desensitization experiments, both groups were injected with CAP (0.5 μg/10 μl) followed by a second injection of CAP (0.5 μg/10 μl) 15 min later. FK-506 (20 mg/10 ml) was administered prior to the first CAP injection, at the same injection site. Nocifensive behaviour was recorded over a 5 min period for all experiments. $^{**}P < 0.01$ and $^{***}P < 0.005$, indicates the significance from the respective genotype vehicle/CAP treatment as measured by one-way ANOVA. $n = 6$–10 animals/group.

One intriguing implication from our investigation is that the loss of AKAP150 significantly inhibited nocifensive responses to CAP application [34]. This evidence may suggest that AKAP150 is required for an appropriate nociceptive response *in vivo* following TRPV1 activation. These findings demonstrate a response to AKAP150 ablation that was not observed in heterologous CHO or cultured TG models. Previous data using thermal stimulation of peripheral nociceptors did not reveal this genetic difference [5]. This may be due to the peripheral expression of other heat-sensitive channels including TRPV3 [35] and TRPV4 [36,37] that may not be dependent upon AKAP150 scaffolding proteins for functionality *in vivo*. In agreement with our *in vitro* results, agonist-mediated behavioural desensitization still occurred in AKAP150$^{-/-}$ animals, supporting the conclusion that AKAP150 does not participate in the pharmacological desensitization of TRPV1.

The results generated from the present study indicate that the expression of AKAP150 is not necessary for pharmacological desensitization of TRPV1 in TG neurons. However, the presence of AKAP150 and association with TRPV1 at the plasma membrane may be necessary for appropriate responses to typical nociceptive stimuli. Continued investigation of additional target proteins that associate with AKAP150 and/or TRPV1 may serve to elucidate additional mechanisms that underlie TRPV1 desensitization.

AUTHOR CONTRIBUTION

Elaine Por and Nathaniel Jeske conceived and designed the project. Elaine Por, Bret Samelson and Sergei Belugin conducted the research. Nathaniel Jeske directed the research. Elaine Por, Armen Akopian, John Scott and Nathaniel Jeske wrote the paper.

ACKNOWLEDGEMENTS

We thank Ruben Gomez for expert technical assistance. We also thank Dr David Julius (University of California at San Francisco, San Francisco, CA, U.S.A.) for providing materials used in the present study.

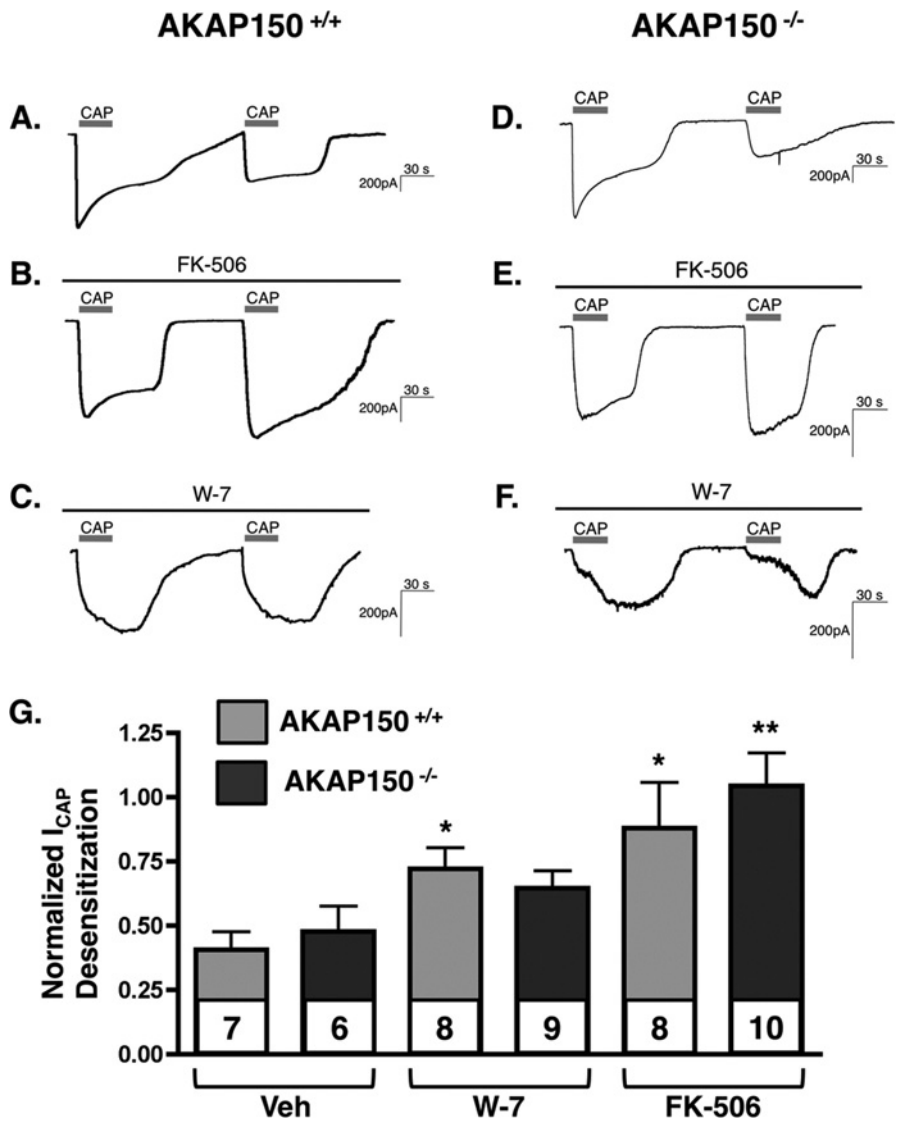

Figure 7 Genetic ablation of AKAP150 does not affect CAP-induced desensitization of TRPV1 activity

Sample CAP (300 nM, 30 s)-current traces from TG neurons isolated from wild-type (AKAP150$^{+/+}$) (**A**–**C**) or AKAP150$^{-/-}$ (**D**–**F**) animals following vehicle (Veh), W-7 (500 mM) or FK-506 (10 μM) co-treatments, as indicated. The time between concurrent CAP applications is 2 min. (**G**) Normalized CAP-desensitization from the total neurons. Normalization of the second CAP applications were carried out against the first CAP application. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significance from vehicle-treated genotype, as determined by one-way ANOVA. *n* values are indicated in the bars of each graph.

FUNDING

This work was funded by the National Institutes of Health [grant numbers DE019311, DE017696 (to A.N.A.), GM48321 (to J.D.S.), NS061884 (to N.A.J.)]; and support from the LeDucq Transatlantic Network (to J.D.S.).

REFERENCES

1 Scott, J. D. and Pawson, T. (2009) Cell signaling in space and time: where proteins come together and when they're apart. Science **326**, 1220–1224
2 Wong, W. and Scott, J. D. (2004) AKAP signalling complexes: focal points in space and time. Nat. Rev. Mol. Cell Biol. **5**, 959–970
3 Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S. and Scott, J. D. (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science **271**, 1589–1592
4 Jeske, N. A., Diogenes, A., Ruparel, N. B., Fehrenbacher, J. C., Henry, M., Akopian, A. N. and Hargreaves, K. M. (2008) A-kinase anchoring protein mediates TRPV1 thermal hyperalgesia through PKA phosphorylation of TRPV1. Pain **138**, 604–616
5 Jeske, N. A., Patwardhan, A. M., Ruparel, N. B., Akopian, A. N., Shapiro, M. S. and Henry, M. A. (2009) A-kinase anchoring protein 150 controls protein kinase C-mediated phosphorylation and sensitization of TRPV1. Pain **146**, 301–307
6 Zhang, X., Li, L. and McNaughton, P. A. (2008) Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. Neuron **59**, 450–461
7 Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D. and Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature **389**, 816–824
8 Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I. and Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron **21**, 531–543
9 Schnizler, K., Shutov, L. P., Van Kanegan, M. J., Merrill, M. A., Nichols, B., McKnight, G. S., Strack, S., Hell, J. W. and Usachev, Y. M. (2008) Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. J. Neurosci. **28**, 4904–4917
10 Mandadi, S., Numazaki, M., Tominaga, M., Bhat, M. B., Armati, P. J. and Roufogalis, B. D. (2004) Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. Cell Calcium **35**, 471–478

11 Mohapatra, D. P. and Nau, C. (2003) Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. J. Biol. Chem. **278**, 50080–50090

12 Novakova-Tousova, K., Vyklicky, L., Susankova, K., Benedikt, J., Samad, A., Teisinger, J. and Vlachova, V. (2007) Functional changes in the vanilloid receptor subtype 1 channel during and after acute desensitization. Neuroscience **149**, 144–154

13 Docherty, R. J., Yeats, J. C., Bevan, S. and Boddeke, H. W. (1996) Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. Pflugers Arch. **431**, 828–837

14 Jeske, N. A., Patwardhan, A. M., Gamper, N., Price, T. J., Akopian, A. N. and Hargreaves, K. M. (2006) Cannabinoid WIN 55,212–2 regulates TRPV1 phosphorylation in sensory neurons. J. Biol. Chem. **281**, 32879–32890

15 Mohapatra, D. P. and Nau, C. (2005) Regulation of Ca^{2+}-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. J. Biol. Chem. **280**, 13424–13432

16 Patwardhan, A. M., Jeske, N. A., Price, T. J., Gamper, N., Akopian, A. N. and Hargreaves, K. M. (2006) The cannabinoid WIN 55,212–2 inhibits transient receptor potential vanilloid 1 (TRPV1) and evokes peripheral antihyperalgesia via calcineurin. Proc. Natl. Acad. Sci. U.S.A. **103**, 11393–11398

17 Klee, C. B., Draetta, G. F. and Hubbard, M. J. (1988) Calcineurin. Adv. Enzymol. Relat. Areas Mol. Biol. **61**, 149–200

18 Hoshi, N., Langeberg, L. K. and Scott, J. D. (2005) Distinct enzyme combinations in AKAP signalling complexes permit functional diversity. Nat. Cell Biol. **7**, 1066–1073

19 Dell'Acqua, M. L., Dodge, K. L., Tavalin, S. J. and Scott, J. D. (2002) Mapping the protein phosphatase-2B anchoring site on AKAP79. Binding and inhibition of phosphatase activity are mediated by residues 315–360. J. Biol. Chem. **277**, 48796–48802

20 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

21 Tunquist, B. J., Hoshi, N., Guire, E. S., Zhang, F., Mullendorff, K., Langeberg, L. K., Raber, J. and Scott, J. D. (2008) Loss of AKAP150 perturbs distinct neuronal processes in mice. Proc. Natl. Acad. Sci. U.S.A. **105**, 12557–12562

22 Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I. and Julius, D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science **288**, 306–313

23 Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D., Cone, R. D. and Scott, J. D. (1992) Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteinsCharacterization of AKAP 79. J. Biol. Chem. **267**, 16816–16823

24 Koplas, P. A., Rosenberg, R. L. and Oxford, G. S. (1997) The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. J. Neurosci. **17**, 3525–3537

25 Bhave, G., Zhu, W., Wang, H., Brasier, D. J., Oxford, G. S. and Gereau, R. W. T. (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron **35**, 721–731

26 Mohapatra, D. P., Wang, S. Y., Wang, G. K. and Nau, C. (2003) A tyrosine residue in TM6 of the vanilloid receptor TRPV1 involved in desensitization and calcium permeability of capsaicin-activated currents. Mol. Cell. Neurosci. **23**, 314–324

27 Tsukamoto, T., Iyo, M., Tani, K., Sekine, Y., Hashimoto, K., Ohashi, Y., Suzuki, K., Iwata, Y. and Mori, N. (2001) The effects of FK506, a specific calcineurin inhibitor, on methamphetamine-induced behavioral change and its sensitization in rats. Psychopharmacology **158**, 107–113

28 Numazaki, M., Tominaga, T., Takeuchi, K., Murayama, N., Toyooka, H. and Tominaga, M. (2003) Structural determinant of TRPV1 desensitization interacts with calmodulin. Proc. Natl. Acad. Sci. U.S.A. **100**, 8002–8006

29 Rosenbaum, T., Gordon-Shaag, A., Munari, M. and Gordon, S. E. (2004) Ca^{2+}/calmodulin modulates TRPV1 activation by capsaicin. J. Gen. Physiol. **123**, 53–62

30 Patwardhan, A. M., Jeske, N. A., Price, T. J., Gamper, N., Akopian, A. N. and Hargreaves, K. M. (2006) The cannabinoid WIN 55,212–2 inhibits transient receptor potential vanilloid 1 (TRPV1) and evokes peripheral antihyperalgesia via calcineurin. Proc. Natl. Acad. Sci. U.S.A. **103**, 11393–11398

31 Ruparel, N. B., Patwardhan, A. M., Akopian, A. N. and Hargreaves, K. M. (2008) Homologous and heterologous desensitization of capsaicin and mustard oil responses utilize different cellular pathways in nociceptors. Pain **135**, 271–279

32 Lishko, P. V., Procko, E., Jin, X., Phelps, C. B. and Gaudet, R. (2007) The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. Neuron **54**, 905–918

33 Rosenbaum, T., Gordon-Shaag, A., Islas, L. D., Cooper, J., Munari, M. and Gordon, S. E. (2004) State-dependent block of CNG channels by dequalinium. J. Gen. Physiol. **123**, 295–304

34 Jeske, N. A., Patwardhan, A. M., Ruparel, N. B., Akopian, A. N., Shapiro, M. S. and Henry, M. A. (2009) A-kinase anchoring protein 150 controls protein kinase C-mediated phosphorylation and sensitization of TRPV1. Pain **146**, 301–307

35 Xu, H., Ramsey, I. S., Kotecha, S. A., Moran, M. M., Chong, J. A., Lawson, D., Ge, P., Lilly, J., Silos-Santiago, I., Xie, Y. et al. (2002) TRPV3 is a calcium-permeable temperature-sensitive cation channel. Nature **418**, 181–186

36 Chung, M. K., Lee, H. and Caterina, M. J. (2003) Warm temperatures activate TRPV4 in mouse 308 keratinocytes. J. Biol. Chem. **278**, 32037–32046

37 Guler, A. D., Lee, H., Iida, T., Shimizu, I., Tominaga, M. and Caterina, M. (2002) Heat-evoked activation of the ion channel, TRPV4. J. Neurosci. **22**, 6408–6414

Received 24 June 2010/22 September 2010; accepted 30 September 2010
Published as BJ Immediate Publication 30 September 2010, doi:10.1042/BJ20100936