Regulation of intracellular Ca^{2+} signaling by inositol 1,4,5-triphosphate receptor-binding protein released with inositol 1,4,5-triphosphate (IRBIT)

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Abstract

Calcium ions play a pivotal role for numerous cellular signaling pathways and alterations of cytoplasmic calcium levels often have profound effects on cellular activity. Inositol 1,4,5-trisphosphate receptors (IP_3R) in the membrane of the endoplasmic reticulum allow for the rapid release of large amounts of Ca^{2+} ions from the endoplasmic reticulum into the cytoplasm upon increases in the cytoplasmic concentration of the second messenger inositol 1,4,5-trisphosphate (IP_3) (Ando et al., 2006; Ando et al., 2014). Recently, inositol 1,4,5-trisphosphate (IRBIT) has been identified as a pseudoligand for IP_3 receptors. Parts of the IRBIT protein bind the IP_3R without activating the receptor, thus acting as a competitive antagonist of release of Ca^{2+} ions by IP_3 receptors (Ando et al., 2006; Ando et al., 2014). High expression levels of IRBIT have been detected in neurons, but few studies have analyzed IRBIT's role in living cells. This research project aims to analyze the role of IRBIT in controlling Ca^{2+}-ion release. The CRISPR/Cas9 gene-editing technology was utilized in an attempt to generate a cell line that lacked expression of IRBIT. This technique failed and so the procedure was adjusted such that cells were transfected with *IRBIT* siRNA to reduce cellular concentrations of IRBIT. Protein expression levels were analyzed using Western blot and confocal microscopy. Effects of decreased IRBIT protein expression on calcium signaling were compared to wild- type cells and were characterized by calcium imaging. The results gleaned from this study aim to provide an in-depth view of IRBIT's function in vitro, allowing us to expand our knowledge of IRBIT's role in dynamic calcium signaling.

Introduction

Calcium ions (Ca^{2+}) are ubiquitous second messengers that are involved in numerous cellular pathways including differentiation, growth, apoptosis, membrane excitability and gene transcription. Within neurons, Ca^{2+} concentration is highly regulated and plays a vital role in the control of synaptic plasticity and memory neurons formation (Pchitskaya et al., 2018). It has been demonstrated that even slight changes in ion concentration can significantly disrupt normal neuronal signaling. Multiple previous studies support the "calcium hypothesis of neurodegeneration," the theory that deregulation of neuronal calcium signaling is a key process in the pathogenesis of diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) (Marambaud et al., 2009; Bezprozvanny, 2010; Pchitskaya et al., 2018). A cure for these diseases still fails to exist, but elucidating mechanisms of Ca^{2+} regulation has emerged a viable strategy in the development of successful therapeutic interventions.

Cytoplasmic Ca^{2+} concentrations hover around 200nM at rest but are subject to more than a 100-fold increase upon cellular stimulation. Neurons utilize a variety of biochemical pathways to properly control levels of cytoplasmic Ca^{2+} levels. The influx of extracellular Ca^{2+} is mediated by ion channels embedded within the plasma membrane, specifically voltage-gated calcium channels (VGCC), AMPA receptors, and NMDA receptors (Marambaud et al., 2009). The main storage unit for Ca^{2+} within the neuron is the endoplasmic reticulum (ER). Activation of inositol 1,4,5- trisphosphate receptors (IP_3R) in the membrane of the ER allows for the rapid release of large amounts of Ca^{2+} from the ER into the cytoplasm.

IP_3Rs are widely expressed tetrameric channels that are only activated when they bind to both the second messenger inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} (Ando et al., 2006; Ando et al., 2014; Taylor and Machaca, 2018). Three IP_3R subtypes have been identified in mammals:

IP_3R1, IP_3R2, and IP_3R3. The subtypes are structurally similar, but IP_3R1 is most abundantly expressed in the brain (Ando et al., 2014). Structural analyses of cytosolic fragments of the IP_3R have provided insight into the IP_3 binding mechanisms. The receptor is mainly composed of two subunits that together form an elongated L-shape structure (Bosanac et al., 2002). The N-terminal domain is composed of 12 β-strands while the C-terminal domain is composed of 8 α-helices. In the center of these two subunits is a deep pore lined with basic amino acid residues, which are responsible for anchoring IP_3 into the receptor (Bosanac et al., 2002). There also exists a suppressor domain (SD) that interacts with both the α and β domains in order to link IP_3 binding to the receptor gating.

With IP_3 bound, the SD associates with the α and β domains, reducing the orientation angle between the α and β domains by approximately 8° (Seo et al., 2012). IP_3 binding thus activates a clam-like gating mechanism which shrinks the entrance of the IP_3 binding pocket, effectively securing the IP_3 in place. Conformational changes in the C-terminal domain were then shown to trigger Ca^{2+} channel opening. Specifically, it was proposed that structural changes in the IP_3 binding domain induced by IP_3 binding were transmitted through the SD to the α domain, thus opening the channel pore (Mikoshiba, 2012).

A pioneering study using confocal microscopy revealed a system of Ca^{2+} release events upon IP_3 binding to the receptor (Parker et al., 1996). It found that Ca^{2+} release is dependent on the strength of a stimulus, and that the calcium response initially highly localized at specific receptor sites but becomes global as cytosolic concentrations of IP_3 increase. The first Ca^{2+} release event is known as a "calcium blip," and most likely reflects the opening of just a few, localized receptors. These blips occur on a very short timescale, typically lasting a few milliseconds (Taylor and Machaca, 2018). "Calcium puffs" result from a more intense stimulus

and reflect the spreading of receptor activity from a few initial areas. These puffs are longer lasting than blips, and have been observed on a 100 ms timescale (Taylor and Machaca, 2018). An intense stimulus produces a regenerative calcium response wave that is propagated throughout the entire cell. It is important to note that the Ca^{2+} response is not all-or-none, and can occur both locally and globally depending on the strength and stimulus and the cytosolic concentration of IP_3 (Parker et al., 1996; Taylor and Machaca, 2018).

As cytosolic concentrations of IP_3 rise above a certain threshold, Ca^{2+} diffuses throughout the cytosol and can bind to the IP_3R as a way of regulating channel activity. Once IP_3 is bound to the receptor, structural changes in the receptor allow Ca^{2+} to bind. A previous mutagenesis study identified two distinct Ca^{2+} binding sites located along the α and β domains (Bosanac et al., 2002). Ca-I is located in the β domain and consists of four different amino acid residues. Ca-II is located across both α and β domains and similarly consists of four amino acid residues. This second Ca^{2+} binding site has been shown to interact with P-II, a conserved region of charged amino acids located within the α domain. P-II is an interaction site with the SD, suggesting that the binding of Ca^{2+} at this site is conformationally coupled to IP_3 gating mechanisms (Bosanac et al., 2002). Binding of Ca^{2+} at these sites has been shown to dilate the receptor pore and allow for the rapid release of Ca^{2+} from the ER lumen to the cytosol (Taylor and Machaca, 2018).

Recently, inositol 1,4,5-trisphosphate binding protein (IRBIT) has been identified as a pseudoligand for IP_3Rs. Parts of the IRBIT protein bind the IP_3R without activating the receptor, thus acting as a competitive antagonist of release of Ca^{2+} by IP_3Rs (Ando et al., 2006; Ando et al., 2014). High expression levels of IRBIT have been detected in neurons, but few studies have

analyzed IRBIT's role in living cells. This project attempts to examine IRBIT's role in HEK293 cells.

There exist two different isoforms of the IRBIT protein: short IRBIT and long IRBIT. For the remainder of this paper, short IRBIT will be referred to simply as IRBIT. The two homologues are extremely similar in terms of amino acid composition but long IRBIT has an extended N terminal domain (Yang et al., 2011). IRBIT is ubiquitous and is highly expressed in neurons, which tend to also have a high density of IP_3Rs. Long IRBIT is often co-expressed with IRBIT, but typically at much lower levels. It is worth noting that long IRBIT was found to have a significantly lower affinity for IP_3Rs compared to IRBIT, and overexpression of long IRBIT had little effect on IP_3R mediated Ca^{2+} release (Ando et al., 2009).

IRBIT is composed of 530 amino acids and includes a 104 amino acid appendage near the N-terminus. A study involving mouse IRBIT showed that this ~ 100 amino acid region (specifically residues 62-103) was serine-rich (Devogelaere et al., 2006). This sequence is crucial because it contains multiple phosphorylation sites that are crucial for binding interactions with the IP_3Rs. The phosphorylation of four different Ser residues (Ser68, 71, 74 and 77) are particularly important for IRBIT's interactions with the IP_3R (Mikoshiba, 2012). Ser68 is perhaps the most functionally important phosphorylation site. In fact, Ser68 mutations have been shown to completely eliminate the ability of IRBIT to regulate the IP_3R (Ando et al., 2006). In vitro kinase assays revealed that Ser68 is phosphorylated first by CaMKIV (calmodulin-dependent protein kinase IV) (Devogelaere et al., 2007). Once Ser68 is phosphorylated, Ser71 becomes a potential phosphorylation site by casein kinase I (CKI). This phosphorylation event then produces multiple CKI phosphorylation sites downstream at Ser74 and Ser77 (Ando et al., 2009).

IRBIT's inhibitory activity is heavily regulated by phosphorylation and dephosphorylation events in the serine-rich region. Specifically, the two-step phosphorylation events of Ser68 by CaMKIV and Ser71, Ser74, Ser77 by CKI allow for tight control over IRBIT's activity. The inhibition of CKI significantly decreases IRBIT's activity in intact cells (Ando et al., 2006). The kinases that phosphorylate Ser68 in vivo remain unknown, but Ser68 is dephosphorylated by protein-phosphatase-1 (PP1) (Devogelaere et al., 2007). Binding activity was further reduced when IRBIT was exposed to alkaline phosphatase, another dephosphorylating agent (Ando et al., 2006). Additionally, the serine-rich region is often subject to proteolytic degradation and is cleaved between Asp73 and Ser74, thus rendering IRBIT unable to bind to the IP_3R.

The amino acids in the binding domains of IRBIT and IP_3 are remarkably similar (Yang et al., 2011; Mikoshiba, 2012). It is therefore not surprising that IRBIT and IP_3 compete for the same binding site on the IP_3R. While IRBIT does not directly interact with IP_3, its activity is inversely correlated with cytosolic concentrations of with IP_3 (Shirakabe et al., 2006). It was found that an IRBIT concentration of approximately 0.1μM was required to inhibit IP_3 binding to the receptor by 50% (Ando et al., 2006). It can then be inferred that an increase in IP_3 concentration subsequently leads to the dephosphorylation and release of IRBIT from the IP_3R.

Since elevated intracellular levels of Ca^{2+} are responsible for a variety of cellular processes, IRBIT's function as a Ca^{2+} regulator is physiologically important. In addition to directly binding the IP_3R, IRBIT has been shown to bind other proteins downstream. Multiple studies over the past 15 years have elucidated some of the specific roles IRBIT plays in cellular regulation. The Arnaoutov group identified IRBIT as a novel regulator of ribonucleotide reductase (RNR) in eukaryotic cells (Arnaoutov and Dasso, 2014). RNR functions by supplying

a pool of deoxynucleotide triphosophates (dNTPs) that is utilized in DNA replication, and unregulated RNR can lead to tumor cell growth. It was found that the serine-rich binding domain of IRBIT interacts with RNR with high affinity in the presence of deoxyadenosine triphosphate (dATP) (Arnaoutov and Dasso, 2014). IRBIT acts as a stabilizer of dATP at the active site of RNR, thus functioning as an allosteric inhibitor of RNR activity. HeLa cells that lacked expression of IRBIT were shown to have an imbalanced pool of dNTPS and a disrupted cell cycle progression (Arnaoutov and Dasso, 2014).

IRBIT may also function as a regulator of Ca^{2+} induced apoptosis via its interactions with Bcl-2 proteins (Bonneau et al., 2016). Bcl-2 proteins have previously been shown to interact with IP_3Rs as a way of regulating Ca^{2+} release. Proper mitochondrial functioning is dependent upon Ca^{2+}, and this supply of Ca^{2+} comes from portions of the ER that are in direct contact with the mitochondria (known as MAMs for mitochondria-associated ER membranes) (Bonneau et al., 2016). If the amount of Ca^{2+} transferred to the mitochondria is too high, cytochrome c is released and apoptotic mechanisms are activated. With regards to this pathway, IRBIT functions by forming a regulatory complex with Bcl2l10 that binds to the IP_3R and prevents excess Ca^{2+} release from the ER to the mitochondria (Bonneau et al., 2016). It was also found that when HeLa cells were treated with apoptosis inducing agents, such as staurosporine (STS), IRBIT underwent dephosphorylation which prevented it from interacting with Bcl2l10, effectively promoting apoptotic mechanisms (Bonneau et al., 2016).

The experiment described in this paper aims to expand off of previous studies and to analyze the role of IRBIT in controlling Ca^{2+}-ion release. High expression levels of IRBIT have been detected in neurons, but few studies have actually analyzed IRBIT's role in living cells. Initially, this experiment attempted to utilize the CRISPR/Cas9 gene-editing technology in order

to generate a cell line that lacked functional expression of IRBIT. By developing an IBRBIT-KO (knockout), we would have been able to eliminate IRBIT's binding activity to the IP_3R. Therefore, it was expected that IP_3 signaling would work with maximum efficiency. We would have been able to test this hypothesis by utilizing confocal microscopy and analyzing the extent of calcium signaling activity in IRBIT-KO cells as well as wild-type (WT) cells. However, sequencing proved our genomic DNA to be invalid. Perhaps the efficiency of Cas9 at the IRBIT locus was too low, and so cellular machinery repaired the DNA to be wild-type.

Since the CRISPR/Cas9 procedure was unsuccessful, we were forced to alter the experiment. The cells were first stimulated with phorbol myristate acetate (PMA) and calyculin A (calyA). Since PMA functions by activating PKD and calyA is a phosphatase inhibitor, it was predicted that treatment with these agonists would activate IRBIT and thus reduce calcium release at the IP_3R. The cells were also transfected with IRBIT siRNA in order to decrease concentrations of the IRBIT protein. It was therefore expected that siRNA treatment would disinhibit calcium release at the IP_3R.

Methods

CRISPR-Cas9 – mediated generation of an IRBIT-knockout cell line

During a summer research project conducted by another Bates student, Brendan Mackey, an *IRBIT*-targeting construct was generated using a plasmid based-procedure. The 20 bp-long guide sequence was designed using the CRISPR Design tool (http://tools.genome-engineering.org) and was cloned into the pSpCas9 (BB)-2A- Puro vector (Addgene, Cambridge, MA) for co-expression with Cas9. This was done by cloning the *IRBIT*-specific guide nucleotides into pSpCas9 (BB)-2A-Puro vector according to the procedure referenced from Ran et al. (2013). The Plasmid Safe treated plasmid was transformed into a chemically competent E. coli strain and the generated CRISPR-*IRBIT* plasmid was validated by sequencing, which was performed at Mount Desert Island Biological Laboratory (MDIBL).

The created *IRBIT*-targeting construct was transfected into human embryonic kidney cells (HEK293) using Lipofectamine 3000 (Thermo Scientific) according to manufacturers' instructions and was treated with Puromycin to select for successfully transfected cells. After 72 hours of Puromycin treatment, surviving cells were serially diluted in EMEM medium without Puromycin to a final concentration of 0.5 cells per well. Wells were inspected for clonal appearance about 1 week after plating and wells with multiple cells were marked off. The cells were then incubated and allowed to expand for 2-3 weeks without Puromycin- selection pressure. Isolated cell clones were tested for IRBIT expression by Western blot.

Cell Culture

HEK293 cells were used because these cells express IRBIT endogenously and can be genetically modified (Ando et al., 2006; Ando et al., 2014). HEK293 cells were cultured in Eagle's Minimum Essential Medium (EMEM) using 10% fetal bovine serum and 100 U/ml

penicillin and 100 µg/ml streptomycin. Cells were passaged bi-weekly upon reaching 70-80% confluency.

Transfection

Cells were transfected with Lipofectamine 3000 (Thermo Scientific) according to manufacturer's instructions. The cells were grown to 60-80% confluency at transfection. 0.3 µg of PH-RFP DNA was added to 2.5 µl of P3000 Reagent in 10 µl Opti-MEM medium and the solution was mixed well. The diluted DNA was added to each tube of 3.75 µl diluted Lipofectamine 3000. The mixture was incubated for 5-10 minutes at room temperature. The DNA-lipid complex was added to the cells and after incubating the cells for 1-2 days, the transfected cells were analyzed.

Protein Isolation

Cytoplasmic proteins were isolated from HEK293-cells by spinning the cells down at 300*g and re-suspending them in 1 ml H_2O. The cell suspensions were frozen in liquid nitrogen and thawed at 37°C in a water bath. This was repeated twice and then the samples were centrifuged for 3 min at 300*g at 4°C. The supernatant was removed and spun again at 20,000*g for 20 minutes at 4°C. The pellet was resuspended in HEPES- lysis buffer (150 mM NaCl, 10 mM HEPES, 0.5% Triton X-100, pH 7.4).

Isolation of Genomic DNA and Polymerase Chain Reaction (PCR)

Genomic DNA of HEK293 cells was isolated with the PureLink Genomic DNA Isolation Kit (Life Technology, Carslbad, CA) according to manufacturer's instructions. The subsequent PCR reaction was performed with an Eppendorf EP Gradient Mastercycler PCR machine

(Eppendorf, Hamburg, Germany). The following pipetting scheme was utilized: 10 µl of Q5 reaction buffer (5X), 1 µl of 10 mM dNTPs, 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 1 µl of DNA, 0.5 µl of Q5 high fidelity DNA Polymerase, and 32.5 µl of nuclease-free H_2O. Samples were denatured at 98°C for thirty seconds. Afterwards, thirty-five cycles with the following profile were run: ten seconds at 98°C, thirty seconds at 64°C, and one minute at 72°C. Reactions were completed by a two-minute-long incubation at 72°C. Samples were stored at 4°C and then analyzed using gel electrophoresis.

SDS-PAGE

Approximately twenty micrograms of protein were separated by SDS-PAGE using Novex Nu-PAGE Bis-Tris 4-12% gels according to manufacturer's instructions (Life Technologies, Carlsbad, CA). Samples will be denatured at 70°C for 10 minutes. 50 ml of 20X NuPAGE MES Running Buffer were added to 950 ml of deionized water to prepare 1X SDS Running Buffer. To prepare the gel, the comb was removed and the gel wells were rinsed three times with 1X Running buffer. The gel wells were filled with 1X Running Buffer. The samples were loaded on the gels, then the protein size standard was loaded. The upper (200 ml) and lower (600 ml) buffer chambers were filled with 1X Running Buffer. Then the gel ran for 90 minutes at 100 V constant in MES Running Buffer at room temperature.

Western Blot Analysis

Western blots were performed to detect IRBIT protein using wet protein transfers as described in Kruse et al (Kruse et al., 2016). A monoclonal anti-IRBIT antibody (Abcam, Cambridge, MA) was used at a dilution of 1:10,000 and was detected using a horseradish peroxidase-coupled secondary antibody (1:5000, Santa Cruz Biotechnology) and

chemiluminescence (Pierce ECL Western Blotting Substrate, Pierce Protein Biology Products, Thermo Scientific).

Preparing Glass Chips for Confocal Microscopy

Glass pieces (#0 thickness, Thermo Scientific, Waltham, MA) of 4x4 mm size were washed once with 70% ethanol and twice with distilled water. Glass chips were incubated for 30 minutes with poly-L-lysine solution (Sigma-Aldrich) were then washed twice with distilled water. The chips were dried under the cell culture hood and sterilized by exposure to UV-light for at least 30 minutes. Lastly, the chips were stored at room temperature.

Plating for Confocal Microscopy

All medium from a 35 mm dish was removed and 400 µl of 0.05% Trypsin was added for 40 seconds. One ml of regular medium containing FBS was added and the cells were then washed at the bottom of the dish. The cell suspension was transferred to a 15 ml tube and spun for 60 seconds at 250*g. The supernatant was removed and the cells were resuspended in 1 ml of regular medium. Then 10-15 chips were placed into a 35 mm dish and 2 ml of the regular medium was added. 150-180 µl of cell suspension was added to the dish and the cells were incubated overnight at 37C.

Confocal Microscopy

Confocal microscopy was used to visualize Ca^{2+} signaling in HEK293 cells *in vitro*. The procedure is described in Jensen et al (Jensen et al., 2009). Briefly, the cells were imaged using a Leica TCS SP8 confocal microscope with a 63x oil-immersion objective. The cells were stimulated with agonists (100µM ATP, 190ng/ml phorbol myristate acetate, 10nM Calyculin A) for endogenously expressed Gαq-coupled GPCRs, and changes in cytoplasmic calcium levels

were detected by usage of calcium-sensitive fluorescent dye Fluo-4 (Life Technologies, Carslbad, CA) according to manufacturer's instructions.

Data Analytics

Image J was used to analyze duration and amplitude of Ca^{2+} signals. Student's t-test or 2-way ANOVA tests was used to test for statistical significance. P-values below 0.05 were considered significant.

Results

A Western blot was conducted with HEK293 cells as well as HT22 cells, an immortalized cell line derived from murine hippocampal neurons, and the results showed expression of IRBIT and GAPDH (Fig. 1). Since neuronal cells were unavailable for this experiment, the Western demonstrated that both HEK293 cells and HT22 cells can function as comparable systems for neuronal cells that endogenously express *IRBIT*. Given their ease of transfection and genetic manipulation, HEK293 cells were chosen as a suitable model for all experiments.

The research for this experiment was initially focused on generating an IRBIT-KO cell line using CRISPR/Cas9. This portion of the experiment was conducted by another Bates student, Brendan Mackey, as part of a summer research grant. 21 potential IRBIT knock-out cell lines were obtained and were subsequently tested for alterations of the *IRBIT* gene locus.

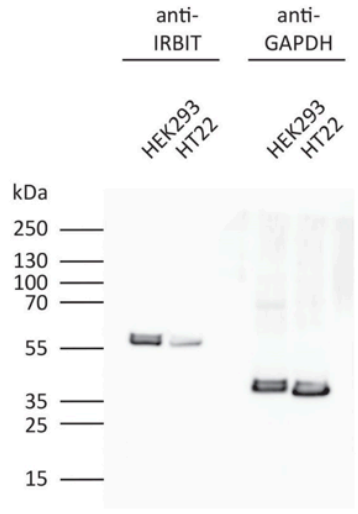

Figure 1. Western blot of HEK293 and HT22 cells showing expression of IRBIT and GAPDH.

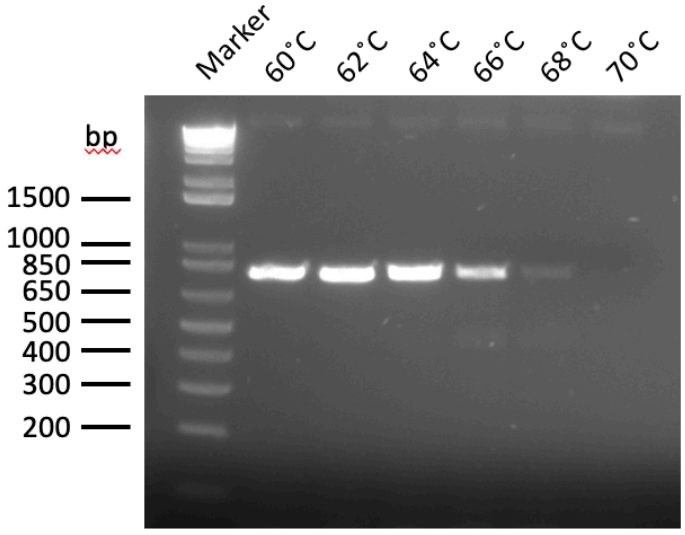

Figure 2. Results of a polymerase-chain reaction (PCR) of genomic IRBIT DNA visualized by gel electrophoresis. Six different annealing temperatures were tested (in degrees Celsius: 60, 62, 64, 66, 68, 70). The target fragment size was 803 base pairs.

In order to do this, a polymerase chain reaction (PCR) was run in order to amplify the fragment of *IRBIT* to be used for DNA sequencing of all 21 clones. To determine the optimal annealing temperature for this reaction, a preliminary PCR was run on genomic *IRBIT* DNA. A gel electrophoresis showed that some digests of genomic DNA produced smears of greater intensity than others (Fig. 2, lanes 3 and 4), and 64°C was the determined optimal temperature. The presence of the *IRBIT* gene was verified in all 21 clones through gel electrophoresis as evidenced by the presence of bands at the target fragment size of 803 base pairs (Fig. 3). Isolation of genomic DNA followed by sequencing of the IRBIT locus revealed that all puromycin-sensitive clones had a wild-type genomic DNA sequence for IRBIT.

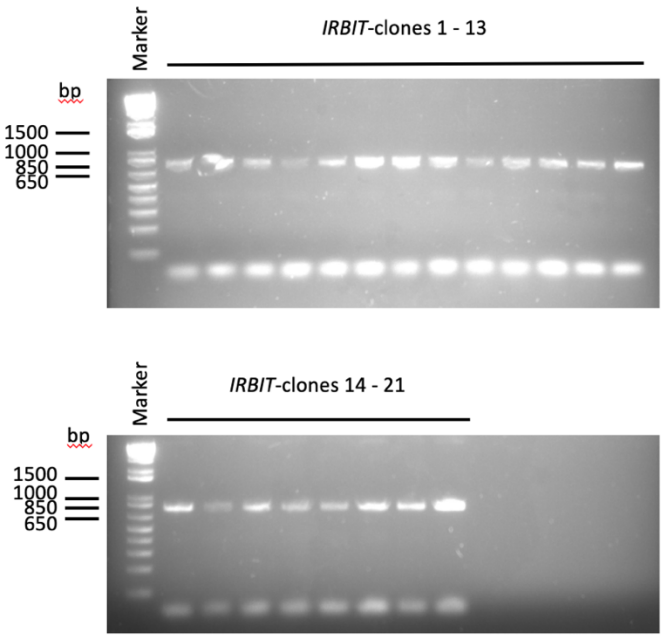

Figure 3. Results of a PCR of 21 *IRBIT* clone samples visualized by gel electrophoresis. The target fragment size was 803 base pairs.

Rather than continuing with the development of a CRISPR/Cas9 mediated *IRBIT*-KO cell line, subsequent experiments focused on manipulating the activity and concentration of the IRBIT protein. Previous studies have shown that phosphorylation events are critical for IRBIT's binding activity at the IP_3R (Ando et al., 2006; Shirakabe et al., 2006). Specifically,

phosphorylation of Ser68 allows for subsequent phosphorylation events at Ser71, Ser74, and Ser77. Dephosphorylation of IRBIT has been shown to reduce IRBIT's ability to bind at the IP_3R. It is hypothesized that protein kinase D (PKD) phosphorylates IRBIT while protein phosphatase I (PPI) dephosphorylates *IRBIT* in vivo.

The first experiment compared the effects of phorbol myristate acetate (PMA) and calyculin A (CalyA) on the intensity of ATP-induced calcium signaling in wild-type cells. PMA is a known activator of PKD, which are hypothesized to function by phosphorylating the serine-rich region of *IRBIT* and activating the protein. Additionally, CalyA is a known PPI inhibitor,

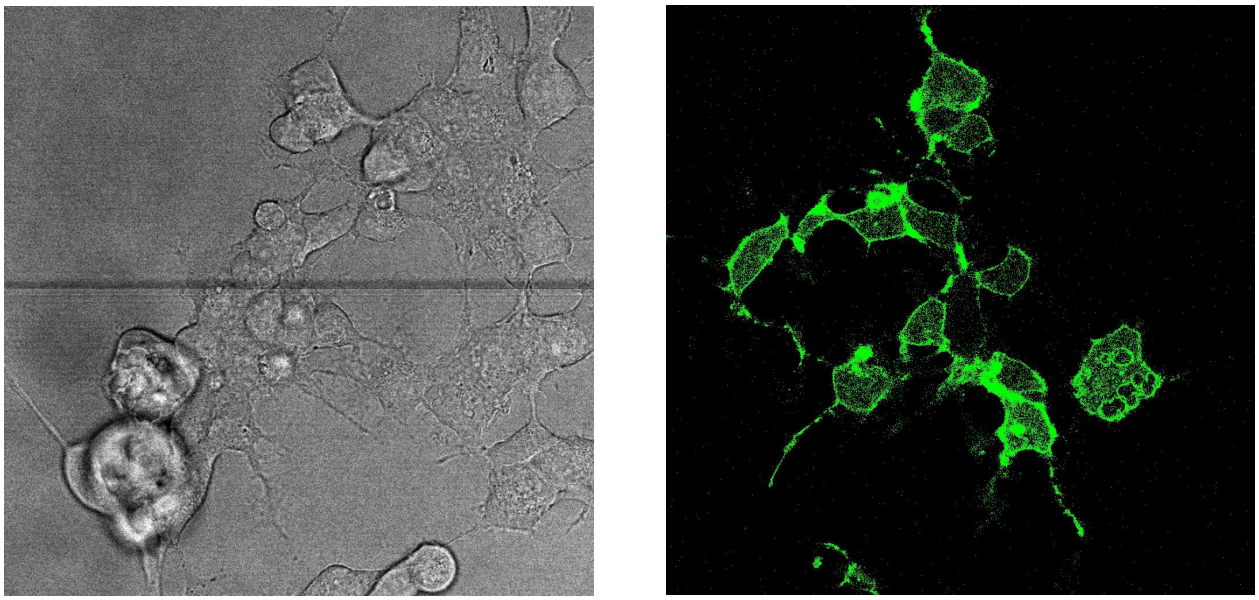

Figure 4. Left: HEK293 cells used in Ca^{2+} imaging confocal microscopy experiments. Right: PH-RFP transfected HEK293 cells visualized using confocal microscopy.

which theoretically would prevent the dephosphorylation, and thus deactivation, of IRBIT. The second experiment analyzed the effect of *IRBIT* siRNA on the intensity of calcium signaling. Since the CRISPR/Cas9 generation of an IRBIT-KO failed, *IRBIT* siRNA was instead utilized in

an effort to reduce cellular concentrations of *IRBIT*. It was expected that the IBRIT siRNAwould target IRBIT mRNA for degradation and thus decrease *IRBIT's* function in vitro. Both of the experiments were conducted with PH-RFP transfected HEK293 cells as a marker for successfully transfected cells (Fig. 4).

The procedure regarding confocal microscopy remained the same for both of the conducted experiments (Fig. 5). In the first experiment, the control cells were treated with ATP to activate endogenous purinergic receptors, thereby resulting in a hydrolysis of $PI(4,5)P_2$ and generation of cytoplasmic IP_3. These cells showed a substantial calcium response, with the peak of calcium signaling intensity occurring approximately 24 seconds after agonist application. The cells treated with PMA/CalyA showed no calcium signaling response following agonist treatment. There was a statistically significant difference between the two conditions in terms of signaling intensity (unpaired t-test; p = .0439; mean +/-SEM; controls n = 24; PMA/CalyA n = 10) (Fig. 6).

In the second experiment, *IRBIT* siRNA cells treated with ATP showed a signaling pattern similar to the control group. However, the standard error of the mean among cell samples for the siRNA group at the peak signal intensity was significantly higher than that of the control group. However, statistical analysis revealed that there was no significant difference in intensity between control and *IRBIT* siRNA cells (unpaired t-test; p = .2658; mean +/-SEM; controls n = 24; *IRBIT* siRNA n = 9) (Fig. 6). *IRBIT* siRNA cells treated with PMA/CalyA showed a weaker and more delayed calcium signaling response compared to both the control group and the *IRBIT* siRNA cells treated with ATP. The peak signal intensity for the *IRBIT* siRNA PMA/CalyA group occurred approximately 36 seconds after onset of agonist application. Statistical analyses revealed that there was no significant difference in intensity between *IRBIT* siRNA and *IRBIT*

siRNA PMA/CalyA cells (unpaired t-test; p = .9810, mean +/- SEM; *IRBIT* siRNA n = 9 ; *IRBIT* siRNA PMA/CalyA n = 10) (Fig. 6).

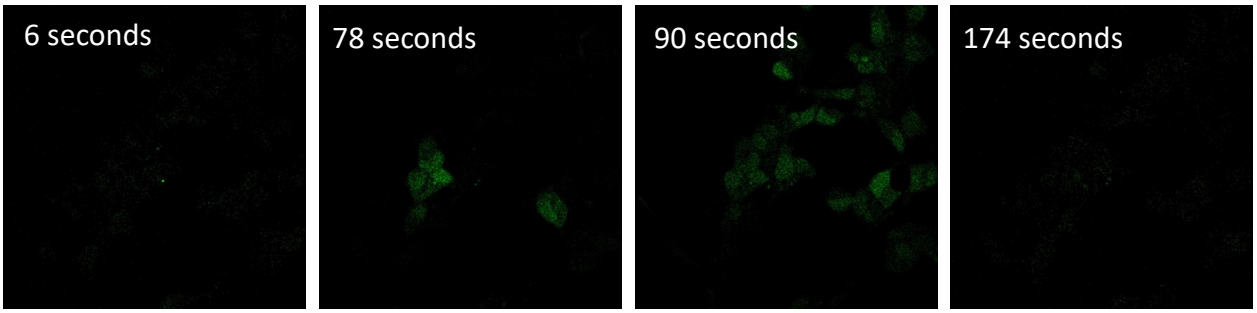

Figure 5. The calcium-sensitive fluorescent dye fluo-4 was used to visualize Ca^{2+} release from the endoplasmic reticulum (ER) in HEK293 cells using confocal microscopy. Ringer's solution was applied for 60 seconds followed by a 20 second application of 100µM ATP. Cells were then rinsed with Ringer's solution for the remainder of the imaging experiment (180 seconds).

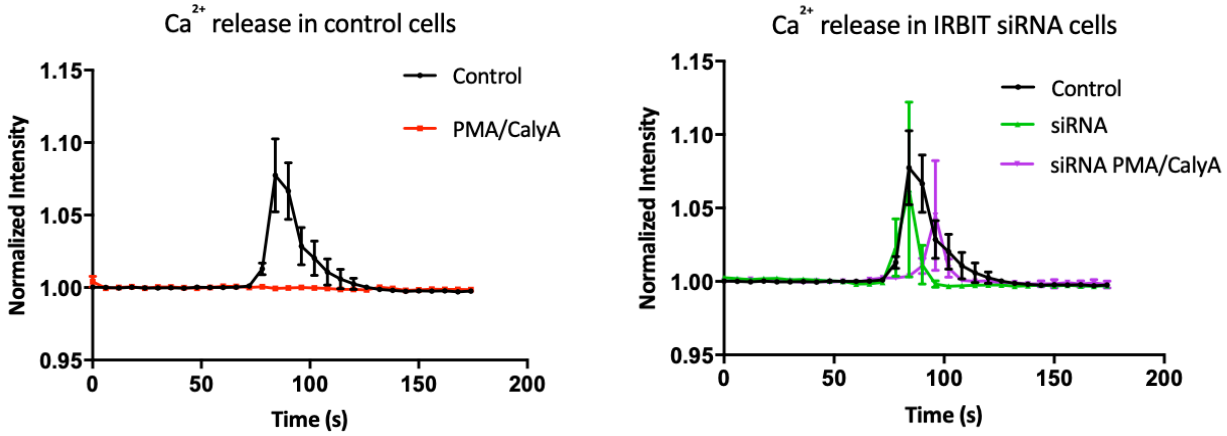

Figure 6. Left: Fluorescence intensity of fluo-4 in control cells and cells treated with PMA/Calyiculin A. There is a significant difference in intensity between control and PMA/CalyA cells (controls n = 24; PMA/calyA n = 10). Right: Fluorescence intensity of fluo-4 in control cells, IRBIT siRNA cells, and IRBIT siRNA cells treated with PMA/CalyA (right). There is no significant difference in intensity between control and *IRBIT* siRNA cells (controls n = 24; *IRBIT* siRNA n = 9) nor between *IRBIT* siRNA and *IRBIT* siRNA PMA/CalyA cells (*IRBIT* siRNA n = 9 ; *IRBIT* siRNA PMA/CalyA n = 10).

Discussion

Regarding the first experiment, it was initially hypothesized that treatment with PMA/CalyA would significantly reduce the intensity of calcium signaling response in the HEK293 cells. Since PMA activates PKD, it was expected that the PKD would phosphorylate IRBIT at Ser68, leading to subsequent phosphorylation events at Ser71, Ser74, and Ser77. This phosphorylation at the serine-rich region of the protein was expected to activate the protein, ultimately allowing IRBIT to bind at the IP_3R and restrict Ca^{2+} release from the ER. Further, it was expected that CalyA, a known inhibitor of PPI, would function by reducing dephosphorylation events at the serine-rich region of the protein, thus increasing IRBIT's ability to bind to the receptor.

The results from the first experiment provide evidence to support this initial hypothesis. Treatment of the HEK293 cells with PMA/CalyA entirely eliminated any sort of calcium signaling event compared to the control cells treated with ATP. On the surface, it is enticing to believe that IRBIT is entirely responsible for the lack of signaling response. However, while these results seem to confirm IRBIT's hypothesized role with respect to the IP_3R, they are somewhat inconclusive. PKD is an evolutionarily conserved protein kinase family and is known to mediate a wide array of biological functions (Rozengurt, 2011). For example, previous studies have demonstrated that PKD may play a role in signal transduction, membrane trafficking, and secretion (Rozengurt, 2011). Additionally, nearly two thirds of all of the protein kinases that are encoded by the human genome are serine/threonine kinases. Therefore, IRBIT is a protein far from unique in that it is activated by serine phosphorylation. Since PKD has been shown to activate a variety of proteins implicated in many different cellular processes, it is possible that results seen from the first experiment cannot be fully explained by a change in IRBIT's activity.

Additionally, PPI is a major eukaryotic protein phosphatase that has been shown to regulate a huge range of cellular functions. It is established that PPI is responsible for the dephosphorylation of thousands of different proteins within the cell (Cohen, 2002). Similar to the grey area described regarding the effects of PKD, it is also possible that the lack of observed calcium signal cannot simply be traced back to IRBIT.

Regarding the second experiment, it was initially hypothesized that cells transfected with *IRBIT* siRNA would show comparable calcium signaling intensity compared to the control cells. siRNAs function by cleaving the mRNA of interest before translation and have 100% complementarity, allowing them highly specific targeting capabilities. It was therefore expected that *IRBIT* siRNA would function by cleaving *IRBIT* mRNA, thus substantially reducing overall intracellular concentrations of IRBIT. A decrease in the levels of the protein would theoretically increase IP_3's ability to bind to the IP_3R, effectively enabling the release of Ca^{2+} from the ER at the receptor.

The results from this experiment provide evidence to support the initial hypothesis. HEK293 cells transfected with *IRBIT* siRNA showed no significant difference in terms of calcium signaling intensity compared to the control cells. However, there was a significant amount of variation among the *IRBIT* siRNA cells used in the confocal experiments with respect to calcium signaling patterns. While the mean calcium signal for these cells closely resembled that of the control cells, nearly half of the *IRBIT* siRNA cells failed to elicit any sort of calcium signal response. It is therefore unreasonable to conclude that the results can be explained by the initial hypothesis. Further, this experiment failed to accurately quantify the effects of *IRBIT* siRNA transfection in reducing intracellular levels of IRBIT. If the *IRBIT* siRNA was successful in reducing translation of *IRBIT* mRNA, we are unsure of the extent to which this occurred.

Perhaps the results shown are consistent with and provide support for the initial hypothesis, but further experiments are required in order to isolate the effect of decreasing intracellular IRBIT concentrations.

It was also hypothesized that cells transfected with *IRBIT* siRNA and treated with PMA/CalyA would show comparable calcium signaling intensity compared to both the *IRBIT* siRNA cells and the control cells. Since *IRBIT* siRNA transfection would theoretically reduce intracellular concentrations of IRBIT, treatment with PMA/CalyA would have little effect on the binding activity of IRBIT due to the sheer decrease in concentration of the protein. IP_3 would therefore successfully outcompete IRBIT and bind to the IP_3R, effectively stimulating the release of Ca^{2+} from the ER.

This hypothesis was supported by the observed results. There was no significant difference in calcium signaling intensity among *IRBIT* siRNA cells treated with PMA/CalyA, *IRBIT* siRNA cells, and control cells. The most notable result from this experiment was that the *IRBIT* siRNA cells treated with PMA/CalyA showed a slight delay (~ 18 seconds) in terms of peak calcium signaling intensity compared to the other two cell groups. Similar to the previous experiment, we were unable to accurately quantify the effects of *IRBIT* siRNA transfection in reducing levels of IRBIT. Assuming that this transfection did not completely eliminate IRBIT, a low concentration of the protein was most likely still present within the cell. It is possible that the observed delay in calcium signaling can partially be explained by the activating effects of PMA/CalyA on the IRBIT protein. Despite decreased levels of IRBIT, application of PMA/CalyA could have stimulated phosphorylation at the serine-rich region of the protein, allowing IRBIT to bind to the IP_3R and prevent the release of Ca^{2+} at the receptor. Upon cellular stimulation, IP_3 would have then been forced to outcompete IRBIT in binding to the IP_3R in order to trigger the release of

Ca^{2+}. This competition between IRBIT and IP_3 at the IP_3R serves as a plausible explanation for the slight delay in calcium signaling response in the *IRBIT* siRNA cells treated with PMA/CalyA.

All of the initial hypotheses for the experiments were loosely supported by the observed results, but the results remain far from conclusive. It is imperative that future research endeavors adopt a more highly targeted approach in order to elucidate IRBIT's role in regulating Ca^{2+} release. In fact, there are a few central questions that remain unanswered regarding the extent of IRBIT's involvement in the IP_3R cascade. It is widely hypothesized that IRBIT binds to the IP_3R at rest to prevent the random release of Ca^{2+} at the receptor, but this has yet to be confirmed *in vivo.* Additionally, the intracellular concentration of IRBIT required to successfully outcompete IP_3 at the IP_3R binding domain is unknown.

In order to improve upon the specificity of this experiment, it is necessary to quantify the effect of *IRBIT* siRNA transfection on changes in intracellular IRBIT concentration. The results from this experiment do not provide sufficient evidence to determine whether or not the transfection was actually successful in preventing translation of *IRBIT* mRNA. If it was successful, the extent to which protein concentrations were reduced remains unknown. Future research could incorporate Western blot procedures as a way of tracking changes in the IRBIT concentrations before and after the siRNA transfection. This would allow researchers to directly compare intracellular concentrations of IRBIT to observed changes in calcium signaling intensity.

A CRISPR/Cas9 generated *IRBIT*-KO cell line would be one of the most effective tools to study IRBIT in vitro. Potential research projects could utilize confocal microscopy to compare calcium signaling intensity in *IRBIT*-KO cells with WT cells. This would allow researchers to

experimentally isolate IRBIT and observe the effect of a loss of IRBIT on calcium patterns. This technique would also help determine IRBIT's role in binding to the IP_3R and restricting Ca^{2+} release at rest. While perhaps unfeasible at the time, the creation of an *IRBIT*-KO mouse would afford researchers the ability to study IRBIT *in vivo*.

Previous studies have demonstrated that IRBIT is most highly expressed in the brain, and immunochemistry in mouse brains revealed that the protein is particularly concentrated in the cerebellum and hippocampus (Ando et al., 2009; Ando et al., 2014). This data does not necessarily provide information about the extent of IRBIT's function, but it does suggest that the protein may serve an important neuronal role. Ca^{2+} has been shown to be involved in multiple cellular signaling pathways such as the regulation of gene expression, synaptic transmission, cellular death, as well as learning and memory (Kawamoto et al., 2012). Extremely precise control of intracellular ion concentration is required to execute these functions properly, and so IRBIT's antagonist binding activity at the IP_3R could potentially serve a very important physiological purpose.

Slight dysfunction in typical Ca^{2+} signaling pathways within the brain can have a profound effect on neuronal processes. Previous research has shown that the development of neurodegenerative diseases (AD, HD, etc.) is often associated with a disruption in the homeostasis of Ca^{2+} related events (Bezprozvanny, 2010). By more closely examining the role of IRBIT in regulating intracellular Ca^{2+} stores, perhaps we can further elucidate the mechanisms of neuronal Ca^{2+} regulation and pave the way for the development of novel therapeutic interventions.

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