Phosphorylation regulates TRPV1 association with β-arrestin-2

Elaine D. POR*, Ruben GOMEZ†, Armen N. AKOPIAN*‡ and Nathaniel A. JESKE*†‡§†
*Department of Pharmacology, The University of Texas Health Science Center, San Antonio, TX 78229, U.S.A., †Department of Oral and Maxillofacial Surgery, The University of Texas Health Science Center, San Antonio, TX 78229, U.S.A., ‡Department of Endodontics, The University of Texas Health Science Center, San Antonio, TX 78229, U.S.A., §Department of Physiology, The University of Texas Health Science Center, San Antonio, TX 78229, U.S.A.

INTRODUCTION

Post-translational modifications in the TRPV1 (transient receptor potential vanilloid 1) channel, specifically phosphorylation, are crucial to the regulation of the receptor [1,2]. Previous studies have demonstrated that phosphorylation of specific serine/threonine residues is positively correlated with sensitization and activation of TRPV1 [3–5]. Importantly, the scaffolding protein AKAP150 (A-kinase anchoring protein 150) effectively co-ordinates TRPV1 phosphorylation [6–8] via its ability to localize PKA (protein kinase A) and PKC (protein kinase C) to the receptor. Anchoring and scaffolding proteins are well-characterized for their role in mediating efficient downstream signalling cascades by organizing specific proteins and enzymes in close proximity to their respective substrates [9,10]. Interestingly, β-arrestins are now well-characterized as critical scaffolding proteins [11]. Historically, β-arrestins were characterized as a small family of cytosolic proteins involved in metabotropic GPCR (G-protein-coupled receptor) regulation. However, previous studies demonstrate novel scaffolding roles for β-arrestins in the regulation of both metabotropic and ionotropic receptors [12,13]. Recently, our group has identified β-arrestin-2 as a scaffolding protein implicated in the desensitization of TRPV1 [14]. Specifically, our study revealed that β-arrestin-2 scaffolds the phosphodiesterase PDE4D5 to the receptor and limits its PKA-phosphorylation status [14]. Our recent findings complement previously identified and well-characterized molecular mechanisms underlying TRPV1 desensitization [15–17]. Taken together these findings demonstrate key proteins implicated in the regulation of TRPV1 via modulation of its phosphorylation status.

Receptor phosphorylation of GPCRs recruits β-arrestins to the plasma membrane [18–20]. Following recruitment and binding to an agonist-stimulated receptor, β-arrestins facilitate receptor desensitization and internalization via association with proteins involved in endocytosis [21,22]. Although β-arrestins were classically defined for mediating receptor internalization via clathrin-dependent endocytosis, their ability to scaffold proteins and localize specific proteins to various substrates contributes to novel roles in regulating receptor desensitization and down-regulation [19,23]. Recently, multiple scaffolding roles for β-arrestins have been identified in which association with the phosphodiesterase PDE4D [24,25], ubiquitin ligase AIP4 [13] and Src kinase [12] is critical in the activation and down-regulation of multiple receptors.

Previous studies demonstrate that ligand-mediated activation of GPCRs and subsequent receptor phosphorylation is required for β-arrestin-2 recruitment [18,26]. Receptor phosphorylation functions as a molecular signal to engage β-arrestin interaction with the activated receptor and subsequent regulation of downstream signalling pathways. Previously, studies have identified β-arrestins and their ability to associate with ionotropic receptors [12,13]; however, the mechanisms underlying recruitment to these receptors have not been investigated.

Abbreviations used: AKAP150, A-kinase anchoring protein 150; 8-Br, 8-Br-cAMP; CAIP, calcineurin autoinhibitory peptide; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CaV, calcineurin; CAP, capsasin; CHO, Chinese-hamster ovary; CK2, casein kinase 2; FOR, forward; GFP, green fluorescent protein; GF109203X; GPCR, G-protein coupled receptor; GRK, GPCR kinase; PDBu, phorbol 12,13-dibutyrate; PDE4D, phosphodiesterase 4D; PKA, protein kinase A; PKC, protein kinase C; REV, reverse; SES, standard extracellular solution; TBB, 4,5,6,7-tetrabromobenzotriazole; TRPV1, transient receptor potential vanilloid 1; WT, wild-type.

To whom correspondence should be addressed (email jeske@uthscsa.edu).
Previous work by our group characterized the functional significance of the association of β-arrestin-2 with TRPV1 and its role in regulating receptor desensitization. As such, we sought to investigate whether TRPV1 phosphorylation dictates the β-arrestin-2 association, as observed for GPCR–β-arrestin complexes. Investigation of the mechanism(s) involved in β-arrestin-2 recruitment to TRPV1 at the plasma membrane may further elucidate the contribution of this association to both normal and pathophysiological conditions.

**EXPERIMENTAL**

**Reagents**

Capsaicin, 8-Br (8-Br-cAMP), H-89 and GFX (GF 109203X) were purchased from Sigma–Aldrich. TBB (4,5,6,7-tetramethylbenzotriazole), KN-93 and KN-92 (inactive derivative) were obtained from Calbiochem.

**Tissue culture**

CHO (Chinese-hamster ovary) cells were utilized for heterologous expression of cDNA constructs. Cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) supplemented with 10% FBS (fetal bovine serum; Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% glutamine (Sigma–Aldrich), and maintained at 37°C and 5% CO₂. For cell-transfection experiments, CHO cells were transfected using Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions. Immunoprecipitation and Western blot analysis were performed with the QuikChange™ XL Site-Directed Mutagenesis kit according to the manufacturer’s instructions (Stratagene). Primers used for rat TRPV1 S116A were: FOR (forward) 5′-GCTCTATGATCGAGGCGCTTCTCGATCGTGCC-3′; and REV (reverse) 3′-GCCACAGATCGAAGATGGCCCTTGAGCATACAGG-5′. Primers used for rat TRPV1 T370A were: FOR 5′-CCATACGAGGAGGATCTGCGATCTGTCG-3′; and REV 3′-CCCATAGCCCATCCTGCGAGACATCTCGATAGG-5′. Primers used for rat TRPV1 S116D were: FOR 5′-GCTCTATGATCGAGGCGCTTCTCGATCGTGCC-3′; and REV (reverse) 3′-GCCACAGATCGAAGATGGCCCTTGAGCATACAGG-5′. Primers used for rat TRPV1 T370D were: FOR 5′-CCATACGAGGAGGATCTGCGATCTGTCG-3′; and REV 3′-CCCATAGCCCATCCTGCGAGACATCTCGATAGG-5′. Primers used for bovine β-arrestin-2 T382A were: FOR 5′-GCCCCGGTGGACGCAAACCTCATTGAATCC-3′; and REV 3′-GAATTCAGAGTTGCGGCCACGAGG-5′. Primers used for bovine β-arrestin-2 T382D were: FOR 5′-GCCCCGGTGGACGCAAACCTCATTGAATCC-3′; and REV 3′-GAATTCAGAGTTGCGGCCACGAGG-5′.

**Site-directed mutagenesis**

TRPV1 and β-arrestin-2 phosphorylation mutants were generated with the QuikChange™ XL Site-Directed Mutagenesis kit according to the manufacturer’s instructions (Stratagene). Primers used for rat TRPV1 S116A were: FOR (forward) 5′-GCTCTATGATCGAGGCGCTTCTCGATCGTGCC-3′; and REV (reverse) 3′-GCCACAGATCGAAGATGGCCCTTGAGCATACAGG-5′.

**Immunoprecipitation and Western blot analysis**

For each experimental condition, cells were treated with the compounds indicated and harvested as described previously [15]. Protein quantification of plasma membrane homogenates was completed using the Bradford assay [27] (Sigma–Aldrich) following the manufacturer’s protocol. Samples (200 mg) were immunoprecipitated with 2 μg of anti-TRPV1 (R-130, Santa Cruz Biotechnology) or anti-GFP (green fluorescent protein) (B-2, Santa Cruz Biotechnology) antibodies. Next, immunoprecipitates were resolved by SDS/PAGE (15% gel), and transferred on to PVDF membrane (Millipore). Western blots were blocked in 5% non-fat dried skimmed milk in TBS (Tris-buffered saline; 15.35 mM Tris/HCl and 136.9 mM NaCl, pH 7.6)/0.1% Tween 20 and visualized using anti-TRPV1, anti-GFP or anti-PDE4D (H-69, Santa Cruz Biotechnology) antibodies followed by the appropriate HRP (horseradish peroxidase)-conjugated secondary antisera (GE Healthcare) and ECL (enhanced chemiluminescence) detection following the manufacturer’s protocol (GE Healthcare). Crude plasma membranes were prepared for immunoprecipitation, SDS/PAGE separation and transfer following UTHSCSA (University of Texas Health Science Center at San Antonio) radiation safety protocols. Autoradiographs were developed after 18 h exposure to blots at −80°C. Densitometry measurements were determined using NIH (National Institutes of Health) 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 Heps, 25 mM sucrose, 1.5 mM MgCl₂ 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 unlysed 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 400 μl of homogenization buffer containing 1% Triton X-100.

**Biotinylation**

Cultured CHO cells were co-transfected with β-arrestin-2–GFP and WT (wild-type) TRPV1, or the indicated TRPV1 mutant cDNA. Next, cells were biotinylated with EZ-Link biotin (0.5 mg/ml; Pierce), harvested and precipitated as described previously [28]. To determine plasma membrane expression of TRPV1, SDS/PAGE was performed followed by transfer on to a PVDF membrane and Western blotting with antibodies specific for TRPV1 (R-130, Santa Cruz Biotechnology) and β1-integrin (M-106, Santa Cruz Biotechnology).

**Calcium imaging**

To measure intracellular Ca²⁺ 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) Fluor 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 fluorescence. To assess for Ca²⁺ accumulation following TRPV1 activation, CAP (capsaicin, 50 nM) was administered for 30 s followed by a 3 min washout with SES (standard extracellular solution) buffer [140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM D-(-)-glucose and 10 mM Heps (pH 7.4)]. For analysis of TRPV1 desensitization, repeated CAP (50 nM; 30 s) applications were interrupted by SES washout for 3 min. Ratiometric data were converted into [Ca²⁺], by using

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Interaction of TRPV1 and β-arrestin-2

Figure 1  PKA and PKC activity regulate the association of TRPV1 and β-arrestin-2

(A) CHO cells transfected with TRPV1 and β-arrestin-2 were assessed for association in normal or serum-free conditions. (B) Quantification of the TRPV1 and β-arrestin-2 association. **P < 0.01, Student’s t test. (C) Transfected cells were serum-starved and treated with vehicle or the PKA and PKC activators 8-Br (10 μM for 5 min) or PDBu (10 μM for 5 min) respectively, followed by co-immunoprecipitation and Western blotting. (D) Quantification of the association following the indicated treatments: ***P < 0.001, one-way ANOVA with Bonferroni post-hoc analysis. (E) Transfected cells were treated with vehicle or the PKA and PKC inhibitors H-89 (20 μM for 5 min) or GFX (10 μM for 5 min) respectively, followed by co-immunoprecipitation and Western blotting. (F) Quantification of the association following the indicated treatments. *P < 0.05 and **P < 0.01, one-way ANOVA with Bonferroni post-hoc analysis. (G) Transfected cells were treated with the CaMKII inhibitor KN-93 (5 μM for 5 min) or the inactive analogue KN-92 (5 μM for 5 min), followed by co-immunoprecipitation and Western blotting. (H) Quantification of the association following the indicated treatments. NS, not significant. For all, results are representative of four independent experiments. IP, immunoprecipitation; WB, Western blot.

the equation $[\text{Ca}^{2+}] = K \times (R - R_{\text{min}})/(R_{\text{max}} - R)$, where $R$ is the 340/380 nm fluorescence ratio.

RESULTS

Previous work by our group has demonstrated β-arrestin-2 association with TRPV1 at the plasma membrane in both primary cultures of trigeminal sensory neurons and a heterologous cell culture model [14]. That study revealed a critical role for β-arrestin-2 in mediating TRPV1 desensitization and maintaining dephosphorylation of the receptor by scaffolding the phosphodiesterase PDE4D5 in close proximity to TRPV1. Furthermore, we identified residues within the TRPV1 sequence modulated by β-arrestin-2 expression. In the present study, we wanted to determine the underlying molecular mechanisms that regulate the association between TRPV1 and β-arrestin-2. Previous findings indicate that ligand-mediated activation of GPCRs leads to β-arrestin-2 recruitment [18,26] and that the phosphorylation status of TRPV1 influences receptor activity [2,7,16]. Thus we investigated whether TRPV1 phosphorylation dictates β-arrestin-2 association. Serum deprivation in cell culture reduces protein phosphorylation [29,30]. Therefore we first assessed the association of TRPV1 and β-arrestin-2 under both normal and serum-free media conditions in transfected CHO cells. As compared with cells cultured in normal medium, serum deprivation resulted in a significant decrease in the association of TRPV1 and β-arrestin-2 at the membrane (Figures 1A and 1B). Various kinases, including GRKs (GPCR kinases), were identified for their role in GPCR phosphorylation and recruitment of β-arrestins to the membrane [26,31]. With regard to TRPV1, PKA- and PKC-mediated phosphorylation is critical to the...
sensitization and subsequent activation of TRPV1 [6–8]. To
determine whether activation of these specific kinases contribute
to the association between β-arrestin-2 and TRPV1, we used
selective activators of PKA and PKC, 8-Br and PDBu (phorbol
12,13-dibutyrate) respectively. As indicated in Figures 1(C) and
1(D), we found that pharmacological activation of both PKA
and PKC increased TRPV1 and β-arrestin-2 association at the
plasma membrane.

To further establish a role for PKA and PKC in regulating
the association of TRPV1 and β-arrestin-2, we used selective
inhibitors of both kinases. Treatment with pharmacological
inhibitors of PKA and PKC, H-89 and GFX respectively,
significantly decreased association of β-arrestin-2 and TRPV1
at the plasma membrane (Figures 1E and 1F). Phosphorylation
of specific residues within the TRPV1 sequence is also modulated
by CaMKII (Ca2+/calmodulin-dependent protein kinase II)
[1,32]. Therefore we investigated whether CaMKII-mediated
phosphorylation contributes to the interaction between β-arrestin-
2 and TRPV1 as well. Pharmacological inhibition of CaMKII with
the selective inhibitor KN-93 did not reveal any differences in
association of these two proteins (Figures 1G and 1H). Taken
together, these findings indicate that cellular PKA and PKC
activity plays a critical role in β-arrestin-2 association with
TRPV1.

Phosphorylation and dephosphorylation of TRPV1 regulates
the activity of the receptor [4,33–35]. CaN (calcineurin)-mediated
TRPV1 dephosphorylation is a well-recognized mechanism under-
lining Ca2+-dependent desensitization of TRPV1 [15,16,32]. Thus
we next assessed whether pharmacological activation or
inhibition of this phosphatase alters the association between
TRPV1 and β-arrestin-2. Following treatment with the Ca2+
ionophore A23187 (1 μM for 10 min), which stimulates the Ca2+-
sensitive phosphatase CaN, a significant decrease in TRPV1 and
β-arrestin-2 was observed at the membrane (Figures 2A and 2B).
In contrast, inhibition of CaN with CAIP (CaN autoinhibitory
peptide; 100 μM for 30 min) increased the association of TRPV1
with β-arrestin-2 at the membrane (Figures 2C and 2D). These
findings demonstrate the contribution of CaN activity to regulating
the association between TRPV1 and β-arrestin-2.

The phosphorylation of certain proteins stimulates the
recruitment of modifiers that control said phosphorylation,
thereby creating a loop of negative feedback. In our cellular
model, we sought to determine whether TRPV1 phosphorylation
at PKA phosphorylation sites Ser116 and Thr370 affect association
of β-arrestin-2 with the receptor. As such, the association would
scaffold PDE4D5 to limit PKA phosphorylation, maintaining
endogenous negative feedback. In Figures 3(A) and 3(B), we
mutated Ser116 and Thr370 to alanine residues, thereby preventing
phosphorylation at these sites, and found a reduced association of
β-arrestin-2 with TRPV1 T370A. In contrast, we mutated these
same sites to aspartate, to mimic a phosphate group, and found
that only TRPV1 T370D stimulated association with β-arrestin-2
(Figures 3C and 3D). Taken together, these data implicate Thr370
of TRPV1 as the amino acid with which phosphorylation determines
β-arrestin-2 recruitment to the receptor. Next, we performed
biotinylation cell-surface expression experiments to confirm that
differences in the association of β-arrestin-2 and TRPV1 are attributed
to the phosphorylation status of specific residues and not to reduced expression at the membrane. Importantly,
no differences in TRPV1 plasma membrane expression were
observed between TRPV1 WT, S116A, T370A, S116D or T370D
(Figure 4). Thus the findings of the present study indicate that
the phosphorylation status of Thr370 in TRPV1 drives receptor
association with β-arrestin-2.

Similar to TRPV1, specific residues within β-arrestin-2 are
also phosphorylated in a regulatory manner. β-Arrestin-2 is
predominantly phosphorylated at Thr382 (bovine), and to a lesser
extent Ser660, by CK2 (casein kinase II) [36,37]. Therefore
we next investigated whether treatment with the selective CK2
inhibitor TBB (25 μM) [38,39] would alter the association
between β-arrestin-2 and TRPV1. Treatment of co-transfected
CHO cells with TBB yielded a significant increase in β-arrestin-
2 and TRPV1 association at the plasma membrane at 30 and
60 min as compared with untreated cells (Figures 5A and 5B).
This is in agreement with previous studies that indicate CK2-
mediated phosphorylation of β-arrestin-2 regulates its ability
to mediate desensitization of ligand-activated receptors [37]. To
confirm our findings, we generated site-specific mutations of
β-arrestin-2, Thr382 to alanine to prevent phosphorylation, and
to aspartate to mimic constitutive phosphorylation. We then assessed
expression of β-arrestin-2 WT and the phosphorylation mutants
in both cytosolic and plasma membrane fractions. As indicated
in Figure 5(C), no differences in β-arrestin-2 plasma membrane
and cytosolic expression were observed between T382A and T382D
as compared with WT. Moreover, treatment with TBB (25 μM
for 30 min) did not alter expression of either β-arrestin-2 WT or
the phosphorylation mutants. Taken together, these data suggest
that β-arrestin-2 phosphorylation is critical to its association
with TRPV1, but does not function to regulate its translocation or
expression at the plasma membrane.

β-Arrestin-2 is constitutively phosphorylated by CK2 and
dephosphorylated following ligand-mediated activation [22,36].
Therefore we next explored whether phosphorylation of β-
arrestin-2 at Thr382 regulates association with TRPV1, since
pharmacological inhibition of CK2 was shown to increase
the association of β-arrestin-2 and TRPV1 (Figure 5A).
We conducted co-immunoprecipitation experiments to evaluate
the association of TRPV1 with β-arrestin-2 WT and the
phosphorylation mutants (T382A and T382D). Mutation of
β-arrestin-2 Thr382 to aspartate (T382D) led to a significant
reduction in the association of TRPV1 and β-arrestin-2 at the
plasma membrane as compared with WT and the T382A mutant.
Interaction of TRPV1 and β-arrestin-2

Site-directed mutagenesis was conducted with TRPV1, in which Ser116 and Thr370 were mutated to alanine (S116A, T370A) or aspartate (S116D, T370D). (A) Association of β-arrestin-2 and TRPV1 alanine mutants determined by co-immunoprecipitation and Western blotting. (B) Quantification of the TRPV1 and β-arrestin-2 association. (C) Association of β-arrestin-2 and TRPV1 aspartate mutants determined by co-immunoprecipitation and Western blotting. (D) Quantification of the TRPV1 and β-arrestin-2 association. *P < 0.05 and **P < 0.01, one-way ANOVA with Bonferroni post-hoc test. Results are representative of four independent experiments. βarr2, β-arrestin-2; IP, immunoprecipitation; NS, not significant; WB, Western blot.

Figure 4 Plasma membrane expression of TRPV1 phosphorylation mutants

CHO cells transfected with the indicated cDNAs were surface biotinylated to identify plasma membrane expression of TRPV1 WT and TRPV1 phosphorylation mutants. (A) Cell-surface expression of TRPV1 WT and alanine mutants, S116A and T370A. (B) Quantification of membrane expression of the TRPV1 alanine mutants as compared with WT. (C) Cell-surface expression of TRPV1 WT and aspartate mutants, S116D and T370D. (D) Quantification of membrane expression of the TRPV1 aspartate mutants as compared with WT. Results are representative of four independent experiments. βarr2, β-arrestin-2; IP, immunoprecipitation; NS, not significant; WB, Western blot.

(Figures 6A and 6B). To assess a functional effect of β-arrestin-2 phosphorylation on the activity of TRPV1, we performed Ca\(^{2+}\) imaging. Importantly, TRPV1 desensitization was significantly reduced in cells transfected with the T382D mutant, but not with the T382A mutant (Figures 6C–6E). This decrease in desensitization may occur as a consequence of a reduced ability of the β-arrestin-2 T382D mutant to effectively associate with TRPV1 at the membrane (Figures 6A and 6B). Conversely, it is plausible that enhanced desensitization observed with the β-arrestin-2 T382A mutant may result from an increased ability to associate with TRPV1. Taken together these data indicate that phosphorylation of Thr382 in β-arrestin-2 functionally limits association with the TRPV1 receptor.

DISCUSSION

TRPV1 phosphorylation by kinases, including PKA and PKC, leads to receptor sensitization [6,7,40–42]. Effective and efficient PKA- and PKC-mediated phosphorylation of TRPV1 occurs...
Thr370 in TRPV1 are critically involved in the activation of the receptor. We demonstrate that the association of TRPV1 and β-arrestin-2 is significantly increased under normal serum media conditions and reduced in serum-free media (Figures 1A and 1B). Moreover, TRPV1 phosphorylation by PKA and PKC increased the association of β-arrestin-2 with TRPV1 at the plasma membrane (Figures 1C–1F). PKA-mediated phosphorylation of key residues Ser16 and Thr370 in TRPV1 are critically involved in the activation and sensitization of the receptor [4,16]. In agreement, functional analysis of TRPV1 PKA phosphorylation mutants revealed that β-arrestin-2 expression enhances receptor desensitization of both TRPV1 S116A and T370A mutants. Furthermore, these effects on T370A are reversed following treatment with the phosphodiesterase inhibitor rolipram [14]. In the present study, we demonstrate that the association of β-arrestin-2 with TRPV1 T370A is considerably reduced, whereas increased association occurs with TRPV1 T370D, as compared with WT (Figure 3). Differences in β-arrestin-2 association, as a consequence of TRPV1 phosphorylation status, further implicate Thr370 as a critical modulatory residue in both the activation and β-arrestin-2-mediated modulation of the receptor.

To date, CaN serves as the predominant mechanism involved in the dephosphorylation and desensitization of TRPV1 [17,44,45]. However, CaN activity does not account for total receptor desensitization, in that pharmacological inhibition of the phosphatase does not completely abolish TRPV1 desensitization [16,46]. Recently, we identified a scaffolding role for β-arrestin-2 in TRPV1 desensitization and its ability to modulate phosphorylation at specific TRPV1 residues, specifically Thr370 [14]. The phosphorylation status of Thr370 in TRPV1 is a critical site involved in Ca2+- and CaN-dependent desensitization of the receptor [15,16]. In agreement, in the present study we provide evidence indicating Thr370 phosphorylation as critical to β-arrestin-2 association with TRPV1. Pharmacological activation and inhibition of CaN with A23187 and the CAIP respectively lead to significant alterations in the association of β-arrestin-2 and TRPV1 (Figure 2). Thus CaN-mediated modulation of TRPV1 contributes to the association between β-arrestin-2 and TRPV1. Collectively, the findings of the present study indicate a concerted action of CaN and β-arrestin-2 in the dephosphorylation and desensitization of TRPV1.

Similar to TRPV1, β-arrestin-2 activity is regulated by phosphorylation at specific serine and threonine residues. Previous reports have indicated that β-arrestin-2 is predominantly phosphorylated at Thr382 (bovine), and to a lesser extent Ser360, by CK2 and undergoes dephosphorylation following ligand-mediated activation at the plasma membrane [36,37]. Inhibition of the constitutively active kinase CK2 with the selective inhibitor TBB significantly increased β-arrestin-2 and TRPV1 association (Figure 5). Thus it appears that CK2-mediated phosphorylation of β-arrestin-2 functions to inhibit its association with TRPV1 at the plasma membrane. Analyses of plasma membrane and cytosolic distribution of both WT and mutant β-arrestin-2 (T382A and T382D) reveal that differences in TRPV1 and β-arrestin-2 association are attributed to the phosphorylation status of Thr382.
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β-arrestin-2 and not its expression (Figure 5C). As indicated in the shift in molecular mass of the β-arrestin-2 T382D mutant (Figure 6A), conformational changes in the protein attributed to its phosphorylation status may diminish affinity for the TRPV1 receptor as compared with WT. Previous studies have investigated the contribution of β-arrestin-2 phosphorylation at this residue with regard to protein activity. Lin et al. [37] demonstrated that the constitutively phosphorylated mutant of β-arrestin-2, T382D, exhibits reduced clathrin binding and deficits in receptor down-regulation and internalization. However, another study suggested that phosphorylation did not affect β-arrestin-2 interactions with endocytotic proteins, but may regulate its ability to form large protein complexes [36]. These findings, in combination with evidence in the present study, implicate specific signalling pathways and phospho-residues within both TRPV1 and β-arrestin-2, which dictate their interaction at the plasma membrane (Figure 7).

Recently, we identified β-arrestin-2 as a scaffolding protein critical to the desensitization of TRPV1. Specifically, we provided multiple lines of evidence detailing the contribution of β-arrestin-2-mediated localization of the phosphodiesterase PDE4D5 to TRPV1 desensitization [14]. As such, in the present study we sought to characterize the mechanism(s) by which β-arrestin-2 is recruited to TRPV1 at the membrane. Our findings indicate that phosphorylation of both proteins influences this interaction and ultimately leads to negative feedback, in which TRPV1 is maintained in a dephosphorylated/desensitized state. In agreement, several divergent studies have demonstrated that recruitment of scaffolding proteins to activated receptors leads to negative regulation of receptor activity. In particular, Rack1 is now identified as a scaffolding protein, in which its association with various proteins inhibits receptor activity and downstream signalling pathways [47,48]. Interestingly, the E3 ubiquitin ligase AIP4 is also identified as a scaffolding protein that inhibits TGFβ (transforming growth factor β) signalling via an ubiquitin-independent mechanism [49]. Moreover, β-arrestin-1-mediated scaffolding of AIP4 to agonist-stimulated TRPV4 receptors is known as a critical mechanism underlying receptor

![Figure 6 β-Arrestin-2 phosphorylation regulates association with TRPV1](image)

Site-directed mutagenesis was conducted on β-arrestin-2, in which Thr382 was mutated to alanine (T382A) or aspartate (T382D). (A) Association of TRPV1 with β-arrestin-2 WT or the β-arrestin-2 threonine mutants was determined by co-immunoprecipitation and Western blotting with CHO cells transfected with the cDNAs indicated. (B) Quantification of the TRPV1 and β-arrestin-2 association with the mutants indicated. (C and D) Representative traces and quantification of Ca2+ accumulation. Ca2+ accumulation following repeat application of CAP (50 nM for 30 s). Shaded grey horizontal bars in (C) indicate the application of CAP. n = 60–92 neurons per transfection group. (E) Normalized second CAP response of CHO cells transfected with the mutants indicated mutants.

*P < 0.05, **P < 0.01 and *** P < 0.001, one-way ANOVA with Bonferroni post-hoc analysis. βarr2, β-arrestin-2; IP, immunoprecipitation; NS, not significant; WB, Western blot.
down-regulation [13]. Collectively, this evidence indicates multiple diverse scaffolding complexes involved in the desensitization and down-regulation of a variety of receptor systems, albeit through unique protein interactions.

With regard to TRPV1, extensive literature has identified a myriad of proteins and molecules that modulate receptor activity. Importantly, studies have implicated scaffolding proteins in the regulation of both TRPV1 sensitization [6–8] and desensitization [14]. The strategic organization of key proteins to TRPV1 at the membrane, via scaffolding proteins, has provided critical insight into receptor regulation. Additional studies, which characterize TRPV1 interaction with scaffolding proteins, may provide a basis for understanding how perturbations of these associations contribute to a vast number of pain and inflammatory conditions. Accordingly, in the present study we identify phosphorylation of both TRPV1 and β-arrestin-2 as important molecular signals, implicated in regulating this interaction.

AUTHOR CONTRIBUTION

Elaine Por and Nathaniel Jeske conceived and designed the project. Elaine Por, Ruben Gomez and Armen Akopian conducted the research. Nathaniel Jeske directed the research. Elaine Por and Nathaniel Jeske wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr David Julius (Department of Physiology, University of California San Francisco, San Francisco, CA, U.S.A.) and Dr Jeffrey Benovic (Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA, U.S.A.) for kindly providing cDNAs.

FUNDING

This work was supported by the National Institutes of Health [grant numbers F31 DE022517-01 (to E.D.P.) and RO1 NS061884 (to N.A.J.)].

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