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Activation of the Transient Receptor Potential Vanilloid-1 (TRPV1) channel mediates Extracellular Signal Regulated Kinase (ERK) phosphorylation via Beta-arrestin-2 signaling

by

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A THESIS

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Abstract

The Transient Receptor Potential Vanilloid 1 (TRPV1) channel plays a pivotal role in pain sensation and transduction under physiological and pathophysiological conditions. Recent work highlighted a possible role for β-arrestin-2, a scaffolding protein that mediates G-protein coupled receptor desensitization, in channel regulation. Interestingly, β-arrestin-2 also acts as a signaling scaffold for the MAPK (ERK1/2) pathway which was described as an important nociceptive marker. In this thesis, several experimental approaches were employed to investigate TRPV1 signaling and to characterize whether β-arrestin-2 as well as ERK play a role downstream of channel activation. The work presented here describes for the first time a unique β-arrestin-2 signaling pathway following TRPV1 channel activation. In particular, we found that calcium influx through TRPV1 channels induced translocation of β-arrestin-2 from the cytosol to the nucleus. In addition, we showed that TRPV1 activation elicited ERK phosphorylation in a β-arrestin-2-dependent manner. Our data suggest that the signaling cascade starts with calcium influx through TRPV1 channels that activates protein kinase C (PKC) and induces its translocation to the plasma membrane. The activation of PKC was necessary for ERK activation as well as β-arrestin-2 nuclear translocation. While this work is the first to describe β-arrestin-2 nuclear translocation downstream of TRPV1 stimulation, the functional relevance of this translocation is yet-to-be unveiled. Given the crucial role of TRPV1 in nociception, understanding its signaling as well as the mechanisms by which the channel is modulated may pave the way to develop a novel class of analgesics.

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Dedication

To my ever dearest Nadia and Mohamed Aboushousha

To my loving Osama and Hamza

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
2fli	2-Furoyl-LIGRLO-amide trifluoroacetate salt
BRET	Bioluminescence Resonance Energy Transfer
CaM	Calcium-modulated protein (calmodulin)
CaMKII	Ca^{2+}/ calmodulin-dependent protein kinase II
CNS	Central nervous system
CREB	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DAG	Diacylglycerol
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
GPCR	G-protein Coupled Receptors
GRK	G protein-coupled receptor kinase
IP3 receptor	Inositol 1,4,5 triSphosphate receptor
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase (MAPKK or MAP2K)

NES	Nuclear export signal
p-38	p-38 mitogen-activated protein kinase
PAR-2	Protease-activated receptor 2
p-ERK	Phosphorylated ERK
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
REAP	Rapid, Efficient And Practical
RLuc	Renilla Luciferase
RTX	Resiniferatoxin
TRP	Transient Receptor Potential channels
TRPA	Transient Receptor Potential cation channel, subfamily A (ankyrin)
TRPC	Transient Receptor Potential cation channel, subfamily C (canonical)
TRPM	Transient Receptor Potential cation channel, subfamily M (melastatin)
TRPML	Transient Receptor Potential cation channel, subfamily ML (mucolipin)
TRPP	Transient Receptor Potential cation channel, subfamily P (polycystic)
TRPV	Transient Receptor Potential cation channel, subfamily V (vanilloid)

Chapter One: **Introduction**

1.1 Pain

Pain is a noxious unpleasant sensory or emotional experience triggered in the nervous system and is associated with actual or potential tissue injury and damage. Detection of painful stimuli occurs at the peripheral terminals of specialized nerve fibers, called the nociceptors (Dubin et al., 2010). These specialized neurons have their cell bodies (soma) in either the dorsal root ganglia (DRG) or the trigeminal ganglia. Nociceptors are small diameter neurons expressing a specific repertoire of ion channels and receptors and are capable of detecting a broad range of noxious potentially harmful physical and chemical stimuli (nociception). Typically, noxious stimuli activate nociceptors that innervate tissues and organs in the periphery. Signals are relayed to the spinal cord then transmitted to higher central nervous system (CNS) structures, where they are perceived as pain (Julius, 2013; Bourinet et al., 2014) (**Figure 1.1**).

Pain is classified as either acute or chronic pain. Under normal physiological conditions, pain sensation lessens as healing progresses until minimal or no pain is detected (acute). Indeed the acute pain is fundamental as it protects from tissue damage. Persistent intense pain however activates the nervous system and can cause allodynia (pain response caused by innocuous stimulus) and hyperalgesia (increased response to noxious stimulus) that can affect normal functioning through the transition to chronic pain. The latter chronic pain does not seem to serve a significant physiological function, but indeed the pain itself turns into a disease process that becomes challenging to treat. Expenditures spent on chronic pain are huge and include both direct costs spent on health care expenses and indirect costs related to loss of productivity. In Canada, chronic pain costs more than cancer, heart diseases and HIV combined (Lynch, 2011),

with estimates placed at $6 billion/year spent on direct healthcare costs and ~ $37 billion/year on productivity loss and sick days (Lynch, 2011).

The etiology of chronic pain is diverse (e.g. physical trauma, chemotherapy, diseases, metabolic disorders, infections, etc) and comprises a wide variety of underlying molecular mechanisms. Therefore, it is one of the most complicated topics to deal with, knowing that targeting one pathway may work for a certain type of chronic pain but not for others. One such important underlying mechanism is represented by the ion channels located in the primary afferent nociceptive neurons that contribute to the detection of noxious stimuli. Several ion channels have been shown to play important roles in nociception (McCleskey et al., 1999; Sousa-Valente et al., 2014); one of which is the transient receptor potential channel (TRP) family of ion channels. These are the focus of intense research because they could represent an important target in the treatment of acute and chronic pain (Moran et al., 2011).

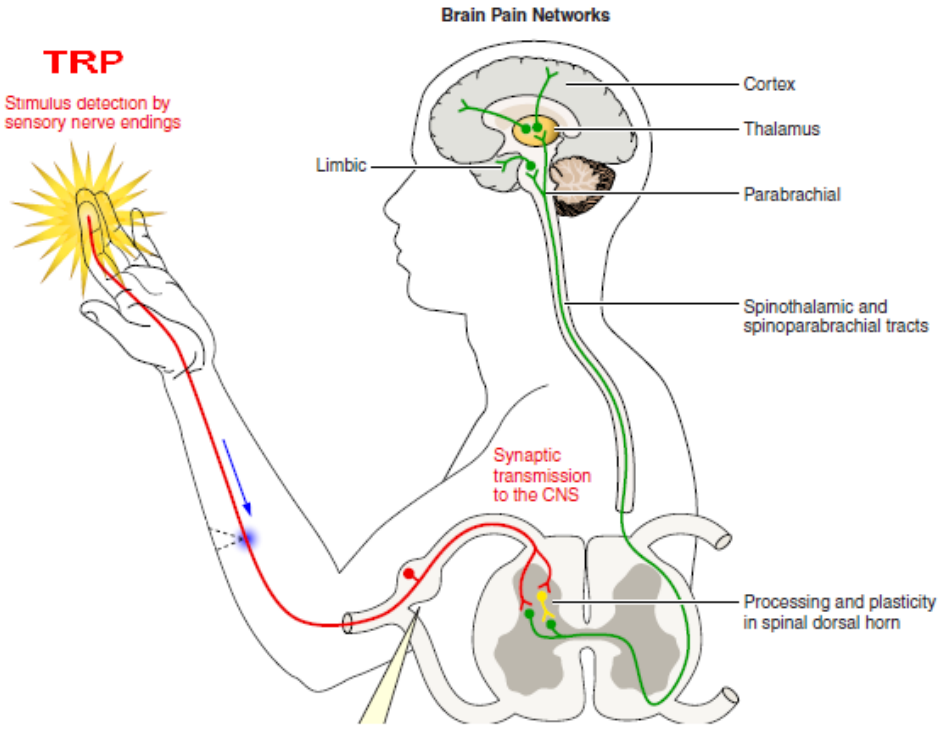


Figure 1.1 Pain signal transmission

Sensory information being transmitted from peripheral nociceptors to higher CNS structures in the brain. An example of ion channels expressed in nociceptors is the Transient Receptor Potential (TRP) family of channels. Adapted from The American Physiological Society Physiological Reviews (Bourinet et al., 2014). No permission was required.

1.2 Transient receptor potential (TRP) channels

Nociceptors express multiple subtypes of TRP channels (Patapoutian et al., 2009). The TRP channel family is one of the largest families of ion channels with representative members across the phylogenic tree from yeast to humans. Members of this family have been classified into six subfamilies; TRPV, TRPA, TRPC, TRPM, TRPML, TRPP (Ramsey et al., 2006). Mammalian TRPs are permeable to cations and their membrane topology is similar to the superfamily of voltage-gated channels (Zheng, 2013) with six transmembrane domains, a pore loop between transmembrane segment 5 and 6 and amino (N) and carboxy (C) terminal regions of variable lengths (**Figure 1.2**). Beyond their general membrane topology, TRP channels display diverse modes of activation (temperature, osmolarity, chemical compounds, mechanical stimulation, light and acids), regulation (phosphorylation, transcription, glycosylation, etc), ion selectivity, broad tissue distribution and different physiological functions (Levine et al., 2007). Within the TRP family, TRPV1 is known to play a pivotal role in inflammatory pain sensation and transduction (Premkumar et al., 2008).

1.3. Transient Receptor Potential Vanilloid 1 Channel (TRPV1)

TRPV1, also known as the capsaicin or vanilloid receptor, is a non-selective cation channel with significant permeability to calcium (Mohapatra et al., 2003). It has a diverse tissue distribution with higher expression in sensory neurons (Tominaga et al., 1998) and is activated by multiple endogenous and exogenous stimuli (Kaszas et al., 2012). Endogenous stimuli include acidic pH, heat and several inflammatory mediators, while capsaicin, the pungent component of chilli pepper, and resiniferatoxin (RTX), a naturally occurring chemical extracted from a cactus-like plant, are representative examples of exogenous stimuli. TRPV1 represents an important

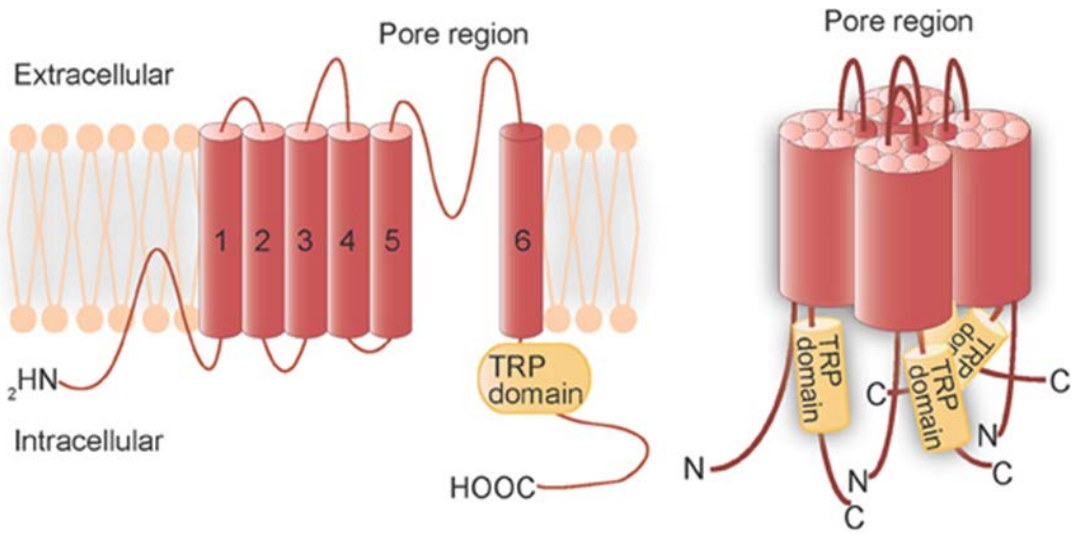


Figure 1.2 General TRP channel topology

In general TRP channels consist of 6 transmembrane segments with intracellular amino (N) and carobxy (C) termini. The cation permeable pore-forming domain is located between transmembrane segments 5 and 6. Adapted from Frontiers in Physiology (Takahashi et al., 2012). No permission was required.

signaling integrator in nociceptors under both normal physiological and pathophysiological conditions including inflammation and chronic pain (Rosenbaum et al., 2007). Several biochemical analyses have confirmed that TRPV1 forms tetramers, with four subunits assembled around a central pore as a functional unit (García-Sanz et al., 2004; Flynn et al., 2014). This oligomerization has been shown to be essential for channel trafficking to the plasma membrane and activation (Ferrandiz-Huertas et al., 2014). Structurally, each TRPV1 channel subunit has six transmembrane segments (S1-S6) with a short pore-forming hydrophobic stretch between S5 and S6 (Tominaga et al., 2005; Bourinet et al., 2014) **(Figure 1.3)**. Like other TRP channels, TRPV1 has long amino and carboxyl termini, both of which are intracellular and contain multiple important sites for channel regulation by protein kinases and binding sites for scaffolding proteins (Btech et al., 2013).

There is accumulating evidence in the literature describing TRPV1 as a critical mediator of inflammatory pain (Devesa et al., 2011; Fernandes et al., 2012). Inflammatory mediators such as PGE2, bradykinin or nerve growth factors can indirectly sensitize TRPV1. Following exposure of sensory neurons to inflammatory mediators, TRPV1 becomes sensitized which consequently causes hypersensitivity to chemical and thermal stimuli to the extent that normal body temperature can be sufficient to activate nociceptors (Huang et al., 2006; Nakanishi et al., 2010).

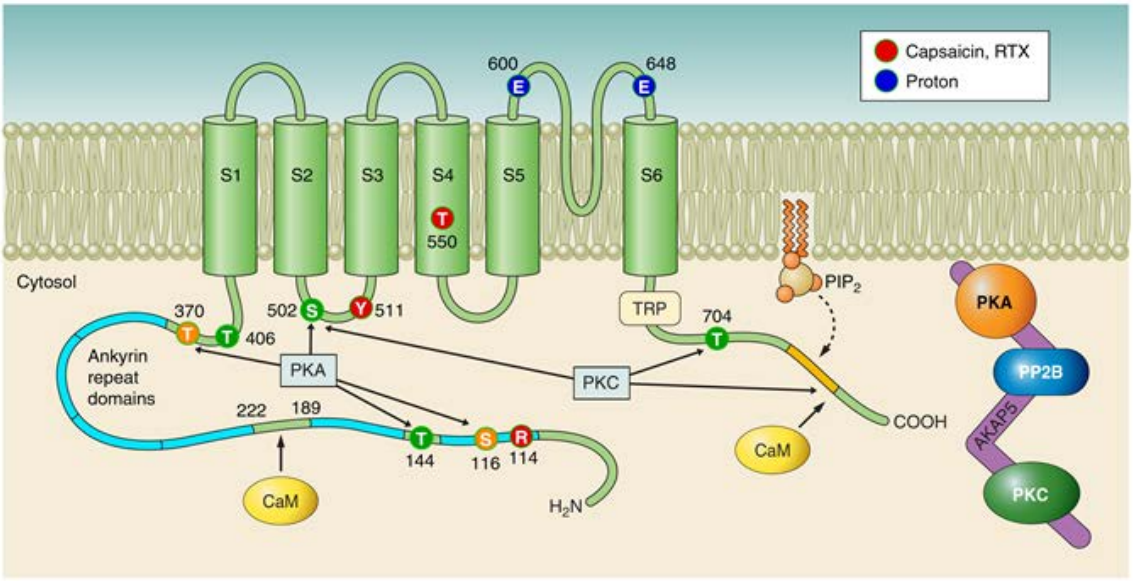


Figure 1.3 TRPV1 structure

Transient Receptor Potential Vanilloid 1 (TRPV1) topology showing its six transmembrane segments (S1-S6). Both amino and carboxyl termini contain sites important for regulating channel function. Highlighted are several key phosphorylation sites such as S502, T370, T144, S116 (PKA sites) and T704, S502, S800 (PKC sites). Adapted from The American Physiological Society Physiological Reviews (Bourinet et al., 2014). No permission was required.

Importantly, the TRPV1 channel is subjected to extensive modulation. The post-translational phosphorylation of channel subunits represents a major regulatory mechanism of TRPV1 function and trafficking (Studer et al., 2006; Lee et al., 2005). Similar to many other ion channels, there are several identified phosphorylation sites in TRPV1 that are targets for protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Winter et al., 2013). These sites are thought to be critical for channel sensitization through an increase in channel function and surface expression (Bhave et al., 2003; Moriyama et al., 2005). Along these lines, previous work has indicated that PKC can regulate TRPV1 activity either by potentiation of TRPV1 current or by reducing the desensitization of the channel, two independent regulatory mechanisms of TRPV1 (Planet et al., 2007; Mandadi et al., 2004). Moreover, desensitization is an important characteristic of the channel. Prolonged exposure to TRPV1 agonists desensitizes the channel, a process partly responsible for the decreased response to repeated application of capsaicin on primary afferent neurons (tachyphylaxis) (Koplas et al., 1997; Caterina et al., 2001; Mohapatra et al., 2003). Recently, an interesting link between the TRPV1 channel and the scaffolding protein, β-arrestin-2 has been proposed (Por et al., 2012) with β-arrestin-2 being a regulator of TRPV1 channel desensitization via a phosphorylation-dependent mechanism (Por et al., 2013).

1.4 Beta-arrestins

Beta-arrestins are anchoring/scaffolding proteins involved in the organization of signaling complexes of proteins and enzymes in close proximity to their respective substrates (Logue et al., 2010). The arrestin family is composed of four members: arrestin-1 (visual arrestin) found in retinal rods and cones, arrestin-4 (x-arrestin) found in retinal cones, and arrestins-2 and -3 (β-arrestin-1 and -2, respectively) which are ubiquitously distributed (Ma et al.,

2007). Beta-arrestins were initially identified as negative regulators of G-protein-coupled receptors (GPCR) (Lefkowitz et al., 2004). Upon ligand binding, GPCRs undergo conformational changes so that they can be phosphorylated by GPCR kinases (GRKs) which allows β-arrestins to bind the agonist-occupied receptor at the membrane. This in turn uncouples the receptor from the G protein and promotes receptor internalization and desensitization (Reiter et al., 2006). However, the current state of knowledge describes β-arrestins as scaffold proteins that guide receptor signals from the plasma membrane to different cellular destinations and facilitate various signaling cascades (Lefkowitz et al., 2005; Ritter et al., 2009).

Beta-arrestin-2 has been shown by Bohn and coworkers to desensitize the anti-nociceptive μ-opioid receptor. Using a knockout mouse model lacking β-arrestin-2, they assessed the latter's role in the modulation of pain sensation through altering opioid receptor activity (Bohn et al., 2004). Another recent study also highlighted this correlation between β-arrestin-2 and opioid receptor activity in primary afferent dorsal root ganglion (DRG) neurons (Lam et al., 2011). Interestingly, β-arrestin-2 recruitment to opioid receptors was recently shown to shift the phosphorylation pattern of extracellular signal-regulated kinases (ERK), suggesting a possible functional interaction that encompasses GPCR, β-arrestin recruitment and ERK activation (Rozenfeld et al., 2007).

Intriguingly, a link between β-arrestins and TRP channel regulation has been recently suggested by Por and coworkers who found that β-arrestin-2 regulates TRPV1 desensitization (Por et al., 2012). Activation of PKA or PKC augmented the association between TRPV1 and β-arrestin-2, thus implicating channel phosphorylation as a regulatory factor of TRPV1/β-arrestin-2 interaction (Por et al., 2013). However, the functional significance of this interaction remains

elusive and it is unknown whether it implicates downstream cellular alterations that modulate nociception.

1.5 The Extracellular Signal-Regulated Kinase

ERK is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases that represents a central pathway involved in transmitting signals from extracellular stimuli to regulate a wide array of physiological functions, such as proliferation, differentiation, cell cycle progression and nociception (Zhang et al., 2002; Avruch, 2007). Different stimuli are capable of activating the ERK pathway, including growth factors, cytokines, several GPCR ligands and carcinogens (Cruz et al., 2007). Classically, once ERK is activated; it translocates to the nucleus and in turn promotes gene transcription thus is involved in regulation of different cellular fates (Plotnikov et al., 2011). Measurement of ERK phosphorylation (p-ERK) is widely used to monitor multiple cellular cascades in which ERK is involved (Yoon et al., 2006). For instance upon an inflammatory insult; ERK is phosphorylated into p-ERK, activating a downstream multitude of signaling pathways that influence nuclear gene transcription and in turn contribute to inflammation (Zhang et al., 2014). Another instance of similar ERK pathway activation has been reported in pain and nociception (Ji et al., 2002; Lee et al., 2012; Cho et al., 2012). In this case, ERK expressed in sensory neurons is phosphorylated in response to noxious stimuli and its inhibition was proved to be antinociceptive (Chen et al., 2009). The ERK activation was shown to be mediated, at least partially, via TRPV1 signaling (Chen et al., 2009). This may suggest a possible correlation between inflammation, ERK activation and TRPV1 channels.

1.6 Rationale and hypothesis

As mentioned above, several lines of evidence highlight β-arrestins as potential key players in nociception. There is also evidence that they play a role in the activation of ERK (nociceptive marker) in association with GPCR activation (Tohgo et al., 2002; Heitzler et al., 2012). However, it is unknown whether the nociceptive TRPV1 channel operates through a similar or distinct signaling pathway.

Since the TRPV1 channel represents a key player in pain pathways, the overall goal of this thesis was to investigate whether β-arrestin-2 and p-ERK play a role downstream of TRPV1 channel activation (**Figure 1.4**). The rationale for this proposal is two-fold: I) at the beginning of this project, I was able to show that TRPV1 channel activation (using the pharmacological agonist, resiniferatoxin) elicits ERK activation; and II) recent work has described a biochemical association between TRPV1 and β-arrestin-2 (Por et al., 2012). Interestingly, my preliminary data proposed an unexpected β-arrestin-2 translocation to the nucleus subsequent to TRPV1 activation. It was within this context that we decided to study the putative functional crosstalk between TRPV1 activation, β-arrestin-2 translocation and ERK phosphorylation.

My working hypothesis states that: ***TRPV1 channel stimulation directly activates the nociceptive marker ERK in a β-arrestin-2-dependent manner.***

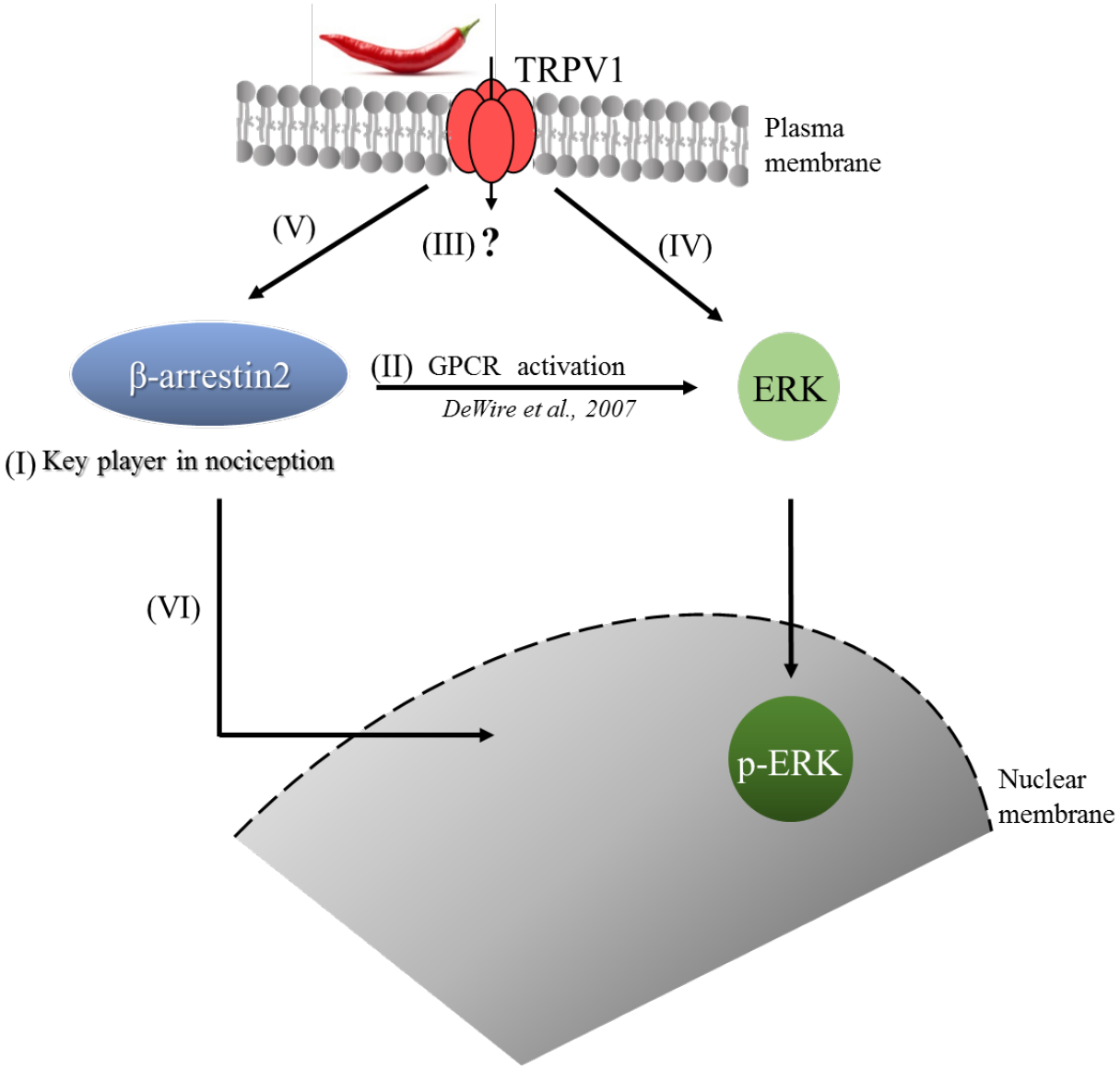

TRPV1
Plasma membrane
(V)
(III) ?
(IV)
β-arrestin2
(II) GPCR activation
DeWire et al., 2007
ERK
(I) Key player in nociception
(VI)
p-ERK
Nuclear membrane

Figure 1.4 The rationale for this study

Schematic diagram depicting the rationale for this thesis. (**I**) β-arrestin-2 has been described as a potential key player in nociception. (**II**) Several studies showed the role played by β-arrestin-2 in facilitating ERK signaling upon GPCR activation. (**III**) Such a relationship has not yet been identified in ion channels also implicated in nociception such as TRPV1. (**IV**) My preliminary results showed that ERK is phosphorylated in response to TRPV1 channel activation. (**V**) Recently, Por and his coworkers, (Por et al., 2012) described a biochemical association between the TRPV1 channel and β-arrestin-2. (**VI**) I observed β-arrestin-2 nuclear translocation following TRPV1 channel activation. Therefore, I decided to study a possible crosstalk between TRPV1 activation, ERK phosphorylation and β-arrestin-2 nuclear translocation.

1.7 Goals and specific aims

My work was focused on two specific aims:

Aim 1 To investigate the dynamics of interaction between TRPV1 and β-arrestin-2, and determine whether TRPV1/β-arrestin-2 signaling activates downstream ERK.

Hypothesis *TRPV1 channel stimulation leads to ERK phosphorylation in a β-arrestin-2-dependent manner*

The first goal of this thesis was to gain a better understanding of the molecular signaling engaged by TRPV1 channel activation, specifically the possible crosstalk between β-arrestin-2 and ERK. Several experiments were conducted to investigate the ensuing signaling cascade downstream of TRPV1 channel stimulation. Moreover, several regulatory proteins were identified to play a role downstream of the proposed TRPV1 signaling.

Aim 2 To investigate whether Ca^{2+} influx through TRPV1 channels is necessary for β-arrestin-2 nuclear translocation and ERK activation.

Hypothesis *β-arrestin-2 nuclear translocation as well as ERK activation that result from TRPV1 channel stimulation depend on extracellular Ca^{2+} entry through the channel.*

Using pharmacological tools to elicit cytosolic calcium rise, I examined the role of calcium in ERK and/or β-arrestin-2 signaling in response to TRPV1 stimulation.

Chapter Two: **Materials and Methods**

2.1 Cell culture and transfection

Human Embryonic Kidney cells (tsA-201) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), L-glutamine (Sigma) and penicillin/streptomycin antibiotics (Invitrogen). Cells were maintained at 37 ^{o}C in a 5% CO_2 incubator (Altier et al., 2011). Cells were transfected using either calcium phosphate or lipofectamine (Invitrogen) transfection reagents according to the manufactures's recommended experimental conditions. In the former (calcium phosphate transfection protocol), cells were seeded at ~40-50% confluency on the day of transfection. The $CaCl_2$/HEBS (HEPES-buffered saline solution)/DNA complex was prepared as follows: For a 35 mm dish or one well of a 6 well plate, two solutions were prepared: solution (A): 1-2 µg of desired DNA was added to 100 µl of 250 mM $CaCl_2$ and gently mixed; solution (B): 100 µl of 2X HEBS solution. Solution A was added drop-wise to solution B and left to stand at room temperature for 15 min. The DNA/$Ca_3(PO_4)_2$ mix was then added to the plate with gentle swirling. Cells were washed 6-10 hours post transfection and fresh growth medium was added. Experiments were started 48 hours post transfection. HEBS recipe: 0.28 M NaCl, 0.05 M HEPES, 1.5 M Na_2HPO_4, H_2O, pH adjusted to 7.05.

Beta-arrestin-2 siRNA was transfected using lipofectamine® 2000 according to the manufacturer's recommendations. Briefly, cells were seeded at ~80% confluency on the day of transfection. For a 35 mm dish or one well of a 6 well plate, 200 pmol siRNA in addition to 1-2 µg of DNA were diluted in 100 µl of antibiotic and serum-free medium (solution A). In another sterile tube, 6-8 µl of lipofectamine 2000 were added to 100 µl of antibiotic and serum-free

medium (solution B). Solution A was mixed gently with solution B by pipetting up and down. The mixture was incubated for 15-30 min at room temperature. Cells were then washed twice with antibiotic and serum-free medium before adding the DNA/siRNA mixture. Five to seven hours post transfection, medium was removed and fresh normal growth medium added. Cells were incubated for an additional 18 hours before replacing the medium with fresh normal growth medium. Experiments were started 24-48 hours after the last step.

2.2 Western blot assay

Two days after transfection, cells were serum-starved for 3 hours. Cells were then treated, harvested and lysed in RIPA lysis buffer (1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, diluted in PBS) supplemented with a protease and phosphatase inhibitor cocktail (Roche). Cell lysates were centrifuged at 15,000 rpm for 15 minutes, supernatants were collected and pellets were discarded. Fresh or previously frozen lysate samples were run on 12% tris-glycine gels and transferred to nitrocellulose membranes. Depending on the antibodies used, membranes were blocked in 5% BSA (Sigma) or milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween-20. pH 7.5) and incubated with primary antibodies overnight at 4 ^{o}C. After washing (3 times, 10 min each), membranes were incubated with secondary antibodies corresponding to the primary antibodies' species. Membranes were developed using ECL chemiluminescent substrates and images were obtained using Bio-Rad or Kodak Gel Chemidoc.

2.3 Confocal microscopy and imaging

For confocal imaging, cells were plated on glass bottom cell culture dishes (Matteks) coated overnight with polyornithine (Sigma). Cells were ~50% confluent on the day of

transfection. Cells were washed 6-10 hours post transfection and left to grow for 48 hours. On the day of experiment, cells were treated, washed twice and fixed with 4% paraformaldehyde (PFA) for 5 min. Images were obtained using a Fluoview (FV1000) laser scanning confocal microscope.

2.4 Immuno-staining

Cells plated on pre-coated glass bottom culture dishes and transfected with the desired DNA were left to grow for two days before the day of experiment. For p-ERK staining, cells were serum starved (3 hours), treated with RTX (10 nM, 5 or 15 min), washed and fixed using 4% PFA. Cells were then blocked (PBS+ 1% FBS) and permeabilized with 0.1% Triton-X100 for 30 min. Samples were incubated (18 hours, room temperature) in diluted anti-p-ERK rabbit antibody (1: 500) in block solution supplemented with 1% BSA. After washing, cells were incubated with Alexa-488-conjugated secondary anti-rabbit IgG (1 hour, room temperature). Confocal images were obtained using Fluoview (FV1000) confocal microscopy. For HA-Ras staining, the same protocol was used; however, the anti-HA rat primary antibody (Roche) was used at 1: 1000 (1 hour, room temperature). Rat-FITC secondary antibody was used for 1 hour at room temperature before images were obtained.

2.5 Co-immunoprecipitation of β-arrestin-2

Cell lysates were prepared using the same western blotting protocol described above. Lysates were then added to protein A and protein G beads mixture (ratio 1:1, GE Health Care), incubated with β-arrestin-2 antibody and left to rotate overnight at 4 ^{o}C. Lysates with beads were later centrifuged (2000 rpm, 1 min) and washed 3 times in RIPA buffer. Lysis buffer was

discarded and pellets were re-suspended in 60 µl RIPA+20 µl gel loading buffer. Fresh samples were finally run on SDS-PAGE 12% gels and transferred to nitrocellulose membranes. Membranes were incubated with ERK/p-ERK/GFP primary antibodies followed by the secondary antibody of choice. Since β-arrestin-2 and p-ERK antibodies are both of the same species (mouse), we used Mouse TrueBlot Ultra secondary antibody to overcome the detection of immunoprecipitating immunoglobulin heavy and light chains.

2.6 Plasmids and phosphorylation mutants

TRPV1 and TRPA1-mCherry tags were produced by cloning the TRPV1 and TRPA1 sequences into sticky-ended annealed oligonucleotides coding for mCherry. Beta-arrestin-2-YFP tag was made by cloning its sequence into sticky-ended annealed oligonucleotides coding for YFP. Beta-arrestin-1-YFP was purchased from Addgene plasmid repository. HA-tagged Ras was purchased from Missouri S& T cDNA Resource Center. PKC-βII-GFP was generously provided by Dr. Stephen S. G. Ferguson. Site-directed mutagenesis of TRPV1-calmodulin (CaM) binding sites (K155, W787) was achieved by converting lysine (K) or tryptophan (W) into alanine (A) to inhibit CaM binding. These mutants were previously prepared in our laboratory.

2.7 Subcellular fractionation

Separation of cytoplasmic and nuclear fractions was achieved using the Rapid, Efficient and Practical (REAP) protocol (Suzuki et al., 2010). Briefly, culture media were removed from cell culture dishes and cells were washed twice using ice-cold PBS. One ml of PBS was added to each 10 cm dish, cells were scraped and collected in microcentrifuge tubes. Samples were centrifuged (10 s, 2000 x g) and supernatants were discarded. One ml of 0.1% NP40 was added

to each pellet with pipetting up and down several times. Small aliquots were set aside as whole cell lysates. The rest of the samples were centrifuged (10 s) and supernatants were transferred to new tubes as the cytoplasmic fractions. The pellet portions were re-suspended in 1 ml 0.1% NP40 and centrifuged (10 s, 2000 x g). After discarding the supernatants, the nuclear fractions were obtained. Before running samples on SDS-PAGE, whole lysates were mixed with 4X Laemmli buffer (ratio 3:1), sonicated on ice at level 2 twice (5 min) and boiled (4 min). Additionally, nuclear fractions were re-suspended in 200 µl of 1X Laemmli buffer, sonicated on ice twice (5 min) and boiled (4 min). Cytoplasmic fractions were mixed with 4X Laemmli buffer (ratio 3:1) and boiled (4 min). Samples were then run on 12% gels. 0.1% NP40: 10X PBS, 10% NP40. 4X Laemmli buffer: 0.25% 0.25M Tris-HCl (pH 6.8), 20% β-mercaptoethanol, 8% SDS, 20% glycerol, 0.008% bromophenol blue.

2.8 Bioluminescence Resonance Energy Transfer (BRET) assay

HEK cells were co-transfected with TRPV1-Rluc/β-arrestin-2-YFP or TRPV1-Rluc/TRPV1-YFP (positive control). Forty eight hours after transfection, cells were washed in PBS and detached in Versene solution (PBS+10 mM EDTA). Cells in Versene were centrifuged at 2000 rpm (5 min), supernatant discarded and pellets collected. Pellets were then re-suspended in 400 µl PBS and plated in a black 96-well plate (Greiner Bio-one) to measure the total YFP fluorescence or a white 96-well plate (Brand) for BRET assay. Total YFP fluorescence was measured in a Victor 2 plate reader (Perkin Elmer) using an excitation filter of 485 nm and emission filter of 530 nm. BRET signal was measured in a Mithras LB940 (Berthold Technologies) after incubating the cells with the Rluc substrate, coelanterazine-h (5 µM, 5 min). BRET measurements were calculated as the ratio of YFP emission (530 nm) to the Rluc

emission (460 nm). BRET signal was expressed as net BRET, which is the signal from YFP/Rluc minus the signal from Rluc alone. In the BRET saturation curve, the concentration of transfected TRPV1-Rluc was kept constant (50 ng/well), while YFP ratio was increased (from 100 ng to 1.5 µg).

2.9 Chemicals and drugs

Phospho-CaMK-II, p-44/42 MAPK (ERK 1/2), phospho-p44/42 MAPK (ERK 1/2) and β-arrestin-2 rabbit antibodies were purchased from Cell Signaling. A second β-arrestin-2 mouse antibody (immunoprecipitation experiment), GAPDH antibody in addition to β-arrestin-2 siRNA were purchased from Santa-Cruz Biotechnology. The TRPV1 agonists, RTX and capsaicin, were obtained from Sigma-Aldrich. The MAPK inhibitor (U-0126) was bought from Cell Signaling. The PKC inhibitor (CGP53353) was generously supplied by Dr. Pierre-Yves von der Weid, and GF109203X was purchased from Tocris bioscience. Secondary anti-mouse and anti-rabbit IgG were purchased from GE Healthcare. Coelenterazine-h was ordered from ProLume, while the protease and phosphatase inhibitor cocktails were purchased from Roche. Mouse TrueBlot Ultra secondary anti-mouse IgG was purchased from Rockland antibodies and assays. Phosphorylated p38, p38, JNK as well as phosphorylated JNK antibodies (Cell Signaling) were kind gifts from Dr. Morley Hollenberg. We obtained β-arrestin-2 CRISPR cells as a kind gift from Dr. Rithwik Ramachandran. A23187 was obtained from Sigma-Aldrich, ionomycin from Alomone Labs and the anti-GFP antibody from Torrey Pines Biolabs. Finally, the Amersham ECL select western blotting reagents were ordered from GE Life Sciences.

2.10 Statistical analysis:

Results were expressed as means ± S.E.M. Where appropriate, analysis of variance (ANOVA) followed by Dunnett's *post hoc* test or unpaired t-tests were performed using GraphPad Prism 6. Data were considered statistically significant if P value was ≤ 0.05.

Chapter Three: **Results**

3.1 TRPV1 channel activation induces β-arrestin-2 nuclear translocation

To investigate whether TRPV1 was able to signal to β-arrestin-2, I examined the effect of TRPV1 activation in a recombinant system. HEK cells co-transfected with TRPV1-mCherry and β-arrestin-2-YFP were treated with the potent TRPV1 agonist resiniferatoxin (RTX, 10 nM) for 5, 10 or 15 min. Cells were fixed and imaged using confocal microscopy. Following TRPV1 activation, significant β-arrestin-2 translocation to the nucleus was observed while the TRPV1 channel remained stably anchored at the plasma membrane (**Figure 3.1.A**). Interestingly, this finding is in stark contrast to the current understanding of GPCRs, according to which β-arrestin translocates to the activated receptor at the membrane (Ferguson et al., 2004). This nuclear translocation was time dependent and occurred in ~30% (5 min), ~45% (10 min) and ~80% (15 min) of the TRPV1-expressing cells (**Figure 3.1.B**).

Since live-cell imaging provides more reliable information, being less prone to experimental artifacts, I decided to test our previous findings by conducting live-cell imaging experiment. As shown in **Figure 3.1.C**, the nuclear translocation of β-arrestin-2 was observed in cells co-transfected with TRPV1 channel and β-arrestin-2-YFP. Interestingly, β-arrestin-2 translocation was irreversible; washing with PBS or HBSS did not reverse the observed nuclear translocation. Control experiments were conducted using HEK cells transfected with β-arrestin-2-YFP but not TRPV1. Application of RTX for 15 min did not evoke any detectable translocation of β-arrestin-2 (**Figure 3.1.D**), a finding consistent with a crucial role for TRPV1 activation in the subsequent β-arrestin-2 nuclear translocation. Finally, translocation of β-arrestin-2 to the nucleus was confirmed by employing a nuclear stain that co-localized with the fluorescent β-arrestin-2 signal (**Figure 3.1.E**).

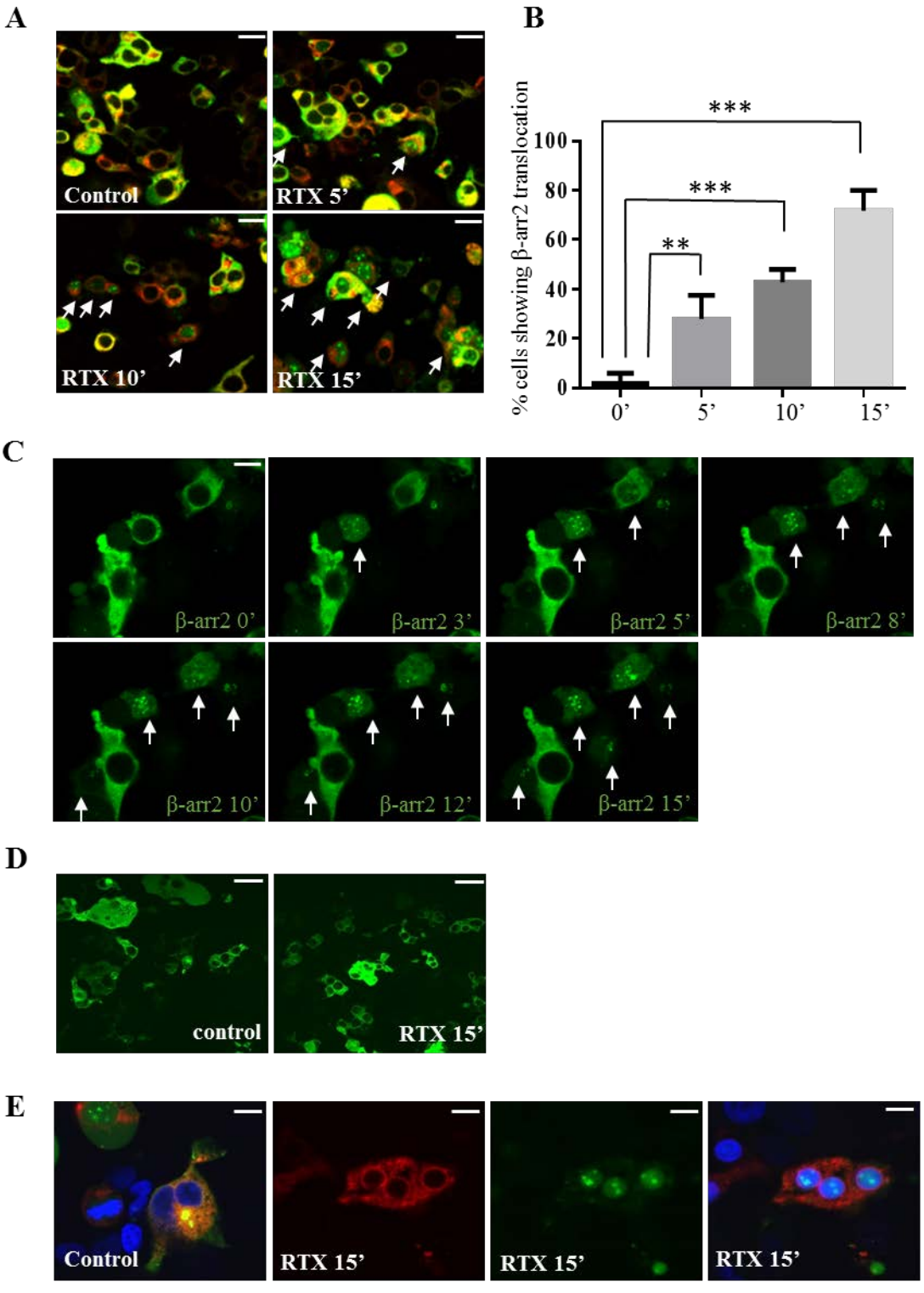

A
Control
RTX 5'
RTX 10'
RTX 15'
B
% cells showing β-arr2 translocation
***
***
**
100
80
60
40
20
0
0'
5'
10'
15'
C
β-arr2 0'
β-arr2 3'
β-arr2 5'
β-arr2 8'
β-arr2 10'
β-arr2 12'
β-arr2 15'
D
control
RTX 15'
E
Control
RTX 15'
RTX 15'
RTX 15'

Figure 3.1 TRPV1 channel activation triggers β-arrestin-2 nuclear translocation

(A) Representative confocal images showing β-arrestin-2 nuclear translocation in response to TRPV1 channel activation by RTX (10 nM). TRPV1-mCherry appears in *red* and β-arrestin-2-YFP in *green. (Scale bar = 20 µm).* **(B)** Percentage of cells showing β-arrestin-2 (β-arr2) nuclear translocation following RTX treatment (10 nM) at 5, 10 or 15 *min (n = 10 fields per condition, RTX 0 min 2 ± 2, RTX 5 min 27.9 ± 4.7, RTX 10 min 42.7 ± 2.3, RTX 15 min 71.7 ± 2.6 p: ** <0.001, *** <0.0001).* **(C)** Confocal live cell imaging showed β-arrestin-2 nuclear translocation in response to RTX (10 nM). *(Scale bar = 10 µm).* **(D)** Cells transfected with β-arrestin-2-YFP alone and treated with RTX (10 nM, 15 min) did not exhibit nuclear β-arrestin-2 translocation. *(Scale bar = 35 µm).* **(E)** Nuclear Hoechst blue staining confirming β-arrestin-2 (*green*) translocation to the nucleus. *(Scale bar = 10 µm).*

The classic TRPV1 agonist, capsaicin, was used in another set of experiments to confirm that β-arrestin-2 translocation depends on TRPV1 channel activation. **Figure 3.2.A** illustrates the nuclear pattern of β-arrestin-2 localization in response to capsaicin treatment (1 μM, 15 min), indicating that TRPV1-induced translocation of β-arrestin-2 was not restricted to a single modality of channel activation. In addition, I tested if this effect was TRPV1 specific by using cells co-transfected with another calcium permeable TRP channel, the Transient Receptor Potential Ankryin 1 (TRPA1), along with β-arrestin-2-YFP. Activation of the TRPA1 by the classic agonist, mustard oil (100 μM for 15 min), failed to show any detectable changes in β-arrestin-2 cellular localization (**Figure 3.2.B**), confirming the specificity of TRPV1 channels in triggering this effect.

The aforementioned observation of TRPV1-mediated nuclear translocation of β-arrestin-2 reveals a new and opposite pattern compared to the one triggered by classical GPCR activation in which case β-arrestin-2 translocates to the membrane-bound receptor (Ma et al., 2007). To test whether β-arrestin-2 could rapidly but transiently interact with the TRPV1 channel prior to nuclear translocation, I used a bioluminescence resonance energy transfer (BRET) technique (employed to assess protein-protein interaction). In this experiment, two conditions were used: 1) a positive BRET control in which cells were transfected with TRPV1-YFP and TRPV1-Rluc; and 2) a condition in which cells were transfected with β-arrestin-2-YFP and TRPV1-Rluc. **Figure 3.2.C** shows the presence of a positive BRET signal in the control condition (condition 1) that represents the assembly between TRPV1 subunits (two or more subunits), whereas no interaction (BRET signal) was detected in the second condition (condition 2, **Figure 3.2.C**). Furthermore, measuring the BRET signal at saturation revealed that activating TRPV1 channels

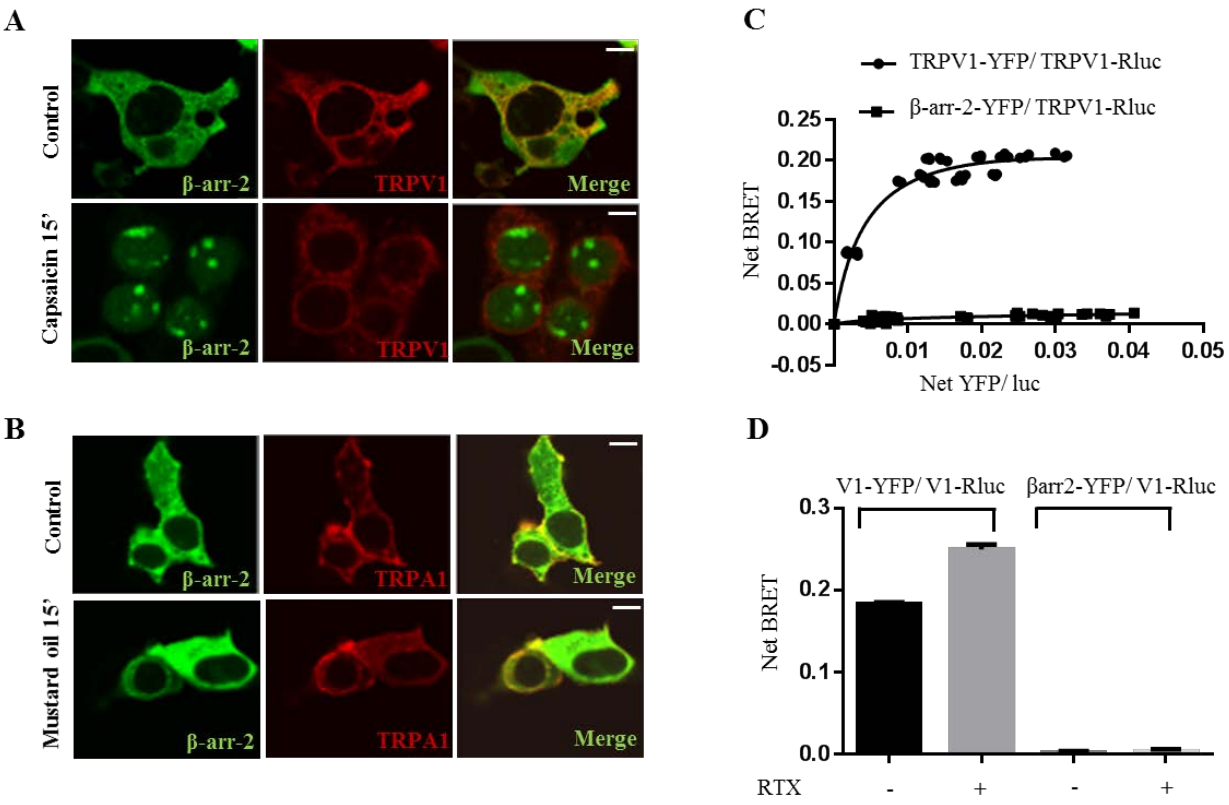

A
Control
Capsaicin 15'
β-arr-2
TRPV1
Merge
B
Control
Mustard oil 15'
β-arr-2
TRPA1
Merge
C
TRPV1-YFP/ TRPV1-Rluc
β-arr-2-YFP/ TRPV1-Rluc
Net BRET
Net YFP/ luc
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0.01
0.02
0.03
0.04
0.05
D
V1-YFP/ V1-Rluc
βarr2-YFP/ V1-Rluc
Net BRET
0.3
0.2
0.1
0.0
RTX
-
+
-
+

Figure 3.2 β-arrestin-2 nuclear translocation is TRPV1 activation specific

(A) Confocal images showing cells transfected with TRPV1-mCherry (*red*) and β-arrestin-2-YFP (*green*). Treatment with capsaicin (TRPV1 agonist, 1 μM) induced β-arrestin-2 nuclear translocation. *(Scale bar = 10 μm).* **(B)** Activation of TRPA1-mCherry (*red*) using mustard oil (100 μM, 15 min) did not alter β-arrestin-2 cellular localization. *(Scale bar = 10 μm).* **(C)** BRET saturation curve of cells co-transfected with increasing amounts of TRPV1-YFP or β-arrestin-2-YFP and a constant amount of TRPV1-Rluc (the energy donor), denoting the specificity of BRET signal. Saturation curve between TRPV1-YFP and TRPV1-Rluc indicated specific TRPV1 subunits interaction, while the low linear BRET signal denoted non-measurable interaction between β-arrestin-2-YFP and TRPV1. **(D)** Activation of TRPV1 channel (RTX, 10 nM 5 min) induced an increase in net BRET signal *(BRET signal – background BRET from TRPV1-RLuc alone*) in cells transfected with TRPV1-YFP/TRPV1-Rluc. However, cells transfected with β-arrestin-2-YFP/TRPV1-Rluc did not display a detectable BRET signal under basal or treated conditions. *(n=2 ,V1-YFP/V1Rluc 0.18 ± 0.0007, V1-YFP/V1-Rluc + RTX 0.24 ± 0.002, β-arr2-YFP/V1-Rluc 0.001 ± 0.0006, β-arr2-YFP/V1-Rluc + RTX 0.002 ± 0.001).*

using RTX enhanced the BRET signal only in the control condition (condition 1), where RTX induced conformational changes in TRPV1 subunits, eliciting the increase in BRET signal. However, cells co-transfected with β-arrestin-2-YFP/TRPV1-Rluc (condition 2) did not show any increase in BRET signal (**Figure 3.2.D**) suggesting that no protein-protein interaction occurs at rest or in response to TRPV1 stimulation.

3.2 Distinctive cellular localization of β-arrestin-1 following TRPV1 activation

To test whether the observed effect is specific to β-arrestin-2, I used HEK cells transfected with β-arrestin-1-YFP alone or TRPV1-mCherry and β-arrestin-1-YFP. In contrast to β-arrestin-2, β-arrestin-1 displayed a basal nuclear expression irrespective of the absence (**Figure 3.3.A**) or presence of TRPV1 (**Figure 3.3.B**). Activation of TRPV1 by RTX (10 nM, 15 min) triggered translocation of cytosolic β-arrestin-1, further increasing its nuclear presence (**Figure 3.3.C**). However, β-arrestin-1 did not exhibit the punctate expression pattern observed for β-arrestin-2 (**Figures 3.1.A** and **E** and **3.2.A**).

3.3 TRPV1 activation elicits ERK phosphorylation

Knowing that there is an established link in the literature between the scaffolding β-arrestin proteins and ERK (Shenoy et al., 2006), I tested whether TRPV1 channel stimulation was able to mediate ERK activation. To examine this possibility, I assessed phosphorylated ERK (p-ERK) using western blot analysis in TRPV1-transfected HEK cells following TRPV1 activation by RTX (**Figure 3.4.B**), capsaicin (**Figure 3.4.C**) as well as low pH (**Figure 3.4.D**), three distinct modalities of channel activation (see Introduction). As shown in **Figure 3.4.D**, the amount of p-ERK increased following TRPV1 activation. Interestingly, the effect was time-dependent and peaked at 5 minutes post channel stimulation. As illustrated in **Figure 3.4.A**, the

activation of p-ERK did not occur in untransfected cells (**Figure 3.4.A**) or cells that were not exposed to agonists or low pH (**Figure 4.4.B-D**). Importantly, the amount of total ERK remained unchanged regardless of whether the cells were stimulated or not (**Figure 3.4.A-D**). In support of our western blot results, immunohistochemical analysis of HEK cells transfected with TRPV1-mCherry shows remarkable nuclear p-ERK punctate staining at 5 minutes following RTX-induced activation of the channel (**Figure 3.4.E**).

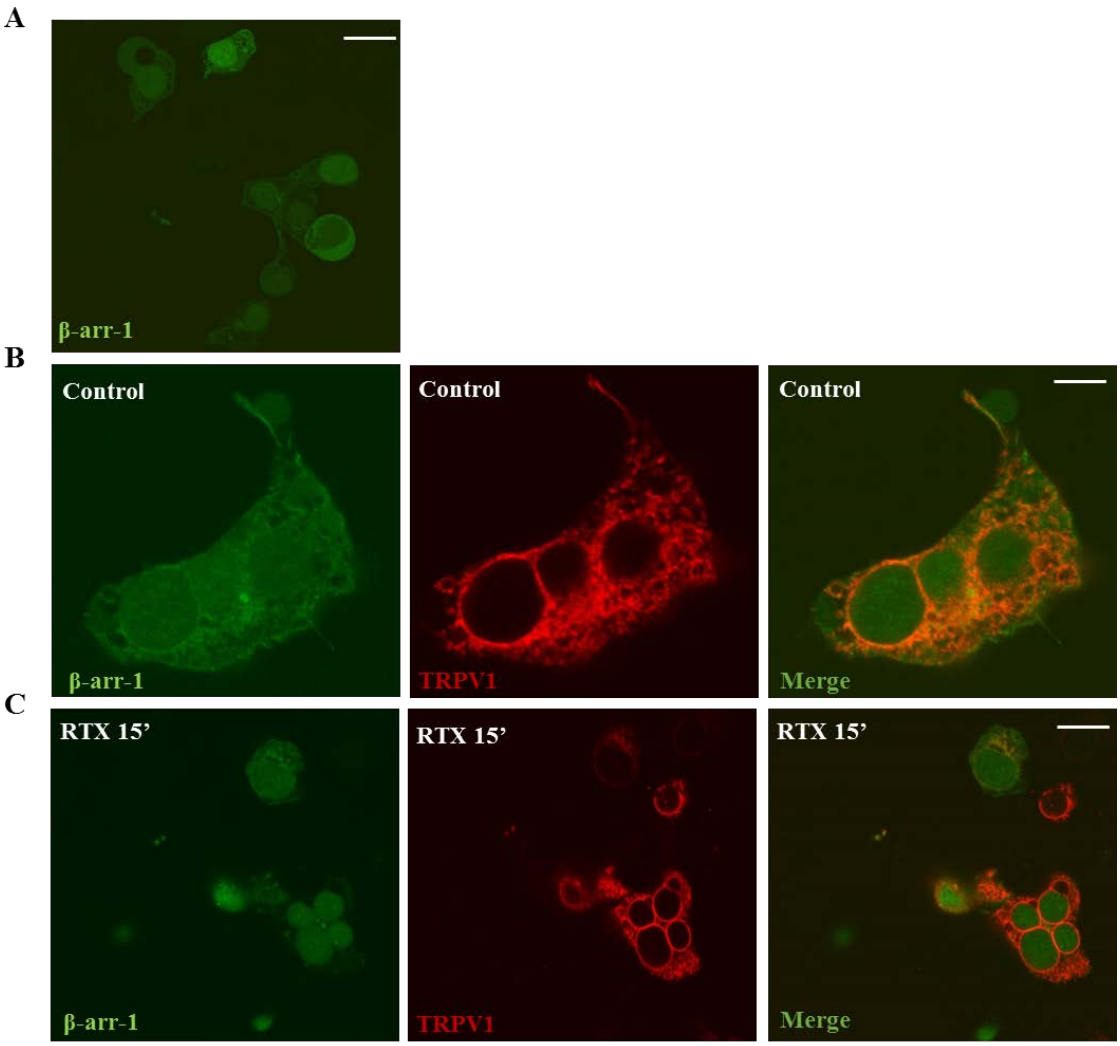


Figure 3.3 TRPV1 activation induced β-arrestin-1 nuclear translocation

(A) In cells transfected with β-arrestin-1-YFP (*green*), β-arrestin-1 was expressed in the nucleus as well as the cytosol. *(Scale bar = 30 µm).* **(B)** Images of control untreated cells transfected with TRPV1-mCherry (*red*) in addition to β-arrestin-1-YFP (*green*). Beta-arrestin-1 did not exhibit any rearrangement when cells were co-transfected with TRPV1-mCherry. *(Scale bar = 10 µm).* **(C)** Activating TRPV1 (RTX, 10 nM, 15 min) evoked further β-arrestin-1 nuclear translocation. *(Scale bar = 30 µm).*

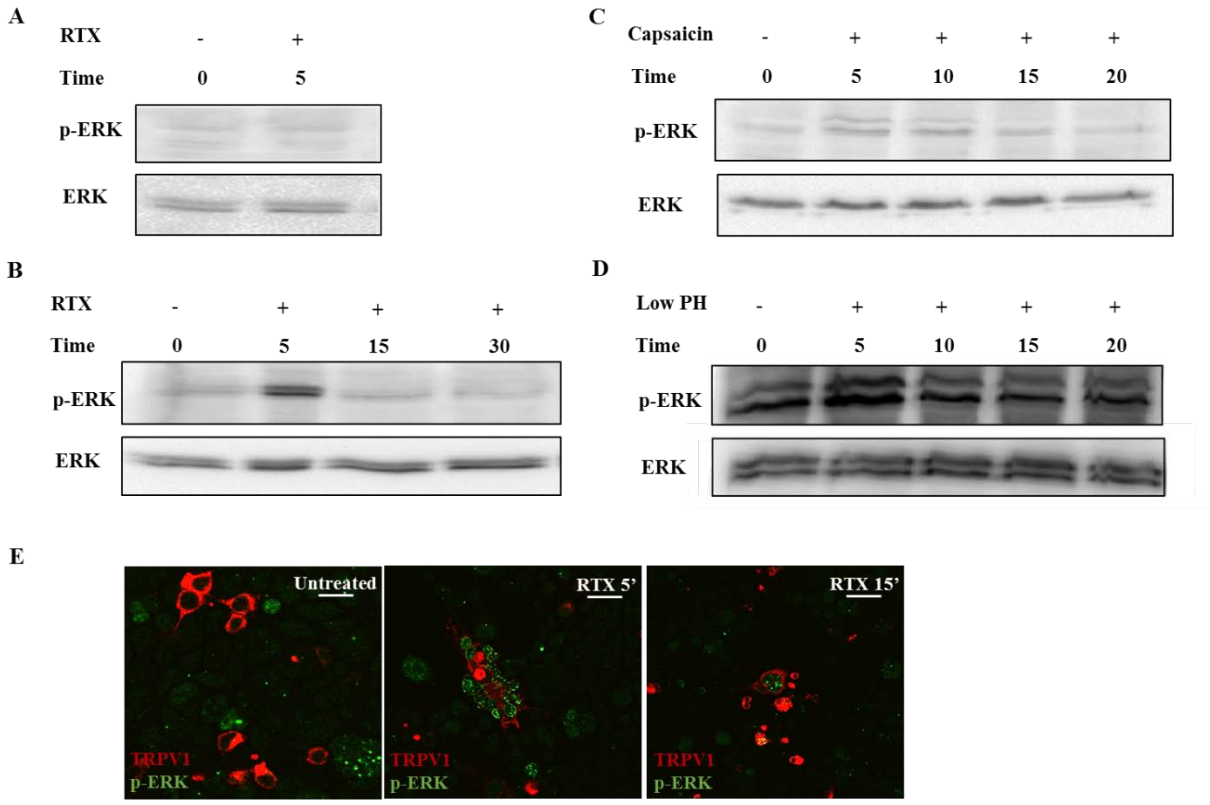


Figure 3.4 TRPV1 activation elicits ERK phosphorylation

(A) Western blot of control untransfected HEK cells in the absence or presence of RTX (100 nM). The TRPV1 agonist RTX had no effect on ERK phosphorylation *(n = 2).* **(B)** Activation of TRPV1 using RTX (100 nM) led to ERK phosphorylation as indicated by the enhanced p-ERK signal. Peak of phosphorylation was observed 5 min post treatment *(n = 3).* **(C)** Capsaicin (1 µM) elicited ERK phosphorylation (maximum effect at 5 min) *(n = 2).* **(D)** Increase in p-ERK band intensity was observed 5 min following TRPV1 activation using an extracellular acidic solution (pH 6) *(n = 2).* **(E)** Immunostaining shows an increase in p-ERK signal 5 min subsequent to TRPV1 activation (RTX, 10 nM) in TRPV1-mCherry transfected cells. (*Scale bars: untreated 20 µm, RTX 5 and 15 min 45 µm).*

3.4 Beta-arrestin-2 translocation does not depend on ERK phosphorylation

Because my data indicate that TRPV1 channel activation triggers not only β-arrestin-2 nuclear translocation but also ERK phosphorylation, I tested whether the phosphorylation of ERK is an upstream event that is necessary for the translocation of β-arrestin-2. In particular, I started by testing if the suppression of ERK activation prior to TRPV1 channel stimulation would be sufficient to inhibit β-arrestin-2 translocation. Indeed, pre-treating cells with the MEK/ERK inhibitor U0126 (10 μM, 30 min) proved sufficient to block TRPV1-mediated ERK phosphorylation (**Figure 3.5.A**), but did not affect TRPV1-induced β-arrestin-2 nuclear translocation (**Figure 3.5.B, C**). This confirms that β-arrestin-2 translocation occurs independently of ERK phosphorylation.

3.5 Beta-arrestin-2 is required for ERK phosphorylation

I then tested whether ERK activation/phosphorylation depends on the presence of β-arrestin-2. I knocked down β-arrestin-2 expression, using an siRNA approach, and assessed the effect on ERK phosphorylation using western blot analysis. At 200 pmol, β-arrestin-2 siRNA proved sufficient to suppress endogenous and over-expressed β-arrestin-2 (**Figure 3.6.A**). Interestingly, cells co-transfected with TRPV1 and β-arrestin-2 siRNA (200 pmol) showed a decreased level of p-ERK in response to RTX compared to cells transfected with TRPV1 alone (**Figure 3.6.B**). Similar results were obtained when RTX was replaced by capsaicin as a TRPV1 agonist (**Figure 3.6.C**). Another approach used was to transfect TRPV1 in a cell line deprived of β-arrestin-2 (β-arrestin-2 CRISPR cells). Not surprisingly, p-ERK was undetectable after TRPV1 activation was induced by RTX (100 nM, 5 min) (**Figure 3.6.D**). These results strongly support a role for β-arrestin-2 in ERK activation following TRPV1 channel stimulation.

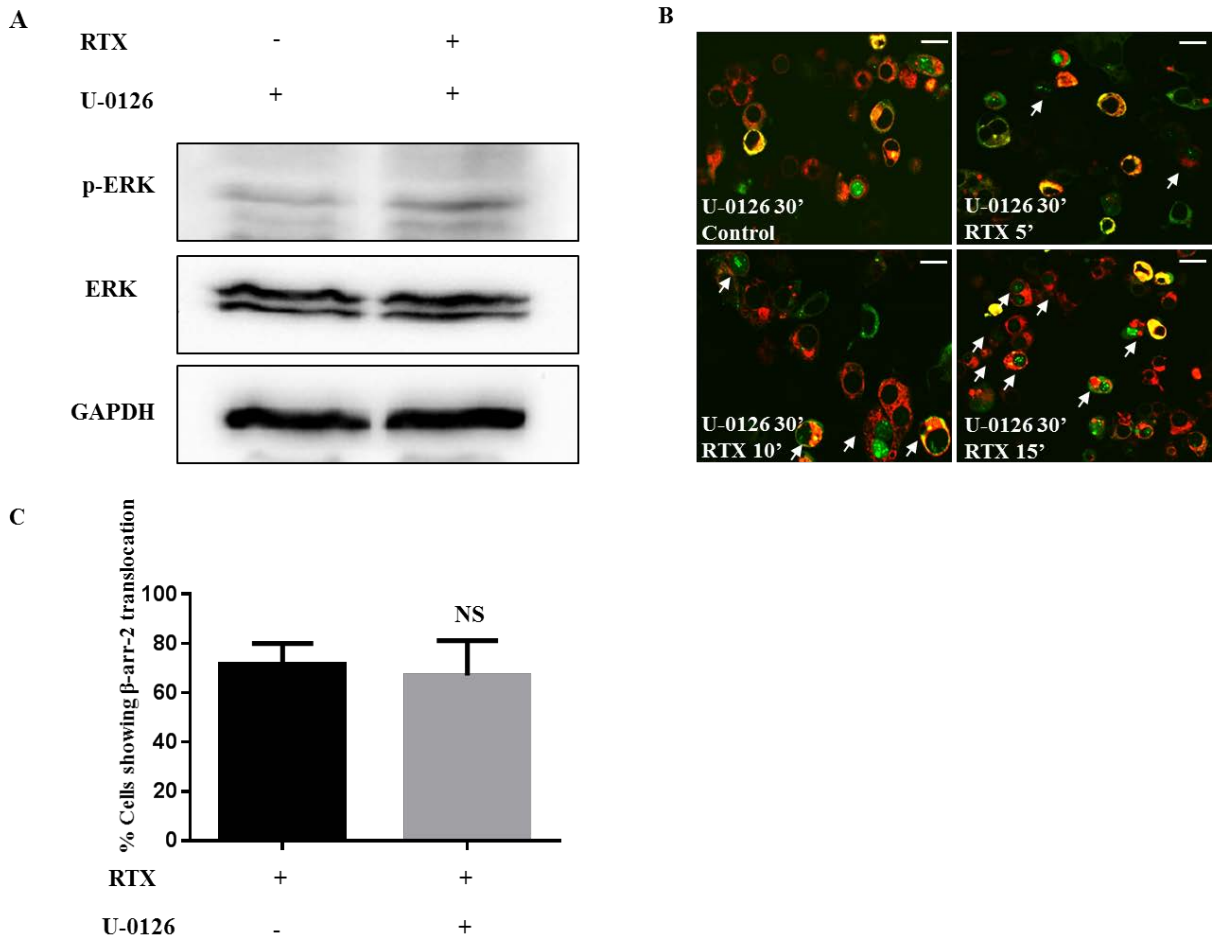


Figure 3.5 Blockade of ERK phosphorylation does not affect β-arrestin-2 translocation

(A) Western blot shows that pre-incubation of cells with 10 µM of the MEK/ERK inhibitor (U-0126, 30 min) was sufficient to block ERK phosphorylation after stimulating the channel with RTX (100 nM, 5 min) *(n = 3).* **(B)** Confocal images of TRPV1-mCherry *(red)*/β-arrestin-2-YFP *(green)* co-transfected cells. Cells were pretreated with U-0126 for 30 min before activating the channel with RTX (10 nM) at 5, 10 or 15 min; this blockade did not affect β-arrestin-2 nuclear translocation. *(Scale bar 20 µm).* **(C)** Percentage of cells showing β-arrestin-2 nuclear translocation in response to RTX treatment in the absence or presence of U-0126. *(Control RTX 71.7 ± 2.6, RTX + U-0126 67 ± 4.4. n = 10 fields per condition).*

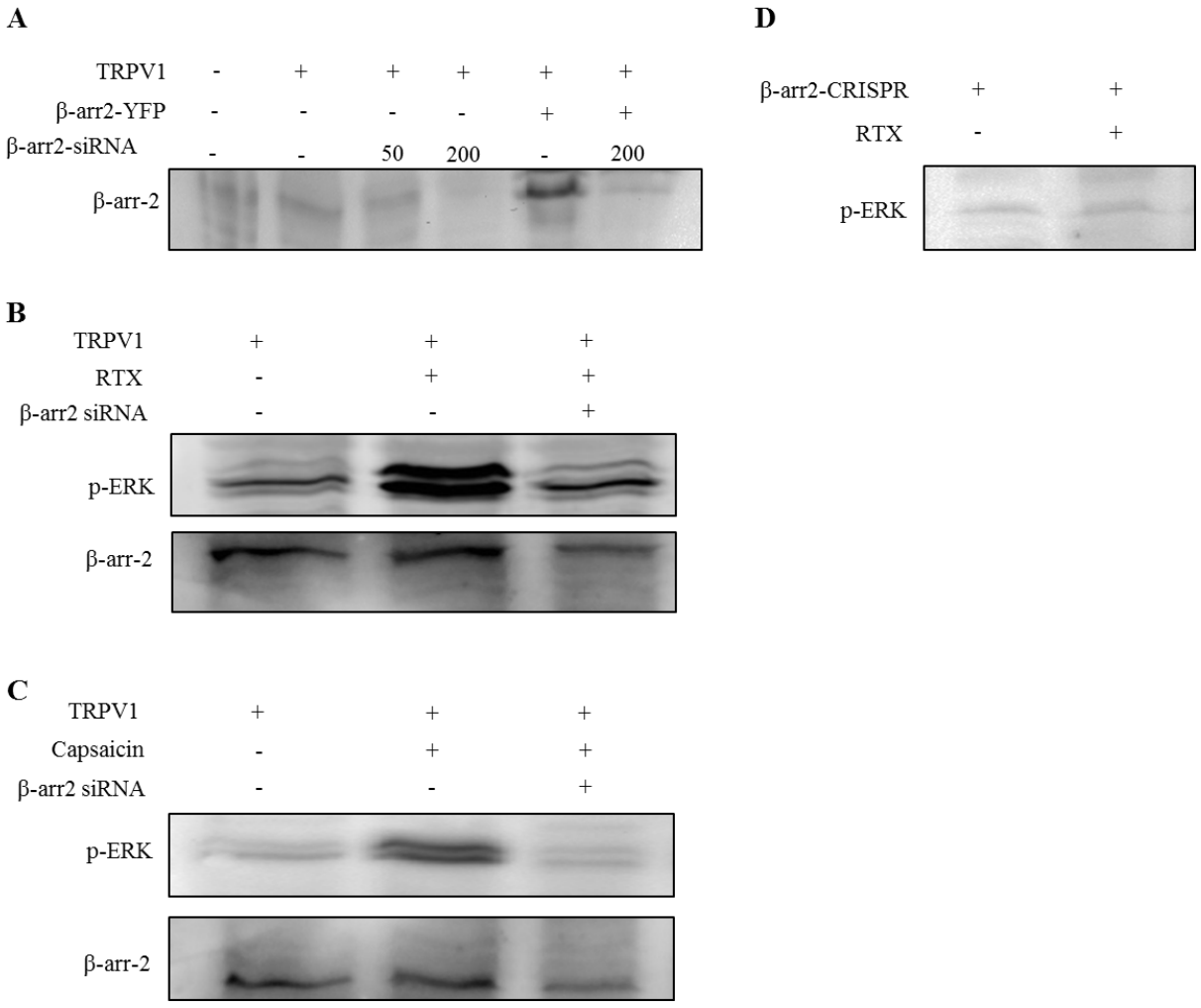


Figure 3.6 Knock down of β-arrestin-2 inhibits ERK phosphorylation

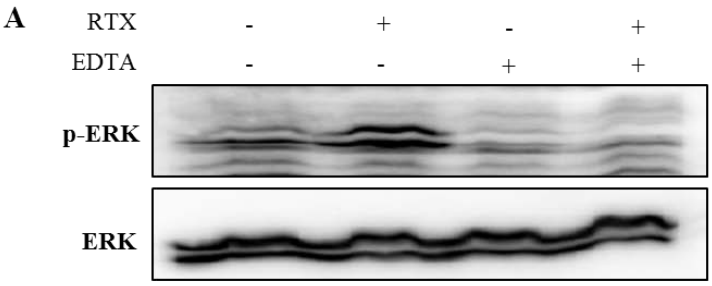
(A) Western blot showing the decrease in endogenous and over-expressed β-arrestin-2 using 200 pmol of the siRNA *(n = 1).* **(B)** Knock down of β-arrestin-2 inhibited the expected increase in p-ERK in response to TRPV1 activation using RTX (100 nM, 5 min) *(n = 3).* **(C)** Capsaicin (1 μM, 5 min) treatment of cells co-transfected with TRPV1 and β-arrestin-2 siRNA did not induce ERK phosphorylation *(n = 1).* **(D)** β-arrestin-2 CRISPR cells transfected with TRPV1 and treated with RTX (100 nM, 5 min) did not show any p-ERK bands *(n = 2).*

3.6 ERK phosphorylation and β-arrestin-2 nuclear translocation depend on calcium influx through TRPV1 channels

Although TRPV1 is a non-selective cation channel, it shows high permeability to Ca^{2+} (Caterina et al., 1997). To verify whether ERK activation and β-arrestin-2 translocation rely on Ca^{2+} influx through TRPV1 channels, we tested the effect of removing extracellular Ca^{2+}. Using 10 mM EDTA or EGTA to chelate extracellular Ca^{2+} we found an inhibition of ERK phosphorylation following TRPV1 activation (**Figure 3.7.A, B**). Furthermore, cells co-transfected with TRPV1-mCherry/β-arrestin-2-YFP and treated with RTX (10 nM, 15 min) did not exhibit detectable nuclear translocation in the presence of EGTA (**Figure 3.7.C, D**).

To further characterize the source of calcium needed for ERK activation and β-arrestin-2 nuclear translocation, I treated TRPV1-expressing HEK cells with either A-23187 (1μM, 5 min) or ionomycin (10 μM, 5 min), ionophores highly selective for calcium. Surprisingly, both failed to induce ERK phosphorylation at 5 min post treatment (**Figure 3.8.A**). The A-23187 ionophore also did not induce β-arrestin-2 translocation in cells co-transfected with TRPV1-mCherry and β-arrestin-2-YFP (**Figure 3.8.B, C**). A transient rise in intracellular [Ca^{2+}] can be evoked by the activation of protease-activated receptors (e.g. PAR2), which are endogenously expressed in HEK cells (McGuire et al., 2004). Upon activation by PAR2 agonist, Gq-coupled signaling triggers the depletion of calcium stores through the activation of IP_3 receptors (Kim et al., 2008). Therefore, we tested whether a PAR2-induced increase in cytosolic [Ca^{2+}] could mediate ERK activation. Unexpectedly, I found that the PAR2 agonist 2-furoyl-LIGRLO-amide (2fli, 5 μM, 5 min) failed to increase p-ERK (**Figure 3.8.D**) or to induce nuclear translocation of β-arrestin-2 (cells transfected with TRPV1-mCherry/ β-arrestin-2-YFP) (**Figure 3.8.E, F**).

These results strongly support not only that there is a calcium-dependence of ERK activation and β-arrestin-2 translocation, but also that the Ca^{2+} influx must be driven through TRPV1 channels to engage this signaling pathway.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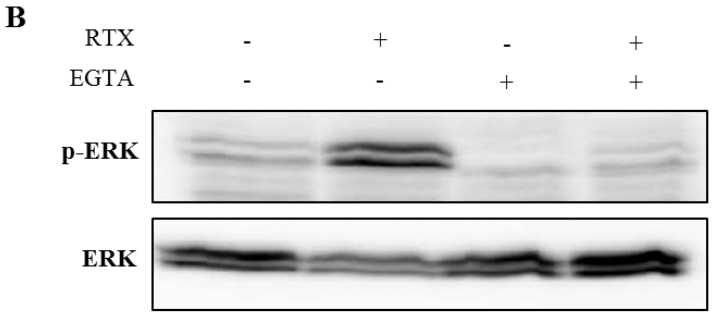
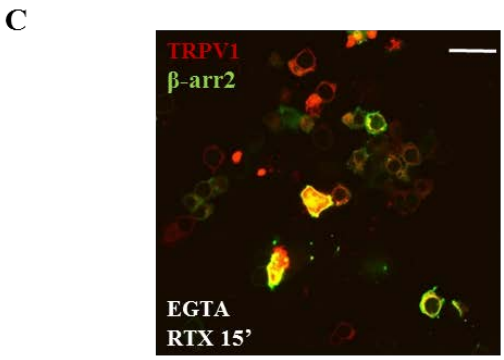
RTX - + - +
EDTA - - + +
p-ERK
ERK


B
RTX - + - +
EGTA - - + +
p-ERK
ERK


C
TRPV1
β-arr2
EGTA
RTX 15'

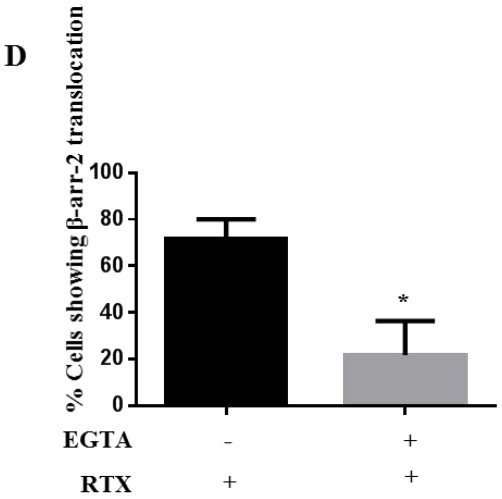

D
% Cells showing β-arr-2 translocation
100
80
60
40
20
0
*
EGTA - +
RTX + +

Figure 3.7 Extracellular calcium is necessary for TRPV1-mediated ERK activation and β-arrestin-2 nuclear translocation

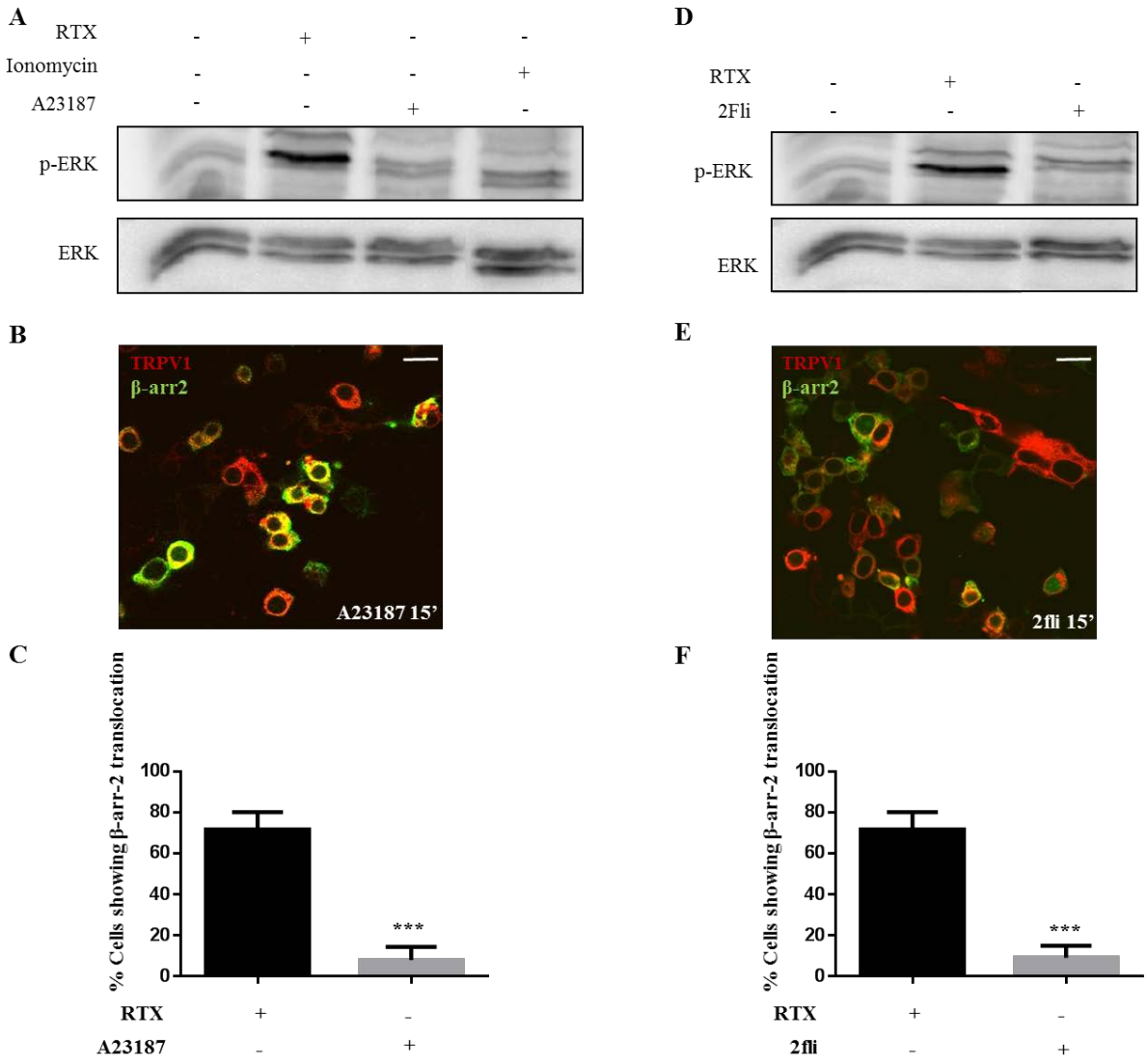
(A) Chelation of Ca^{2+} using 10 mM EDTA prevented the increase in ERK phosphorylation induced by RTX treatment (100 nM, 5 min) *(n = 2).* **(B)** Activation of TRPV1 channels using RTX (100 nM, 5 min) in the presence of 10 mM EGTA did not elicit ERK phosphorylation *(n = 2).* **(C)** Confocal image of TRPV1-mCherry/ β-arrestin-2-YFP transfected cells treated with RTX (10 nM, 15 min) in the presence of EGTA. β-arrestin-2 did not display nuclear translocation. *(Scale bar 45 µm).* **(D)** Bar graph showing percentage of cells exhibiting β-arrestin-2 nuclear translocation in response to RTX (10 nM, 15 min) in the absence or presence of EGTA (10 mM) *(RTX control 71 ± 2.6, RTX+ EGTA 21.8 ± 10 P: * <0.0001).*

3.7 Protein Kinase C (PKC) is activated upon TRPV1 channel stimulation

Protein kinase C (PKC) is another kinase linked to activation of the MEK/ERK pathway (Ueda et al., 1996) which also shows calcium-dependency (Luo et al., 1993) and recently has been reported to be involved in the biochemical association between TRPV1 and β-arrestin-2 (Por et al., 2013). It is therefore possible that PKC plays a role downstream of TRPV1 activation. Treatment with RTX (10 nM, for 2 and 5 min) of cells co-transfected with TRPV1-mCherry and PKC-βII-GFP, one of the conventional calcium-dependent PKC isoforms, showed a transient translocation of the PKC to the membrane (a hallmark of its activation (Nishizuka et al., 1995; Hoque et al., 2014)). This translocation to the membrane appeared as early as 2 min post TRPV1 activation and returned to its pre-treatment level 5 min after RTX treatment (**Figure 3.9.A**).

3.8 PKC activation is required for ERK phosphorylation and β-arrestin-2 translocation

TRPV1-transfected cells were treated with either the PKC βI, II inhibitor CGP53353 (10 μM) or the PKC α, βI inhibitor GF109203X (10 μM) for 30 min prior to TRPV1 activation by RTX. Blocking PKC-βII (CGP53353) significantly decreased ERK phosphorylation while the PKC inhibitor GF109203X had no significant effect (**Figure 3.9.B, C**). Interestingly, cells treated with either CGP53353 or GF109203X and subsequently challenged with RTX (10 nM) failed to show any additional β-arrestin-2 nuclear translocation compared to basal conditions (**Figure 3.9.D**). The percentage of cells showing β-arrestin-2 translocation in response to RTX in the presence of PKC inhibitors was significantly different than cells treated with RTX alone for 15 minutes (~30% compared to ~80%, **Figure 3.9.E**). This provides strong evidence that TRPV1-induced ERK phosphorylation and β-arrestin-2 nuclear translocation are PKC mediated.


A
RTX
Ionomycin
A23187
p-ERK
ERK
B
TRPV1
β-arr2
A23187 15'
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2Fli
p-ERK
ERK
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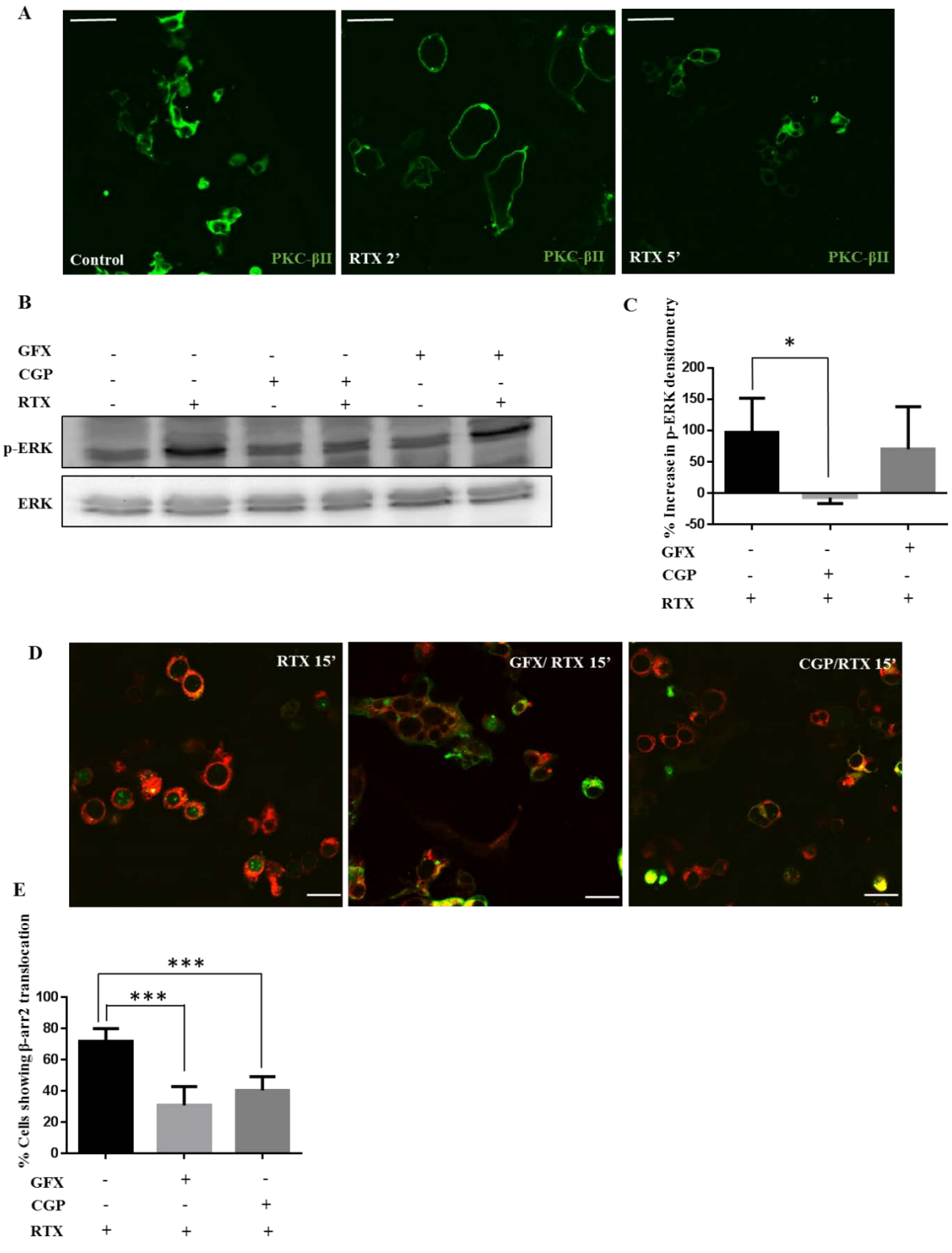
TRPV1
β-arr2
2fli 15'
F
% Cells showing β-arr-2 translocation
100
80
60
40
20
0
***
RTX
2fli

Figure 3.8 TRPV1-mediated Ca^{2+} influx is required for ERK phosphorylation/β-arrestin-2 nuclear translocation

(A) Western blot compares the increase in ERK phosphorylation in response to RTX (100 nM), A23187 (1 μM) or ionomycin (10 μM) *(n = 2).* **(B)** Confocal image shows no β-arrestin-2 nuclear translocation after treating cells with A23187 (1 μM, 15 min). *(Scale bar 30 μm).* **(C)** Bar graph shows the significant difference in percentage of cells exhibiting β-arrestin-2 translocation between RTX (10 nM) and A23187 (1 μM) treated cells *(RTX treatment 71.7 ±2.6, A23187 treatment 7.8 ±2.03, n = 10 fields per condition, P: *** < 0.0001).* **(D)** Activation of endogenous PAR2 (2fli, 5 μM, 5 min) induced less ERK activation compared to RTX (100 nM, 5 min) treatment *(n = 2).* **(E)** In cells transfected with TRPV1-mCherry (*red*) and β-arrestin-2-YFP (*green*), 2fli (5 μM, 15 min) did not induce β-arrestin-2 nuclear translocation. *(Scale bar 30 μm).* **(F)** Percentage of cells showing β-arrestin-2 nuclear translocation in response to 2fli (5 μM, 15 min) is significantly lower than that treated with RTX (10 nM, 15 min) *(RTX 71.7 ± 2.6, 2fli 8.9 ± 1.8, n = 10 fields per condition, P: *** < 0.0001).*


A
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RTX 2'
PKC-βII
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PKC-βII
B
GFX
CGP
RTX
p-ERK
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C
% Increase in p-ERK densitometry
200
150
100
50
0
-50
*
GFX
CGP
RTX
D
RTX 15'
GFX/ RTX 15'
CGP/RTX 15'
E
% Cells showing β-arr2 translocation
100
80
60
40
20
0
***
***
GFX
CGP
RTX

Figure 3.9 PKC activation is required for ERK phosphorylation and β-arrestin-2 nuclear translocation following TRPV1 stimulation

(A) Images of cells co-transfected with TRPV1/ PKC-βII-GFP and treated with RTX (10 nM) for 2 or 5 min. Two minutes following TRPV1 activation PKC showed membrane translocation of PKC-β-II (*green*). *(Scale bar 50 µm).* **(B)** Blot of p-ERK in cells transfected with TRPV1 and treated with RTX (100 nM) under control conditions or in the presence of CGP53353 (10 µM) or GF109203X (10 µM) *(n = 3).* **(C)** Bar graph shows the percentage increase in p-ERK densitometry following RTX (100 nM, 5 min) treatment in the presence or absence of PKC blockers *(RTX 96.4 ± 22.5, RTX+ CGP -7.4 ± 5.3, RTX+ GFX 70.2 ± 47.8, P: * < 0.05).* **(D)** Confocal images of TRPV1-mCherry/ β-arrestin-2-YFP transfected cells treated with RTX (10 nM, 15 min). Cells were treated with CGP53353 (10 µM) or GF109203X (10 µM) for 30 min prior to TRPV1 activation. *(Scale bar 30 µm).* **(E)** Bar graph shows the inhibition in percentage of cells exhibiting β-arrestin-2 nuclear translocation in the presence of PKC blockers *(n= 3, RTX 71.7 ± 2.6, RTX+ CGP 40.4 ± 2.4, RTX+ GFX 30.9 ± 4.5, P: *** < 0.0001).*

3.9 The Ras pathway is activated downstream of TRPV1 activation

The Ras-Raf-MEK pathway is one of the signaling cascades that leads to ERK activation. The flux of Ca^{2+} has been shown to activate a small membrane-bound guanine nucleotide binding protein Ras (Agell et al., 2002). Immunostaining shows that TRPV1 stimulation with RTX (10 nM) in cells transfected with TRPV1-mCherry/ HA-tagged Ras, showed Ras membrane translocation (**Figure 3.10**). This indicates that Ras is activated downstream of TRPV1 activation. The membrane translocation of Ras was transient and reached the peak at 5 min post RTX treatment (**Figure 3.10**).

3.10 Co-immunoprecipitation of β-arrestin-2/ERK

Based on previous results, the idea of pulling down endogenous β-arrestin-2 to detect possible interaction with p-ERK seemed appealing. Under basal conditions, there was an interaction between β-arrestin-2 and ERK. Following TRPV1 channel activation by RTX (100 nM), there was a remarkable increase in ERK/ β-arrestin-2 interaction which peaked at 10 min post RTX treatment and returned to its basal level by 15 min (**Figure 3.11**), the same time point at which maximum β-arrestin-2 translocation was previously observed (**Figure 3.1**). However no interaction between β-arrestin-2 and p-ERK was observed.

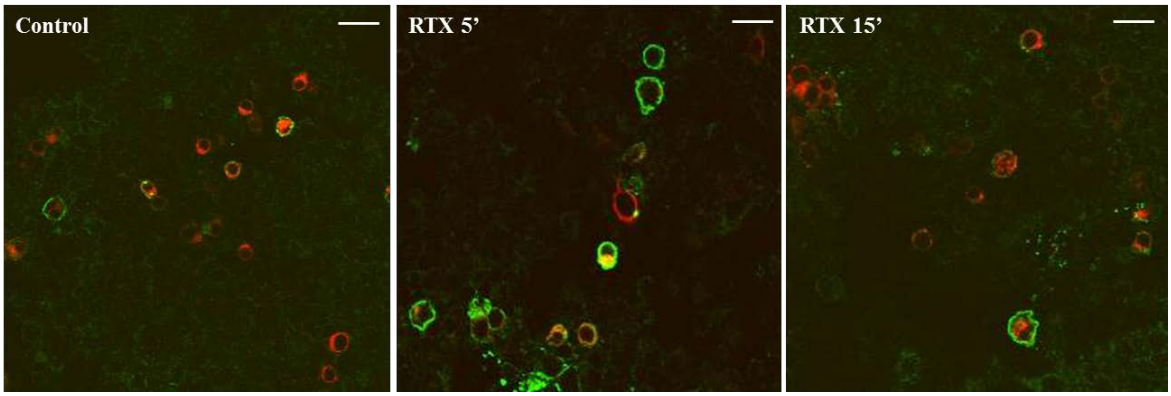


Figure 3.10 RAS is activated upon TRPV1 channel stimulation

Confocal images of cells co-transfected with TRPV1-mCherry and Ras-HA. Immunostaining of Ras-HA with FITC *(green)* showed its activation and membrane translocation following RTX (10 nM) treatment. Translocation peaked 5 min following TRPV1 activation. *(Scale Bar 30 µm).*

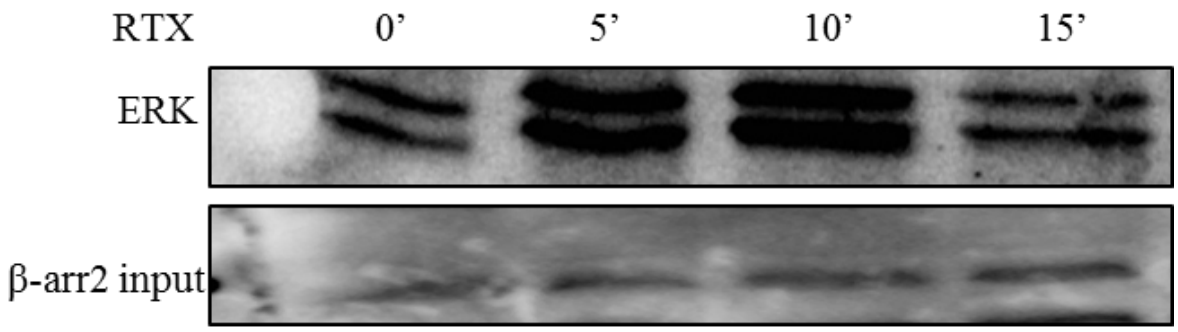


Figure 3.11 Immunoprecipitation of β-arrestin-2 and ERK

Western blot showing interaction between β-arrestin-2 and ERK. There was an increase in the interaction subsequent to TRPV1 activation. Peak of β-arrestin-2/ ERK interaction occurred 10 min post RTX treatment (100 nM). Membrane was later stripped and blotted for β-arrestin-2 input control *(n = 3).*

3.11 Phosphorylated ERK is retained in the cytoplasmic fraction

Upon activation p-ERK can translocate to the nucleus or remain in the cytoplasm with ultimate p-ERK location determining its differential cellular effects (Roskoski, 2012). I hence carried out a cellular fractionation experiment in order to detect p-ERK subcellular location. Following REAP fractionation protocol to separate the nuclear from cytoplasmic fractions (Suzuki et al., 2010), p-ERK was shown to be retained in the cytoplasm following TRPV1 activation (RTX 100 nM, 5 min) (**Figure 3.12**). However by using PARP (nuclear polymerase protein involved in DNA repair) and GAPDH (constitutively expressed housekeeping protein) antibodies as nuclear and cytoplasmic markers, respectively, I showed that the fractionation was neither complete nor efficient enough to draw any conclusions (**Figure 3.12**).

3.12 CaMKII is not phosphorylated following TRPV1 activation

Beyond their classical function as scaffolding proteins, β-arrestins have been reported to bind CaMKII and regulate its activity (Mangmool et al., 2010). Additionally, CaMKII modulates TRPV1 channel activity and there are several CaMKII phosphorylation sites on TRPV1 channel. Indeed, phosphorylation of TRPV1 by CaMKII seems to be a prerequisite for capsaicin-evoked current following channel stimulation (Jung et al., 2004). Based on the preceding information, I examined if CaMKII was implicated in the proposed TRPV1-β-arrestin-2 signaling. Western blot analysis revealed that CaMKII was not activated subsequent to TRPV1 stimulation by capsaicin (1 μM, 5 to 30 min) (**Figure 3.13.A**). Furthermore, immunoprecipitates from HEK cells transfected with TRPV1 and CaMKII-GFP showed constitutive interaction between β-arrestin-2 and CaMK-II. In addition, no changes in the interaction levels were detected upon TRPV1 channel activation (**Figure 3.13.B**).

3.13 ERK phosphorylation and β-arrestin-2 nuclear translocation are not CaM-dependent

It has been shown that calmodulin (CaM) binds to TRPV1 at sites K155 and W787 (Lau et al., 2012), and mutation of these sites to alanine inhibits CaM binding to the channel. By comparing the TRPV1-CaM mutants, K155A and W787A, to the wild-type TRPV1, no differences in ERK phosphorylation were detected following RTX (100 nM, 5 min, **Figure 3.13.C**) treatment. Additionally, neither K155A nor W787A mutants inhibited β-arrestin-2 nuclear translocation following TRPV1 channel activation (**Figure 3.13.D**). These results suggest that CaM-binding to the channel is not required for TRPV1-induced ERK and β-arresetin-2 signaling.

3.14 Mitogen-activated protein kinases p38 and JNK are not activated early after TRPV1 stimulation

In a final set of experiments, I tested if other known MAPK members, such as p-38 or JNK, were activated upon TRPV1 stimulation. When TRPV1-transfected cells were stimulated with capsaicin (1 μM) for different durations (from 5 to 30 min), there was no significant change in the levels of phosphorylated p38 or JNK (**Figure 3.14.A, B**) indicating no involvement of these MAPKs downstream of TRPV1 activation.

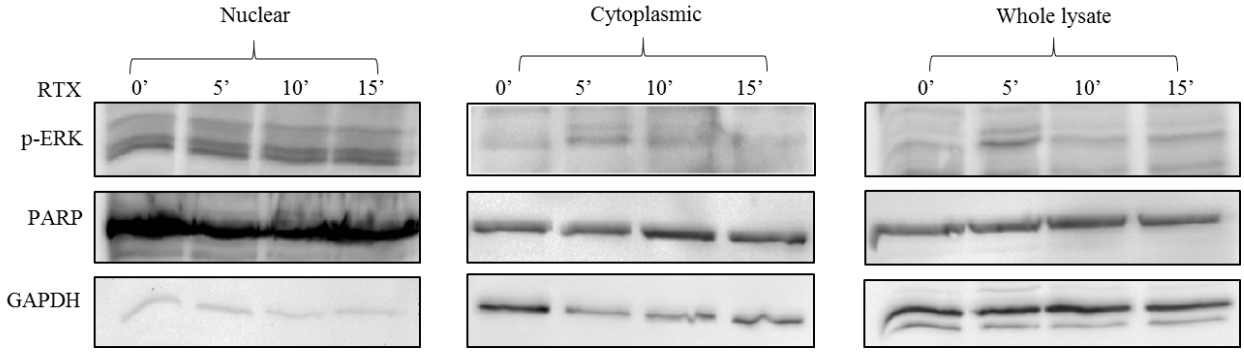


Figure 3.12 Subcellular fractionation of phosphorylated ERK (p-ERK)

REAP protocol was used to separate the cytoplasmic and nuclear fractions. Peak of ERK activation was obvious 5 min after RTX (100 nM) in whole lysates. The nuclear fraction of p-ERK did not show any changes. There was a slight increase in the cytoplasmic p-ERK 5 min post channel activation. Although PARP and GAPDH antibodies were used as nuclear and cytoplasmic markers, they revealed that the fractionation was incomplete *(n = 3).*

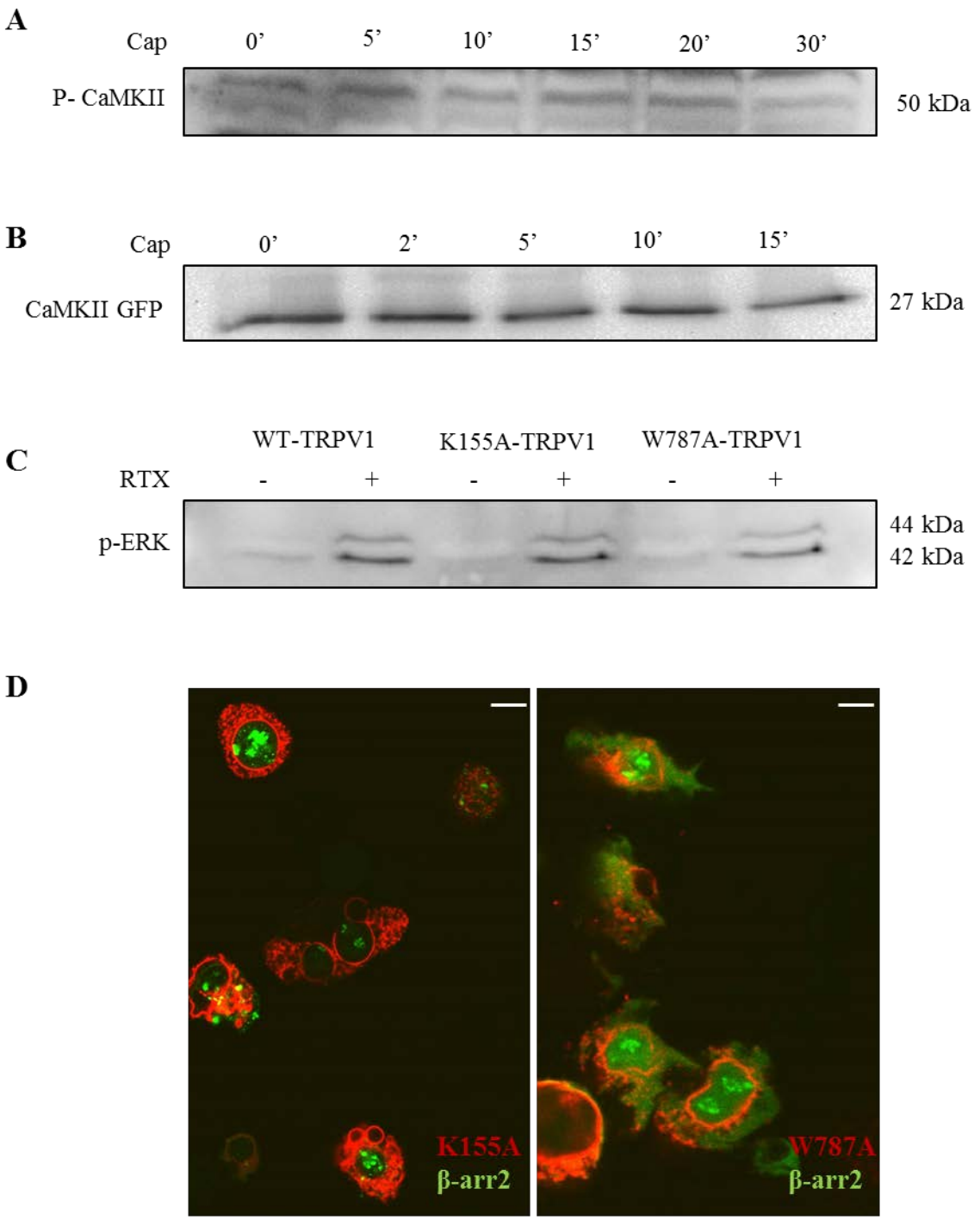

A
Cap
0'
5'
10'
15'
20'
30'
P- CaMKII
50 kDa
B
Cap
0'
2'
5'
10'
15'
CaMKII GFP
27 kDa
C
WT-TRPV1
K155A-TRPV1
W787A-TRPV1
RTX
-
+
-
+
-
+
p-ERK
44 kDa
42 kDa
D
K155A
β-arr2
W787A
β-arr2

Figure 3.13 CaMKII activation and CaM-TRPV1 binding did not occur downstream of TRPV1 activation

(A) Activation of TRPV1 channels (capsaicin, 1μM, 5 to 30 min) did not evoke CaMKII phosphorylation *(n = 2).* **(B)** Cells were co-transfected with TRPV1/CaMKII-GFP and treated with capsaicin (1 μM) two days post transfection. Pulling down endogenous β-arrestin-2 and blotting samples using the GFP antibody, showed a constitutive but non-altering interaction between β-arrestin-2 and CaMKII *(n = 1).* **(C)** Two TRPV1 mutants, K155A-TRPV1 and W787A-TRPV1, were transfected and treated with capsaicin (1 μM) after 48 hours. Neither mutant showed any difference in ERK phosphorylation compared to wild-type TRPV1 *(n = 3).* **(D)** Confocal images showed β-arrestin-2 nuclear translocation 15 min following RTX (10 nM) treatment in cells co-transfected with K155A-TRPV1-mCherry and β-arrestin-2-YFP (*image on the left*) or W787A-TRPV1-mCherry and β-arrestin-2-YFP (*image on the right*). *(Scale bar 25 μm).*

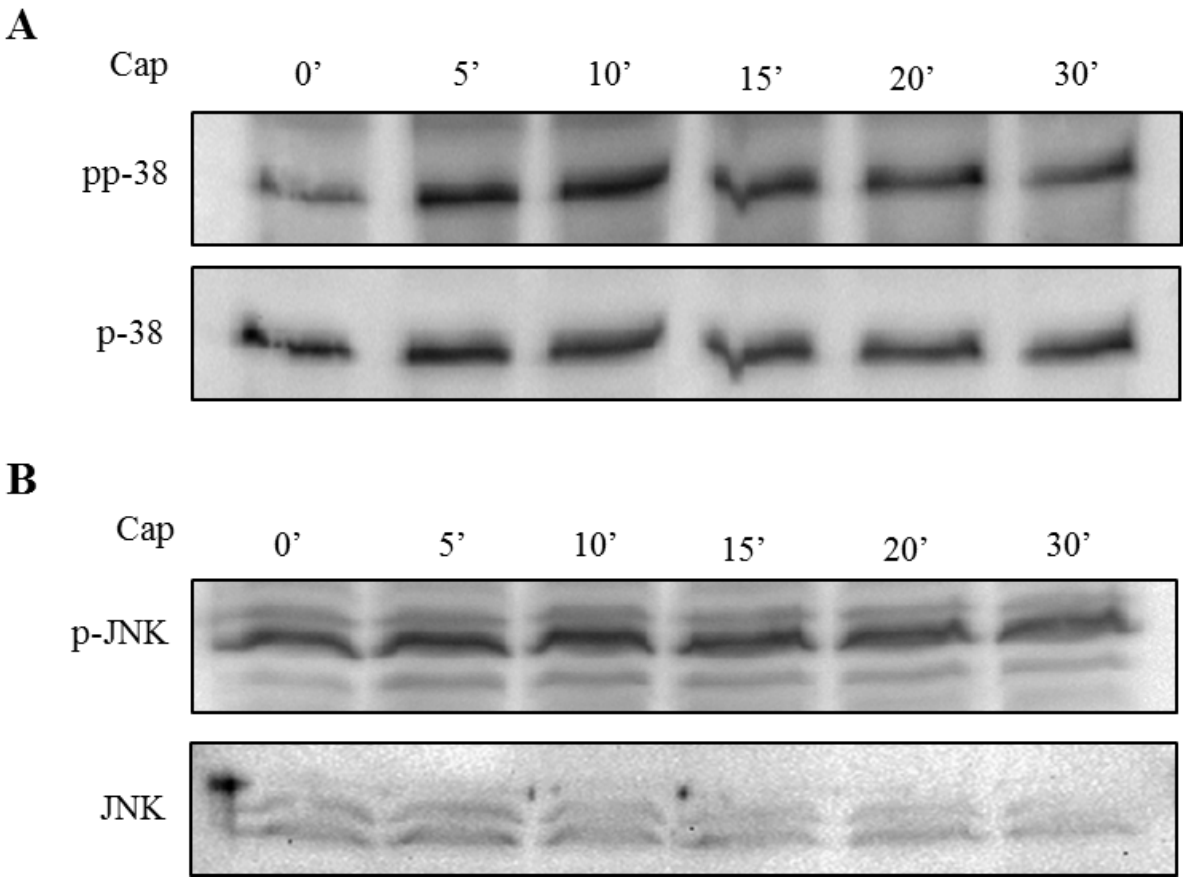


Figure 3.14 p38 and JNK are not activated in early TRPV1 signaling

(A) Western blot shows that activating TRPV1-transfected cells with capsaicin (1 µM, for 30 min) did not induce changes in p-38 phosphorylation (pp-38) *(n = 2).* **(B)** Cells transfected with TRPV1 and activated by capsaicin (1 µM, for 30 min) did not show changes in p-JNK expression *(n = 2).*

Chapter Four: **Discussion**

This thesis explores the molecular aspects of the signaling events occurring downstream of TRPV1 channel activation. This is the first description of the signaling cascade that starts with calcium influx through TRPV1 channels, an event that leads to subsequent activation of protein kinase C (specifically, the PKC-βII isoform) and its translocation to the plasma membrane. The activation of PKC causes ERK phosphorylation and additionally elicits β-arrestin-2 nuclear translocation. Intriguingly, the scaffolding protein β-arrestin-2 seems to be implicated in and necessary for TRPV1-mediated ERK activation prior to its translocation to the nucleus. To the best of our knowledge, this work is the first to describe β-arrestin-2 nuclear translocation downstream of TRPV1 activation. This unique translocation pattern may be involved in yet-to-be unravelled downstream effectors of TRPV1 signaling.

The Transient Receptor Potential Vanilloid 1 (TRPV1) channel has already been described to play a critical role in nociception and is further implicated in several inflammatory conditions (e.g. rheumatoid arthritis, inflammatory bowel disease and neuropathic pain) (Terenzi et al., 2013; Akbar et al., 2008; Zakir et al., 2012). The scaffolding proteins β-arrestins, while first known to be involved in GPCR desensitization and internalization, are also crucial players in nociception via modulation of opioid receptor activity (Bohn et al., 1999; Rowan et al., 2014). Interestingly, recent work has implicated potential cross-talk between TRPV1 and β-arrestin-2 (Por et al. 2012), whereby an interaction between the TRPV1 channel and β-arrestin-2 was reported to occur at the plasma membrane and was suggested to contribute to desensitization of the channel (Por et al., 2013).

It was within these emerging observations that we decided to explore the cell signaling events that occur downstream of TRPV1 activation. Using different approaches

(immunostaining, confocal imaging and BRET assays), my results demonstrated that TRPV1 channel activation by resiniferatoxin induced β-arrestin-2 translocation to the nucleus and far away from the channel. Additional experiments, employing a different TRPV1 agonist (capsaicin), revealed similar observations. Importantly, control experiments in cells transfected with another TRP subtype (TRPA1) did not replicate β-arrestin-2 nuclear translocation, supporting the conclusion that this cellular event is specific to TRPV1 signaling. These observations were unexpected since β-arrestin-2 is classically known to translocate to the activated receptor at the plasma membrane following GPCR activation (Ferguson, 2001).

Beta arrestins are a family of scaffolding proteins that include β-arrestin-1 and β-arrestin-2. Previously reported differences between these subtypes include subcellular localization as well as binding affinity to GPCRs (Wang et al., 2003). While β-arrestin-1 is expressed in the cytoplasm and the nucleus (Hoeppner et al., 2012), β-arrestin-2 is predominantly found in the cytoplasm (Wang et al., 2003) (**Figures 3.2** and **3.3**). The N-terminal domain is necessary for nuclear localization of both isoforms. Iterestingly, and distinct from β-arrestin-1, β-arrestin-2 has been reported to possess a leucine-rich nuclear export signal (NES) sequence that is responsible for its cytoplasmic localization, impeding nuclear translocation in unstimulated cells (Scott et al., 2002). Results in **Figure 3.3** illustrated that the basal nuclear β-arrestin-1 localization was enhanced following TRPV1 channel activation, suggesting a possible recruitment of β-arrestin-1 to the nucleus as observed with β-arrestin-2. However, the nuclear punctate pattern of β-arrestin-2 was strikingly different from the more diffuse distribution of β-arrestin-1. In this thesis, experimental investigation was focused on the intriguing TRPV1/β-arrestin-2 signaling.

Over the past decade, arrestin proteins have been implicated as scaffolding cellular proteins thus facilitating specific signaling pathways (Nogués et al., 2011). Mitogen-activated

protein kinases (MAPKs) are well-described signaling molecules regulated by β-arrestins (DeWire et al., 2007). The MAPK family comprises five distinct groups: 1) ERK1 and ERK2; 2) c-Jun- N-terminal kinase (JNK-1, JNK-2, JNK-3); 3) p38 isoforms (α, β, γ and δ); 4) ERK3 and ERK4; and 5) ERK5 (Roux et al., 2004). Notably, ERK1-2, JNKs and p38 kinases are the most extensively studied MAPKs. Beta-arrestin-2 facilitates ERK signaling in response to activation of various GPCRs such as the protease-activated receptor 2 (PAR2) and the angiotensin receptor subtype 1A (Luttrell et al., 2002; Tohgo et al., 2002). Two distinctive ERK activation pathways have been reported following GPCR activation: the classical G-protein-dependent pathway that is rapid and transient, and the slower and more persistent β-arrestin-dependent activation (Gesty-Palmer et al., 2006). Interestingly, a link between TRPV1 channel and ERK signaling has been extensively studied; for instance, TRPV1 contributes to arthritis pain hypersensitivity and inflammation in an ERK-dependent pathway (Chen et al., 2009). Given these observations, I investigated if ERK is activated early on during TRPV1 signaling and whether a crosstalk between TRPV1, β-arrestin-2 and ERK exists.

Data in **Chapter 3** showed that following TRPV1 channel stimulation, ERK was transiently activated (peaked at 5 min). In addition, results showed a more delayed ERK phosphorylation ~90 min following TRPV1 activation (**Figure 4.1**). While the delayed effect was interesting, several factors and processes (such as proliferation, differentiation and apoptosis (Zhang et al., 2002)) other than direct TRPV1 activation may contribute to ERK phosphorylation at such late time points. Therefore, the main focus of this thesis was to investigate the early signaling events that can be linked solely to TRPV1 activation. It is worth mentioning that my experiments showed that neither JNKs nor p38 MAP kinases were activated downstream of TRPV1 channel activation, suggesting that the ERK1/2 was specifically engaged by TRPV1-

mediated calcium influx. In addressing potential crosstalk between TRPV1, β-arrestin-2 and ERK, data revealed that β-arrestin-2 nuclear translocation evoked by TRPV1 channel activation occurred independently of ERK phosphorylation. On the other hand, knocking down β-arrestin-2 inhibited ERK phosphorylation at 5 min following TRPV1 activation. It is conceivable that β-arrestin-2 may act as a scaffold for a macromolecular signaling complex involved in phosphorylating ERK and that the absence of β-arrestin-2 would compromise ERK activation (**Figure 4.2**). Importantly, our results demonstrated that the crosstalk between β-arrestin-2 and ERK activation occurs prior to β-arrestin-2 nuclear translocation.

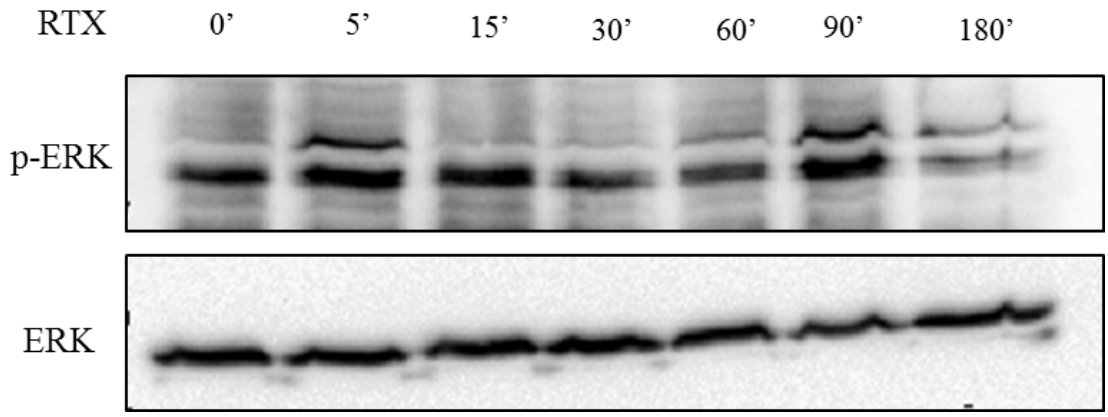


Figure 4.1 Biphasic ERK phosphorylation following TRPV1 activation

Western blot showing that treatment of TRPV1-transfected HEK cells with RTX (100 nM) for different durations (5 to 180 min) induced an early (5 min) and a later (90 min) ERK activation *(n = 2).*

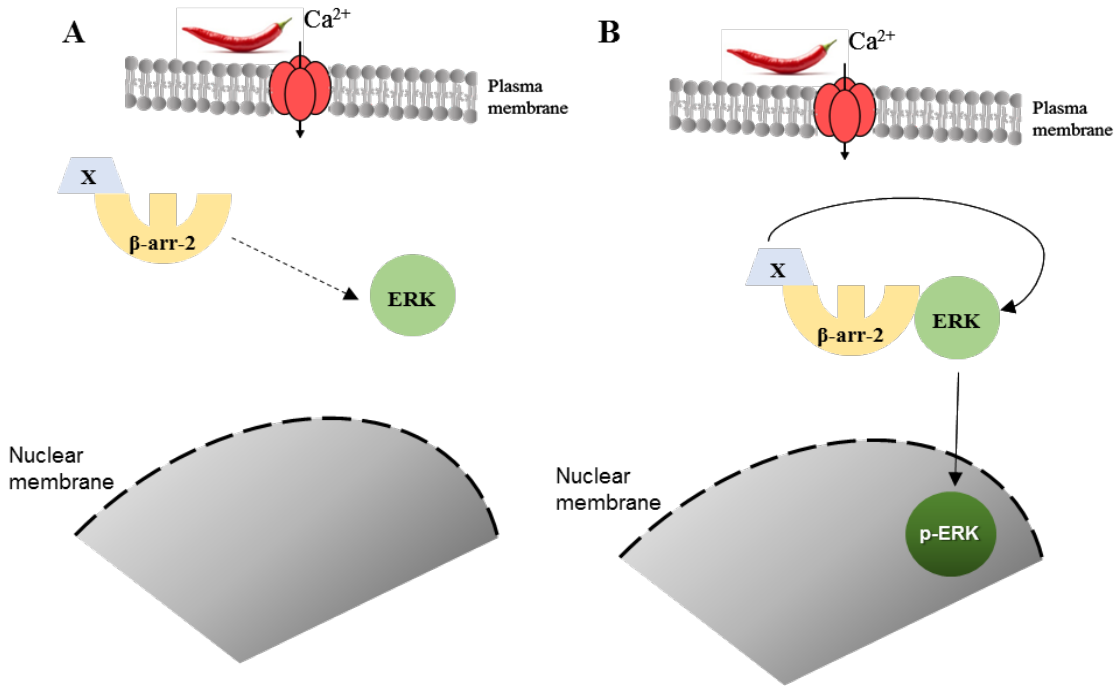


Figure 4.2 A possible explanation for β-arrestin-2–dependent ERK activation

(A) Beta-arrestin-2 may act as a scaffold bringing a signaling protein (X) into close proximity to ERK in response to TRPV1 channel activation. **(B)** This signaling protein (X) is in turn responsible for ERK activation.

Considering the proposed crosstalk between β-arrestin-2 and p-ERK following TRPV1 activation, we sought to co-immunoprecipitate β-arrestin-2 and p-ERK to identify a possible dynamic interaction in response to channel activation. Unexpectedly, data described in **Figure 3.11** revealed an enhanced interaction between β-arrestin-2 and ERK, but not p-ERK, after the pharmacological activation of TRPV1. In particular, immunoprecipitation of β-arrestin-2 showed a constitutive interaction between β-arrestin-2 and ERK that increased upon TRPV1 channel activation (peak at 10 min). It is possible that β-arrestin-2 may have a higher binding affinity for ERK than p-ERK. This may account for the increase in β-arrestin-2/ERK interaction following p-ERK dephosphorylation. A second possible explanation is that p-ERK could be responsible for cytosolic β-arrestin-2 phosphorylation, retaining it in the cytosol and delaying its translocation to the nucleus. This proposition is consistent with what was previously described between ERK and β-arrestin-1 (Lin et al., 1999; DeFea et al., 2000). This phosphorylation could be quick and transient leading to a hardly detectable β-arrestin-2/p-ERK interaction. It follows that once β-arrestin-2 is dephosphorylated, it could translocate to the nucleus (**Figure 4.3**). While an interesting hypothesis, a critical limitation to test this hypothesis is represented by the lack of available phosphorylated β-arrestin-2 antibodies.

For the previous proposition to be true, p-ERK would be expected to be retained in the cytosol. The REAP subcellular fractionation protocol revealed an increase in cytoplasmic p-ERK (5 min), while nuclear p-ERK showed no changes. The latter fractionation results support our previous assumption that maybe β-arrestin-2 is phosphorylated by cytoplasmic p-ERK before it translocates to the nucleus. However, it should be noted that the nuclear and cytoplasmic markers (PARP and GAPDH, respectively) used to confirm efficient subcellular fractionation showed that the fractionation was incomplete. In contrast to fractionation results, immunohistochemistry

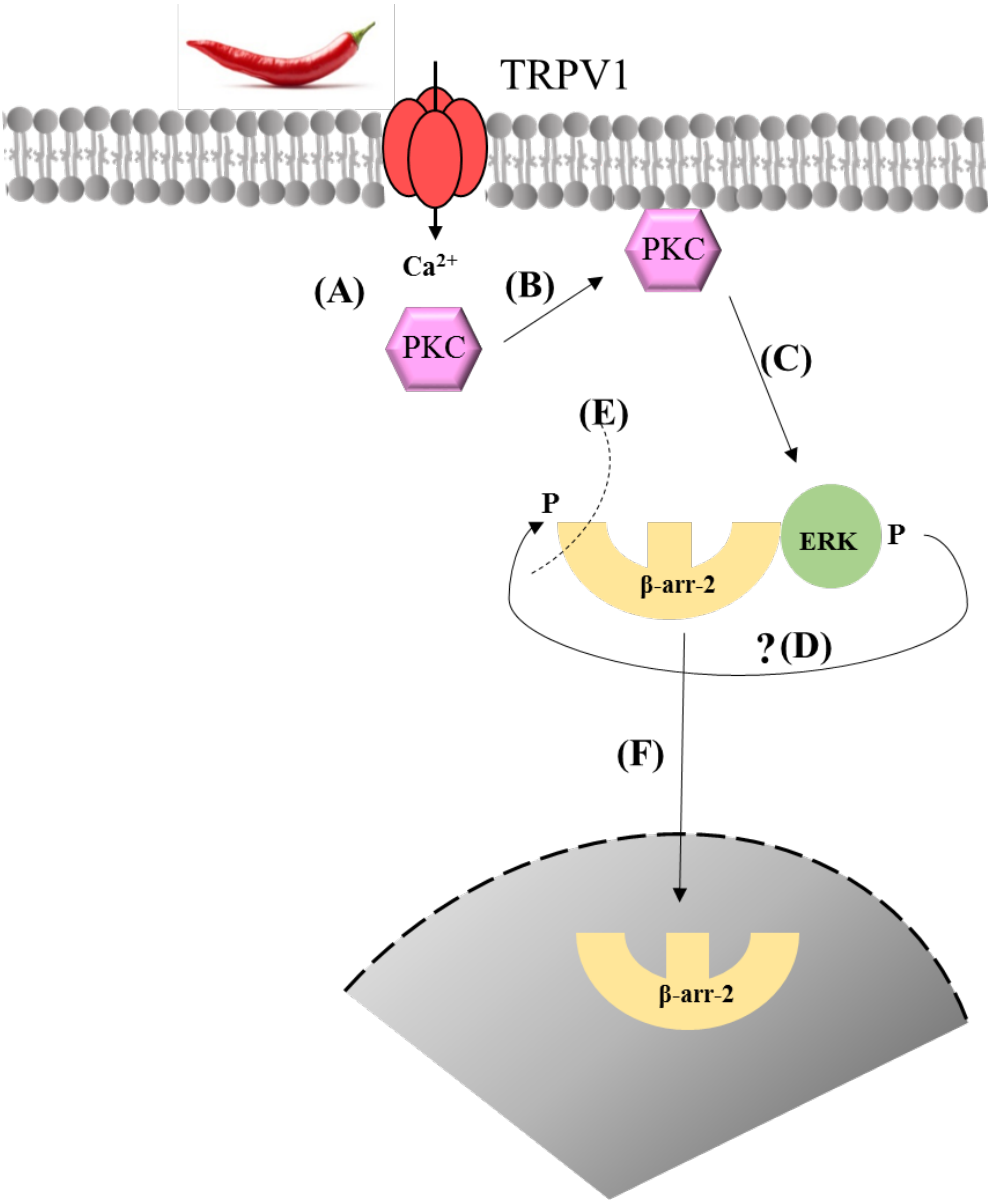

TRPV1
Ca2+
(A)
PKC
(B)
PKC
(C)
(E)
P
β-arr-2
ERK
P
?(D)
(F)
β-arr-2

Figure 4.3 Proposed β-arrestin-2 signaling following TRPV1 channel stimulation

(**A**) Binding of TRPV1 agonist such as RTX or capsaicin leads to channel activation and Ca^{2+} influx. (**B**) This calcium entry is necessary for PKC activation and translocation to the membrane. (**C**) PKC then phosphorylates ERK. (**D**) p-ERK could promote β-arrestin-2 phosphorylation causing its retention in the cytosol. (**E, F**) Phosphorylated β-arrestin-2 is dephosphorylated before nuclear translocation.

showed an increase in nuclear p-ERK following TRPV1 activation. Therefore, further work is required to resolve these discrepancies.

Upon TRPV1 channel activation, Ca^{2+} passes through the channel's pore to the cytoplasm. I thus tested the hypothesis that TRPV1-mediated Ca^{2+} influx was necessary and sufficient for ERK activation and β-arrestin-2 translocation. Our data showed multiple lines of evidence to support this hypothesis. First, extracellular calcium chelation inhibited both effects. Second, experimental analysis showed that it is not the absolute non-specific rise of intracellular [Ca^{2+}] that induced these cellular events. In particular, increasing cytosolic [Ca^{2+}] using ionophores (e.g. A23187, ionomycin) or through the activation of other plasma membrane proteins (e.g. TRPA1 channel, PAR2) failed to elicit ERK phosphorylation or β-arrestin-2 nuclear translocation. This is supported by multiple previous reports clarifying the role of calcium compartmentalization and localization in inducing differential signaling (Laude et al., 2009). Our observations, as well as what is described in the literature, suggest signaling events that, in order to proceed, specifically utilize calcium influx through TRPV1 channel pores. Whether mobilization of Ca^{2+} from intracellular stores, dependent or independent of TRPV1 activity, contributes to the reported findings remains to be tested.

One classical means of ERK activation that is calcium-dependent occurs via the protein kinase C (PKC) pathway (Ueda et al., 1996). The latter kinase comprises a family of approximately 12 known PKC isoforms categorized into three main sub-families: 1) conventional PKC isoforms (α, βI, βII and γ) that are both calcium- and diacylglycerol (DAG)-dependent; 2) novel PKC isoforms (δ, ε, η and θ) which are calcium-independent but require DAG for their activation; and 3) atypical PKCs (ζ and ι/λ) which are calcium- and DAG-independent (Way et al., 2000). Interestingly, PKC is an important modulator of TRPV1

channels through enhancing its sensitization (Sikand et al., 2007). Considering the fact that TRPV1 is calcium permeable in addition to my previous results showing the dependency of ERK phosphorylation and β-arrestin-2 nuclear translocation on calcium, I investigated the consequences of TRPV1 activation on the conventional calcium-dependent PKC isoforms.

Data in **Figure 3.9** showed that stimulation of TRPV1 channels elicited a fast and transient translocation of PKC-βII to the plasma membrane. Activation of this conventional PKC isoform requires calcium as well as DAG. While Ca^{2+} appears to be readily available following TRPV1 activation, the source of DAG is not obvious and may, directly or indirectly, be linked to TRPV1 activation. Calcium influx through TRPV1 following channel activation has been previously shown to activate phospholipase C (PLC) (Rohacs et al., 2008) which in turn hydrolyses the precursor phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and inositol trisphosphate (IP_3). Alternatively, DAG availability may be attributed to other receptors and proteins presumably close to TRPV1 at the plasma membrane. Other conventional PKC isoforms, besides PKC-βII, were not investigated in this thesis and remain to be tested in the future. Using pharmacological (different PKC inhibitors), biochemical (western blotting) as well as confocal imaging approaches, I was able to reveal a critical role for PKC-βII, but not PKC-βI or PKC-α, in ERK phosphorylation. On the other hand, blockade of PKC-α, -βI and -βII abolished β-arrestin-2 nuclear translocation.

A second classical cascade that can lead to ERK phosphorylation is through the Ras/Raf/MEK pathway (McCubrey et al., 2007). This signaling cascade plays pivotal roles in biological processes (e.g. cell cycle progression, differentiation and apoptosis) and is triggered by a diverse range of extracellular stimuli (e.g. hormones and growth factors) acting through cell membrane receptors. This pathway starts with the conversion of Ras-GDP into Ras-GTP which

in turn binds Raf that subsequently phosphorylates MEK1 and MEK2 finally leading to the activation/phosphorylation of ERK (**Figure 4.4**). Interestingly, a link between Ras, ERK and TRPV1 has been previously reported, whereby activated Ras plays a crucial role in the regulation of TRPV1 expression in dorsal root ganglia (DRGs). This modulatory role of Ras was at least partially mediated by ERK (Bron et al., 2003). Preliminary immunostaining results (Chapter 3) showed a rapid and prominent increase in Ras membrane localization following TRPV1 channel stimulation. Further investigations will be required to determine whether Ras signaling leads to ERK phosphorylation downstream of TRPV1 activation. If this proves correct, it will be worthwhile to test whether the Ras and PKC signaling cascades simultaneously or independently trigger TRPV1-induced ERK activation.

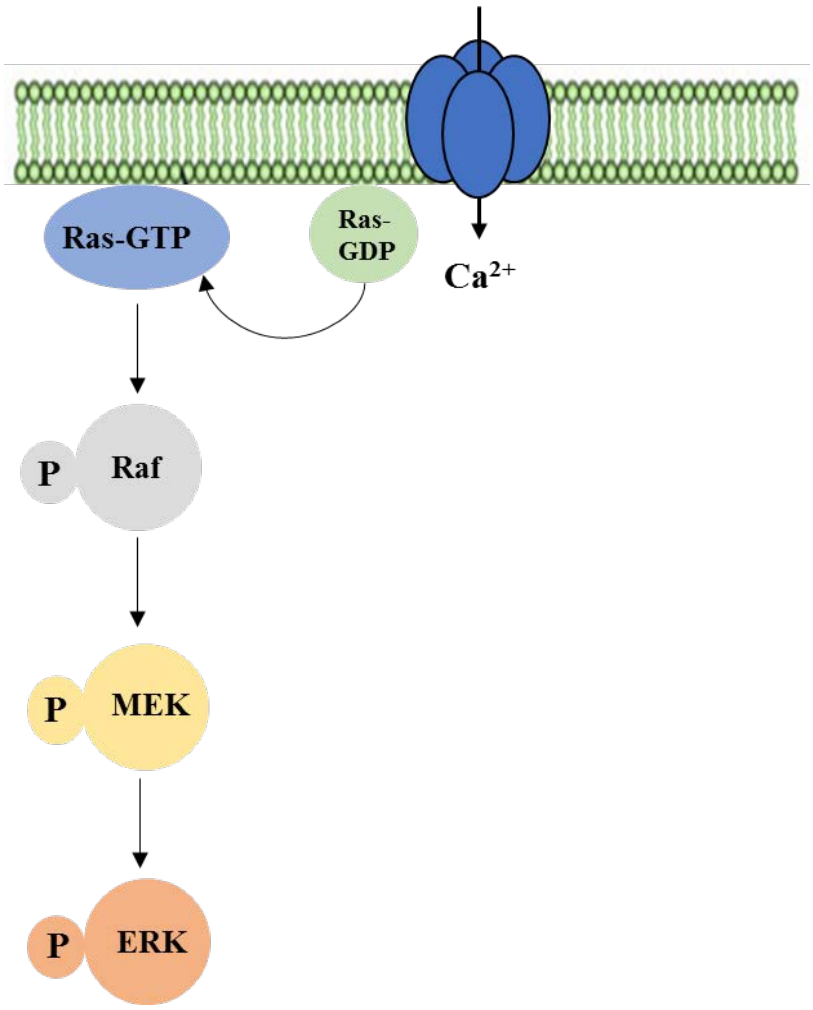


Figure 4.4 The Ras/Raf/MEK/ERK pathway

Upon Ca^{2+} influx, Ras is converted from its inactive form (GDP-bound) to the active GTP-bound form. Activated Ras then stimulates the protein kinase Raf. This in turn phosphorylates MEK (1 and 2) and leads to ERK phosphorylation and activation.

This thesis highlights several molecular insights into TRPV1 signaling (**Figure 4.5**) based on experiments carried out in a heterologous expression system. A yet-unanswered question is whether these results can be replicated in a native system that endogenously expresses TRPV1 channels. While trying to investigate the described signaling cascade in DRG cultures, we encountered several experimental problems or limitations. For instance, western blotting and immunohistochemistry noted high background p-ERK. Similarly, the transcription factor p-CREB (cAMP response-element binding protein), which is a possible β-arrestin-2 nuclear target (Manson et al., 2011), displayed high basal expression that made it unfeasible to quantify changes in response to a given treatment. High background of these phosphorylated proteins (p-ERK, p-CREB) may be attributed, at least in part, to the protocol used for DRG isolation and processing (Cenac et al., 2010). Furthermore, the presence of other cell types in cultures that express p-ERK and p-CREB (e.g. glial cells, Schwann cells) made it very difficult to quantify changes evoked by TRPV1 activation. Due to the limitations encountered while studying DRG cultures, we decided to alternatively advance the reported signaling pathway using an *in vivo* animal model.

There are many interesting, yet unanswered questions pertaining to the consequences of β-arrestin-2 nuclear translocation. For instance: Does the nuclear translocation induce TRPV1 channel desensitization? What are the nuclear targets of β-arrestin-2? Recently, an interesting field emerged focusing on discovering the biological significance of β-arrestin nuclear distribution. The cofactor P300, transcription factors NF-κ-B, p27 and FOS are examples of some identified β-arrestin nuclear targets (Witherow et al., 2004; Kang et al., 2005; Lefkowitz et al., 2006) that have been shown to affect gene transcription. Another interesting nuclear target that may specifically be implicated in TRPV1-induced β-arrestin-2 translocation is the

transcription factor CREB. Notably, calcium influx through TRPV1 stimulates various kinases (e.g. PKC, PKA and CaMK-II), that could be involved in CREB activation. In addition, p-CREB/TRPV1 signaling has been previously described in inflammatory conditions (Nakanishi et al., 2010). It is within this context that ongoing work is beginning to examine the potential effects of β-arrestin-2 nuclear translocation on CREB activation.

Since β-arrestin-2 has been shown to regulate TRPV1 channel desensitization (Por et al., 2012) and TRPV1 is a crucial player in nociception, a key experiment that I started in collaboration with other colleagues (Dr. Tamia K. Lapointe and Mr. Kevin Chapman) is to test a possible functional relevance of TRPV1/β-arrestin-2 signaling. To test this, we will examine the effect of knocking down β-arrestin-2 on TRPV1 signaling *in vivo* by measuring nociceptive responses to capsaicin. Briefly, three groups of mice will be injected intrathecally on three consecutive days (Njoo et al., 2014). The first group will receive PBS (control), the second will be injected with β-arrestin-2 siRNA and the third will receive scrambled siRNA. These mice will be tested for behavioral changes in response to two doses of capsaicin injections into the paw. Dynamic Plantar Aesthesiometer will be used to assess tactile sensitivity following capsaicin injections (**Figure 4.6A**). Expected results are as follow: 1) control mice to exhibit less pain (i.e. more force needed to cause paw withdrawal) in response to second capsaicin dose due to TRPV1 desensitization; 2) β-arrestin-2 knockdown mice to exhibit less desensitization (less force to induce paw withdrawal) compared to control mice following second capsaicin injection; and 3) scrambled siRNA group to behave similarly to the control group (**Figure 4.6**, **Table 4.1**). Next, mice will be sacrificed and DRGs will be collected, sliced and stained for p-CREB, β-arrestin-2 and p-ERK. Preliminary data using western blotting analysis show a decrease in β-arrestin-2 expression in DRGs of mice treated with siRNA (**Figure 4.6.B**). They additionally show the

specificity of p-CREB staining in DRG slices obtained from control untreated animals (**Figure 4.6.C**). Finally, preliminary behavioral analysis showed a decrease in mechanical hypersensitivity as measured by an increase in the force needed to cause paw withdrawal in control mice in response to the second injection of capsaicin. These results indicate a desensitization of TRPV1 mechanosensitive primary afferents observed following the first injection of capsaicin (**Figure 4.6.D**). We will compare these results to nociceptive behaviors from mice that receive intrathecal injection of β-arrestin-2 siRNA. While data collection is undergoing, expected results are summarized in **Table 4.1**.

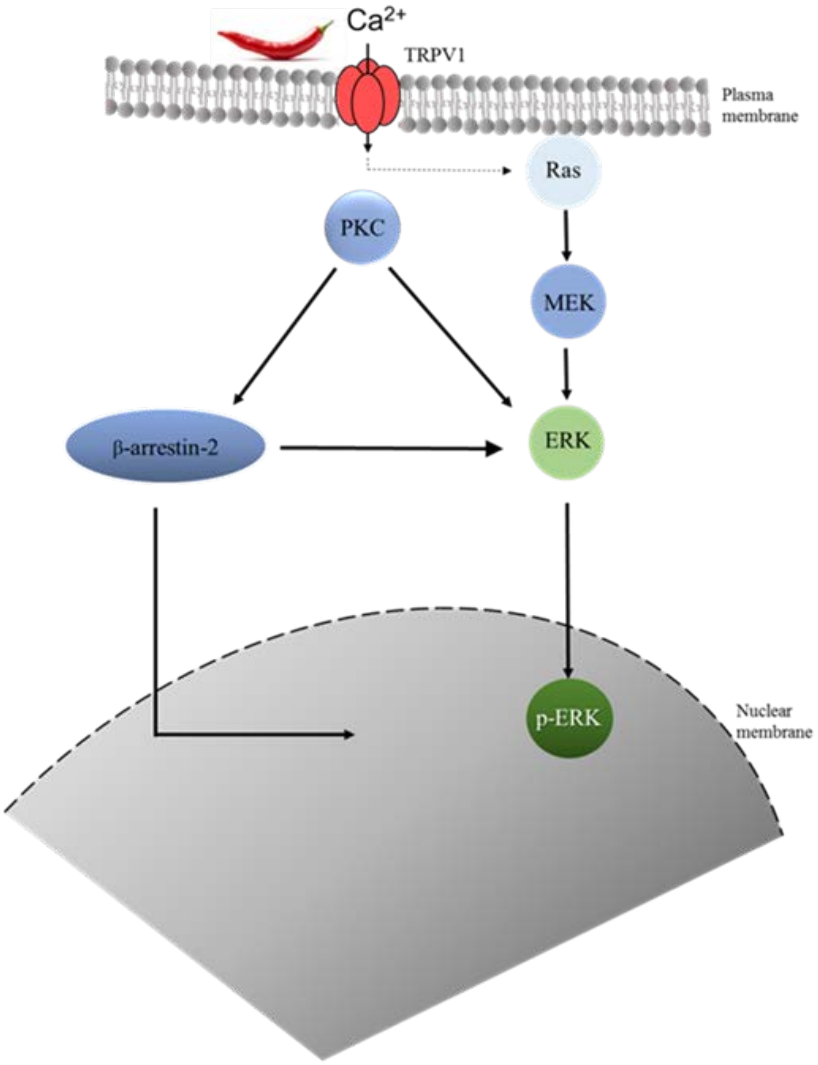
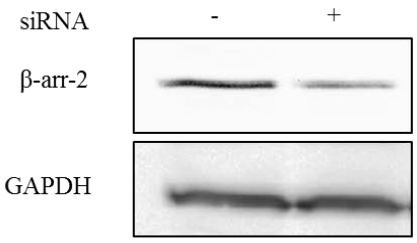


Figure 4.5 Summary of signaling events that occur downstream of TRPV1 channel activation

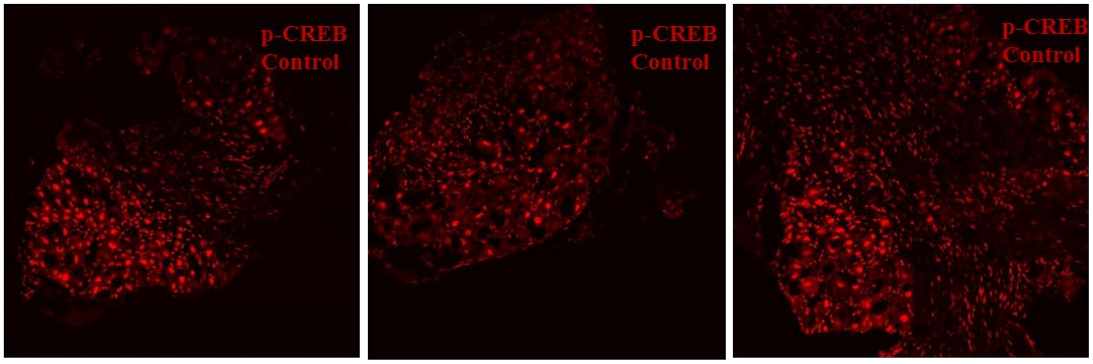
TRPV1 channel stimulation induces Ca^{2+} influx that activates PKC. The latter kinase then phosphorylates ERK and induces β-arrestin-2 nuclear translocation. In addition, β-arrestin-2 is required for ERK activation, likely by serving as a scaffold for the phosphorylation of ERK. My data also suggest that the Ras signaling cascade is activated following TRPV1 channel stimulation.

A

B


siRNA
-
+
β-arr-2
GAPDH

C


p-CREB
Control
p-CREB
Control
p-CREB
Control

D

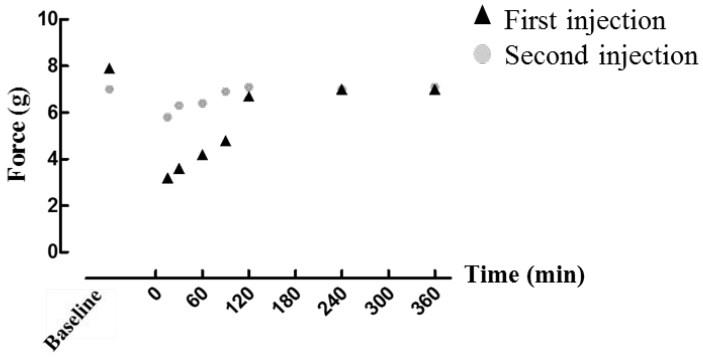

First injection
Second injection
Force (g)
10
8
6
4
2
0
Time (min)
Baseline
0
60
120
180
240
300
360

Figure 4.6 Preliminary results of experiments testing nociceptive behavior responses to capsaicin injection in mice administered with β-arrestin-2 siRNA

(**A**) Dynamic Plantar Aesthesiometer (Ugo Basile 21036 GEMONIO- Varese- ITALY) used to assess mechanical sensitivity in mice. (**B**) Western blot of DRG lysates from mice injected intrathecally with PBS (control) or β-arrestin-2 siRNA and blotted for β-arrestin-2. Note that a decrease in β-arrestin-2 protein expression resulted from siRNA administration (*n=1*). (**C**) DRG slices obtained from different control mice were immunostained for p-CREB. (**D**) Graph showing the force needed to induce paw withdrawal in control mice treated with capsaicin at two different time points (0.5% w/v in 80% saline, 10% tween 80, 10% ethanol, 24 hr apart). The force needed to induce withdrawal increased upon second capsaicin dose implicating desensitization of the response. Intrathecal injections, DRG isolation and slicing were performed by Dr. Tamia K. Lapointe. Tissue processing, western blotting and immunostaining were done by Reem Aboushousha. Behavioral experiments were done by Kevin Chapman.

	Control PBS	β-arrestin-2 siRNA	Scrambled siRNA
Behavioral	**Less pain sensation (high force to cause paw withdrawal) upon second capsaicin injection- desensitization**	**High pain sensation (lower force to cause paw withdrawal) upon second capsaicin injection- Less desensitization**	**Less pain sensation (high force to cause paw withdrawal) upon second capsaicin injection- desensitization**
Immunohistochemistry: DRG slices from control or capsaicin injected mice, stained for p-ERK, p-CREB and β-arrestin-2	**Control (no capsaicin injected): normal p-ERK, p-CREB and β-arrestin-2 staining** **Capsaicin injected: p-ERK over-expression, p-CREB over-expression, nuclear β-arrestin-2**	**Control (no capsaicin injected): Lower p-ERK and p-CREB. Lower β-arrestin-2 expression.** **Capsaicin injected: p-ERK and p-CREB lower expression, lower nuclear β-arrestin-2 expression**	**Control (no capsaicin injected): normal p-ERK, p-CREB and β-arrestin-2 staining** **Capsaicin injected: p-ERK over-expression, p-CREB over-expression, nuclear β-arrestin-2**

Table 4.1. Expected outcomes from *in vivo* β-arrestin-2 siRNA experiment

Given the fact that TRPV1 is implicated in nociception and is up-regulated in several inflammatory conditions, it is important to increase our knowledge regarding its regulation and the signaling pathways engaged by the channel. On the basis of the present and proposed data, this thesis highlights part of the TRPV1 signaling that occurs following channel activation. Our results may have implications for channel desensitization and regulation of gene expression in the context of pain sensitization. The rapidly increasing list of signaling pathways involving β-arrestins points to the importance of these scaffolding proteins in widely diverse cellular processes. In conclusion, this thesis describes for the first time the signaling cascade that starts with TRPV1-mediated calcium influx leading to subsequent activation and translocation of protein kinase C-βII to the plasma membrane, ERK phosphorylation and β-arrestin-2 nuclear localization. A better understanding of this signaling mechanism may provide useful information and advance our understanding of the mechanisms underlying nociception, inflammation and other physiological and pathophysiological processes as well as providing new potential targets for prospective drug design.

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