Camkii-Mediated Phosphorylation of the Inositol 1,4,5-Trisphosphate Receptor at Serine-150 Results in Decreased Channel Activity

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CAMKII-MEDIATED PHOSPHORYLATION OF THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR AT SERINE 150 RESULTS IN DECREASED CHANNEL ACTIVITY

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN CELL AND MOLECULAR PHYSIOLOGY

BY

JOSHUA T MAXWELL

CHICAGO, IL

DECEMBER 2010
ACKNOWLEDGEMENTS

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<td>[ATP]_FREE</td>
<td>Free ATP concentration</td>
</tr>
<tr>
<td>[Ca^{2+}]</td>
<td>Ca^{2+} concentration</td>
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<tr>
<td>[Ca^{2+}]_FREE</td>
<td>Free Ca^{2+} concentration</td>
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<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular Ca^{2+} concentration</td>
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<td>[InsP_3]</td>
<td>D-myo-inositol 1,4,5-trisphosphate concentration</td>
</tr>
<tr>
<td>2-APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>AdA</td>
<td>Adenophostin A</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>AIP</td>
<td>Autocamtide-2 inhibitoty peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Ba^{2+}</td>
<td>Barium ion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ion</td>
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<tr>
<td>Ca^{2+}/CaM</td>
<td>Ca^{2+}-Calmodulin complex</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca^{2+}-calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CDF</td>
<td>Ca^{2+}-dependent facilitation</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
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<tr>
<td>Cs^+</td>
<td>Cesium ion</td>
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<tr>
<td>C-terminus</td>
<td>Carboxyl terminus</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed after depolarization</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>ECC</td>
<td>Excitation-contraction coupling</td>
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<td>ECL</td>
<td>Enhanced chemi-luminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ETC</td>
<td>Excitation-transcription coupling</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FIRE</td>
<td>Fluorescent InsP_3-Responsive Element</td>
</tr>
<tr>
<td>FLAG</td>
<td>DYKDDDDK epitope</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N′-(4-butanesulfonic acid)</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBC</td>
<td>InsP&lt;sub&gt;3&lt;/sub&gt; binding core</td>
</tr>
<tr>
<td>IICR</td>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;-induced calcium release</td>
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<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>D-myo-inositol 1,4,5-trisphosphate</td>
</tr>
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<td>Ins(1,4,5)P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>D-myo-inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>D-myo-inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R1</td>
<td>Type 1 isoform</td>
</tr>
<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R2</td>
<td>Type 2 isoform</td>
</tr>
<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R2-KO</td>
<td>Type 2 isoform knock-out</td>
</tr>
<tr>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R3</td>
<td>Type 3 isoform</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Disassociation constant</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>NXC</td>
<td>Sodium/calcium exchanger</td>
</tr>
<tr>
<td>P&lt;sub&gt;o&lt;/sub&gt;</td>
<td>Open probability</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent Protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT2</td>
<td>InsP&lt;sub&gt;3&lt;/sub&gt;R2 baculovirus</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S150A</td>
<td>Serine-150 to Alanine mutation (non-phosphorylatable)</td>
</tr>
<tr>
<td>S150E</td>
<td>Serine-150 to Glutamate mutation (phosphomimetic)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/endoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase</td>
</tr>
<tr>
<td>SD</td>
<td>Suppressor domain</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SR/ER</td>
<td>Sarcoplasmic reticulum/endoplasmic reticulum</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Salmon sperm DNA</td>
</tr>
<tr>
<td>T1NH</td>
<td>Amino terminal type 1 antibody</td>
</tr>
<tr>
<td>T2NH</td>
<td>Amino terminal type 2 antibody</td>
</tr>
<tr>
<td>T2C</td>
<td>Carboxyl terminal type 2 antibody</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline Tween-20</td>
</tr>
<tr>
<td>TG</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>TMR</td>
<td>Transmembrane region</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>T-octylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Calcium plays an important role in the physiology of the cell, impinging upon a multitude of cellular events ranging from fertilization to cell proliferation and even cellular death (Berridge et al., 2000). Therefore, intracellular calcium storage and regulated release is an important and physiologically relevant topic to address. One mechanism by which calcium is mobilized from intracellular stores is via the activation of inositol 1,4,5-trisphosphate receptors (InsP₃Rs) by the second messenger, inositol 1,4,5-trisphosphate (InsP₃). InsP₃ is produced when one of many neurotransmitters, hormones, or growth factors bind to G protein-coupled receptors on the plasma membrane, causing activation of phospholipase C. Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate into InsP₃ and diacylglycerol, thus allowing InsP₃ to diffuse through the cytoplasm to bind to its receptor (Mignery et al., 1992). InsP₃Rs have a broad tissue and cellular distribution, with the receptor most commonly localized to the endoplasmic reticulum (ER) membrane. InsP₃Rs can be of three isoforms (type 1-3), and are structurally very similar to each other. Activation of InsP₃Rs leads to release of calcium into the cytoplasm of the cell, where it can activate a multitude of signal transduction cascades and a variety of local and global cellular events.

One such enzyme activated by a rise in intracellular $[\text{Ca}^{2+}]$ is $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II (CaMKII). CaMKII is a multifunctional Serine/Threonine
protein kinase involved in many signaling pathways in various cell types (reviewed in Maier and Bers, 2002). The enzyme is composed of homomultimers or heteromultimers of 6-12 subunits, with the subunit isoforms being derived from a family of four closely related genes ($\alpha$, $\beta$, $\delta$, $\gamma$) (Hudmon et al., 2005). All isoforms contain an amino-terminal catalytic domain where ATP binds, a central regulatory domain containing an auto-inhibitory and calmodulin binding sites, and a carboxyl-terminal association domain.

The $\delta$ and $\gamma$ isoforms of CaMKII are the predominant isoforms found in the heart where they have been implicated in regulating cardiac gene expression. Recent results from our lab have shown that the predominant InsP$_3$R isoform in the heart, the InsP$_3$R2, is primarily targeted to the nuclear envelope in ventricular myocytes. Here it forms a macromolecular complex with the nuclear-localized cardiac isoform of CaMKII, CaMKII$\delta_B$ (Bare et al., 2005). Upon stimulation of InsP$_3$ production, Ca$^{2+}$ released through the InsP$_3$R2 activates CaMKII$\delta_B$, allowing it to act on downstream targets, such as histone deacetylases 4 & 5 (HDAC4 & HDAC5) (Wu et al., 2006; Little et al., 2007).

The results of this study and others (Zhu et al., 1996; Matifat et al., 2001; Wu et al., 2006) suggest that the activity of InsP$_3$Rs can be inhibited by CaMKII-mediated phosphorylation. Furthermore, the N-terminal 1078 amino acids of the InsP$_3$R2 have been shown to interact with, as well as be phosphorylated by CaMKII (Bare et al., 2005). However, it has not yet been established what amino acid(s) of InsP$_3$Rs is/are phosphorylated.

In this study, I use exogenously expressed fragments of the InsP$_3$R2 and site-directed mutagenesis to show that CaMKII can phosphorylate the InsP$_3$R2 at Serine-150
and determine that phosphorylation of this residue is responsible for modulation of channel activity. Non-phosphorylatable (S150A) and phospho-mimetic (S150E) mutations were constructed in the full-length InsP₃R2, expressed in COS cells and studied in planar lipid bilayers. Upon treatment with CaMKII, the non-phosphorylatable channel showed no decrease in activity. Conversely, the phosphomimetic channel displayed a very low $P_o$ under normal recording conditions in the absence of CaMKII (2 μM InsP₃ and 250 nM [Ca$^{2+}$]_FREE), thus mimicking a channel that has been phosphorylated by CaMKII. The results of this study show for the first time that Serine-150 of the InsP₃R2 is phosphorylated by CaMKII and results in a decrease in the channel’s open probability. My hypothesis for the mechanism of the regulation of the InsP₃R2 is that phosphorylation either alters the affinity for InsP₃ at the ligand binding site and/or perturbs the intramolecular interactions necessary for proper ligand-induced gating, and this hypothesis is currently being tested.
CHAPTER ONE
REVIEW OF RELEVANT LITERATURE

A. INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR

1. InsP₃R Identification and Structure

Inositol 1,4,5-trisphosphate receptors (InsP₃Rs) are a family of highly conserved intracellular Ca²⁺ release channels, with each channel being a tetramer of four ~313 kDa subunits (reviewed in Bezprozvanny, 2005; Berridge, 2009). This protein was first purified from rat cerebellum and determined to have a high affinity and specificity for inositol 1,4,5-trisphosphate (InsP₃) (K_d ~100nM) when compared to other inositol phosphates (Worley et al., 1987; Supattapone et al., 1988). The type 1 inositol 1,4,5-trisphosphate receptor was cloned from mouse cerebellum and described by two groups simultaneously (Furuichi et al., 1989; Mignery et al., 1989), while functional studies showed that the membrane fraction of cells overexpressing the recombinant protein had enhanced InsP₃ binding and InsP₃-induced Ca²⁺ release capabilities (Miyawaki et al., 1990). The ligand InsP₃ is produced when one of many neurotransmitters, hormones, or growth factors bind G protein-coupled receptors on the plasma membrane, causing activation of phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-
bisphosphate into InsP$_3$ and diacylglycerol. Diacylglycerol modulates protein kinase C activity, and InsP$_3$ can diffuse through the cytoplasm to bind to its receptor (Mignery et al., 1992). This pathway is commonly referred to as the PLC-InsP$_3$ pathway. For example, endothelin-1 (ET-1) is a physiological hypertrophic agonist that activates plasma membrane G protein-coupled receptors, producing the soluble second messenger InsP$_3$ (Mignery et al., 1990). InsP$_3$ diffuses through the cytoplasm to activate InsP$_3$R isoforms found in the cell and also has been shown to diffuse into the nucleus (Remus et al., 2006).

InsP$_3$Rs can be of three isoforms (type 1-3), and are structurally very similar to each other (Mignery et al., 1992). The three isoforms of the InsP$_3$R are encoded by three separate genes (Furuichi et al., 1989; Mignery et al., 1989), and each isoform of the InsP$_3$R is encoded by a ~10kb RNA sequence leading to an approximately 2700-2800 amino acid protein. This protein along with three other monomers forms the functional InsP$_3$R channel which is a tetramer of four subunits (Nakagawa et al., 1991; Sudhof et al., 1991; Blondel et al., 1993). InsP$_3$Rs have a broad tissue and cellular distribution, with the receptor most commonly localized to the endoplasmic reticulum (ER) membrane. Often, multiple isoforms are expressed within a single cell allowing for isoform specific signaling and redundancy of function.

A three-domain model for InsP$_3$Rs has been proposed based on mutagenesis studies of the receptor (Figure 1). The receptor is divided into an amino-terminal ligand-binding domain, a central coupling or modulatory domain, and a carboxyl-terminal Ca$^{2+}$ channel domain (Mignery et al., 1990). The localization of InsP$_3$ binding to the NH$_2$-terminus was established using recombinant, truncated proteins containing larger and
Figure 1: Three-domain model of InsP$_3$R.

The model shows that the InsP$_3$R contains an N-terminal ligand binding domain which is comprised of an inhibitory region (in red) and the InsP$_3$ binding core, a C-terminal channel domain containing six transmembrane regions, and an intervening $\sim$1400 amino acid modulatory region which couples ligand binding to channel gating and contains many sites for modulation of this interaction. When compared to the type 1 InsP$_3$R isoform, the type 2 and type 3 InsP$_3$Rs are 69 and 64% identical, respectively, and the greatest divergence occurs in the Coupling/Modulatory region.
larger portions of the NH$_2$-terminus (Mignery et al., 1990; Mignery and Sudhof, 1990). These and other studies determined that a truncated portion of the type 1 receptor containing the first 586 residues had the same InsP$_3$ affinity and selectivity as the endogenous, full length type 1 receptor (Newton et al., 1994). Additional observations from deletion mutagenesis studies of this region of the receptor showed that removal of the first 225 residues increased this mutant construct’s affinity for InsP$_3$ 10-100 fold in comparison to the endogenous receptor. However, InsP$_3$Rs lacking this region did not form functional channel units (Yoshikawa et al., 1996). Thus, the region encompassing the amino acids from 1-225 were determined to play a role in the prevention of InsP$_3$ binding and termed the “suppressor domain”, while the amino acids from 226-586 were considered the InsP$_3$ “binding core”. The crystal structures of the suppressor domain (SD) and InsP$_3$ binding core (IBC) of the mouse type 1 receptor have been elucidated (Bosanac et al., 2002; Bosanac et al., 2005). The binding core can be broken into two distinct domains; a β-trefoil domain comprised of amino acids 225-436, and an α-helical domain comprised of amino acids 437-586. InsP$_3$ has been proposed to bind at the interface of the two, with basic residues from each contributing to binding and affinity. Specifically, phosphates 1 and 5 of the InsP$_3$ molecule interact with residues in the α-helical domain, while phosphate 4 interacts with the β-trefoil domain of the IBC (Bosanac et al., 2002). The suppressor domain also contains a β-trefoil domain referred to as the “head”, with an intervening helix-loop-helix region forming the “arm”. The suppressor domain may function to both modulate the binding core and control channel gating through potential intramolecular interactions, as recent studies have shown that the
N-terminal 340 amino acids to interact specifically with the S4-S5 linker region in the type 1 InsP$_3$R (Schug and Joseph, 2006).

The coupling/regulatory domain of the three InsP$_3$R isoforms is the least conserved region, possibly attributing to differences in regulation between the various isoforms (Mignery et al., 1992). This domain also contains ATP-binding sites, PKA phosphorylation sites, and a proposed Ca$^{2+}$-sensing region on the type 1 receptor. Type 2 and type 3 receptors have 69% and 64% overall amino acid sequence identity, respectively, to the type 1 receptor. The receptor subtypes exhibit differential affinities for InsP$_3$, with the type 2 receptor having nearly three fold greater affinity when compared to type 1. The type 3 receptor exhibits the lowest affinity for InsP$_3$ (see Figure 2A).

The C-terminal region of the receptor is comprised of the channel-forming domain. This domain is proposed to be comprised of six transmembrane helices and includes the pore forming region of the InsP$_3$R that has been localized to the S5-S6 linker. Following this region, there is a short cytoplasmic tail at the extreme C-terminus of the InsP$_3$R. Helices 5 and 6 seem to be critical for forming the basic pore structure and selectivity filter of the InsP$_3$R. Deletion of trans-membrane regions (TMR) 1-4 leaves only TMR-5 and 6, which are still able to form channels with normal conductance and selectivity characteristics (Ramos-Franco et al., 1999). Ligand binding to the N-terminal ligand binding domain is translated into gating of the InsP$_3$R channel (Mignery and Sudhof, 1990), opening the channel pore and releasing Ca$^{2+}$ from its intracellular stores as described above. Targeting of the InsP$_3$R1 to the endoplasmic reticulum membrane has been localized to transmembrane regions 1 and 2 of the receptor, however, any
Figure 2: Summary of InsP$_3$- and Ca$^{2+}$-dependence of the three InsP$_3$R isoforms.

(A) The type 2 receptor has the highest affinity for InsP$_3$, with the type 1 at approximately 4 fold lower than that and the type 3 about at least 10 x lower than the others. These measurements were done at 250 nM cytosolic Ca$^{2+}$. (B) The type 1 InsP$_3$R exhibits a bell-shaped Ca$^{2+}$ dependence, with highest channel activity at ~1 μM Ca$^{2+}$. The type 2 and type 3 receptors show little inhibition of channel activity at higher [Ca$^{2+}$]. Note that for (A) and (B) single channel data for the type 1, 2, and 3 InsP$_3$R channels is from Ramos-Franco et al., 1998b (red and blue curves) and Hagar et al., 1998 and Hagar & Ehrlich, 2000 (black dashed curves). Open probability (P$_o$) for [InsP$_3$]-dependence of type 3 is scaled up 5.5 х so the indicated ratio of maximum P$_o$ for type 3 / type 1 is as reported by Ehrlich’s group (measured maximum P$_o$ was 0.08, from Hagar & Ehrlich, 2000). (Figure modified from Bers D.M., Excitation-Contraction Coupling and Cardiac Contractile Force, 2003)
combination of two tandem TMRs linked by their luminal loops are sufficient to ensure proper targeting to the ER membrane (Pantazaka and Taylor, 2009). This allows the N-terminal ligand-binding domain to fold in the cytosol into a functional unit before the protein is anchored in the ER membrane. Important oligomerization determinants were also shown to reside in the TMRs 5 and 6 of the carboxy terminus, with possible contributions from the cytoplasmic tail region (Galvan et al., 1999; Galvan and Mignery, 2002).

The three dimensional structure of the tetrameric InsP$_3$R1 isolated from rat cerebellum has been determined by electron cryomicroscopy and single-particle reconstruction. The protein structure has four-fold symmetry resembling a pinwheel with four blades connected by a central region, presumably comprised of the TMRs and the pore forming region of the channel. A large cytoplasmic domain protrudes from the structure to form the blades of the pinwheel, and the IP$_3$-binding core of each subunit has been localized to this region (Serysheva et al., 2003).

2. Function and Regulation

In all tissues, InsP$_3$Rs can be regulated by a variety of intracellular molecules such as protein kinases, numerous proteinaceous binding partners (reviewed in Bezprozvanny, 2005), and also by intracellular ions such as K$^+$ (Marchenko et al., 2005), Ba$^{2+}$, Mg$^{2+}$ (Striggow and Ehrlich, 1996), and Ca$^{2+}$ (Mignery et al., 1992; Perez et al., 1997; Ramos-Franco et al., 2000). Together, Ca$^{2+}$ and InsP$_3$ act to regulate InsP$_3$R channel activity. Reports of spontaneous InsP$_3$R activity of very low $P_o$ have been shown (Ramos-Franco et al., 1998a and 1998b); however, both InsP$_3$ and Ca$^{2+}$ must be present.
on the cytoplasmic side of the channel protein to activate it to appreciable levels. A calcium sensor intrinsic to the InsP₃R1 has been identified based on sequence alignment with the calcium sensing region of the RyR (Miyakawa et al., 2001). It was shown that glutamate 2100 (E2100) of the rat InsP₃R1 is a key residue involved in Ca²⁺-sensing of the channel. Mutagenesis of this residue to aspartate, another acidic amino acid, resulted in a 10-fold decrease in the Ca²⁺-sensitivity of the receptor without affecting other properties of the channel. Furthermore, substitution of glutamate 2100 by non-acidic residues (alanine & glutamine) decreased the Ca²⁺-sensitivity below the range of detection in this study (Miyakawa et al., 2001). These data suggest E2100 is a critical residue in conferring Ca²⁺-sensitivity to the channel, however, this does not exclude other potential Ca²⁺-sensors from being part of this or other regions of the InsP₃R because they may even be contained in other proteins associated with the receptor.

Regulation of InsP₃Rs by Ca²⁺ and InsP₃ is very complex, with the open probability of the channel being dependent on both the intracellular [Ca²⁺] ([Ca²⁺]ᵢ) and the [InsP₃]. Previous work has shown that the InsP₃R isoforms exhibit varying sensitivities to regulation by [Ca²⁺]ᵢ (Ramos-Franco et al., 1998b; see Figure 2B). In general for the InsP₃R1, a small rise in cytoplasmic [Ca²⁺] serves to activate the channel and increase $P_o$, while a higher cytoplasmic [Ca²⁺] inhibits channel activity. Due to the interplay of Ca²⁺ and InsP₃ on channel gating, a range of open probabilities can be observed when either Ca²⁺ or InsP₃ is held constant and the other varied. When examining cytoplasmic Ca²⁺-sensitivities of single InsP₃R1 and InsP₃R2 at a fixed [InsP₃] (1 µM), it was shown that the Ca²⁺ regulation of type 1 and type 2 channels are very different. The Ca²⁺-dependence of the InsP₃R1 channel was bell-shaped, with maximum channel
activity at 1 µM Ca\(^{2+}\). The InsP\(_3\)R2 Ca\(^{2+}\)-dependence, however, had a sigmoidal shape. Channels were active at relatively low [Ca\(^{2+}\)] (~25 nM) and maintained activity over a broad range of Ca\(^{2+}\) concentrations (Ramos-Franco et al., 1998b). The InsP\(_3\)R3 channel Ca\(^{2+}\)-dependence has been reported to be similar to that of the type 2 channel. At a constant [InsP\(_3\)], the channel activates at relatively low [Ca\(^{2+}\)] and slowly reaches a maximal \(P_o\) over a large range of Ca\(^{2+}\) concentrations, reaching a plateau at ~10 µM Ca\(^{2+}\). Like the type 2 channel, no Ca\(^{2+}\)-feedback inhibition is observed (Hagar et al., 1998).

The unique Ca\(^{2+}\)- and InsP\(_3\)-sensitivities of the three types of InsP\(_3\)R may have physiological relevance in mediating different types of intracellular Ca\(^{2+}\) signals depending on which isoforms are expressed in a given tissue. For example, the bell-shaped Ca\(^{2+}\)-dependence of the type 1 channel makes it ideal for mediating small, transient and oscillatory Ca\(^{2+}\) signals since Ca\(^{2+}\) can feedback and turn off the channel. Type 2 InsP\(_3\)R-mediated signals have a sigmoidal Ca\(^{2+}\)-dependence meaning termination of this signal is not intrinsically controlled by a Ca\(^{2+}\)-dependent inactivation mechanism. Of significance in cardiac tissues where type 2 receptors are present, this sigmoidal Ca\(^{2+}\)-dependence makes the InsP\(_3\)R2 resistant to the RyR-mediated Ca\(^{2+}\) signals driving the cardiac contractile cycle and allows InsP\(_3\)-dependent intracellular signaling cascades to operate independent of the global Ca\(^{2+}\) fluxes associated with contraction. Termination of the type 2 response will occur upon depletion of the Ca\(^{2+}\) store, removal of the InsP\(_3\) signal, or regulation through a modulatory protein or factor; whereas the type 1 receptor would be inhibited by the global Ca\(^{2+}\)-fluxes of the contractile cycle. The type 3 InsP\(_3\)R, like the type 2 receptor, shows a lack of negative feedback from cytoplasmic Ca\(^{2+}\). This allows the receptor to stay open in the presence of high [Ca\(^{2+}\)] and initiate a rapid and
almost total release of Ca\(^{2+}\) from intracellular stores as long as InsP\(_3\) is present (Hagar et al., 1998; O’Neill et al., 2002; Hagar and Ehrlich, 2000). This property of the type 3 receptor makes it more suited to signal initiation rather than signal regeneration like the type 1 receptor, and this function has been shown in certain cell types (Nathanson et al., 1994). Thus, expression of multiple InsP\(_3\)R isoforms in the same cells can produce complex patterns of Ca\(^{2+}\)-signaling depending on the type and amount of each isoform present.

Although not necessary for channel gating, the nucleotide adenosine-5’-triphosphate (ATP) can also positively modulate InsP\(_3\)Rs (Meyer et al., 1988; Smith et al., 1985; Taylor and Putney, 1985). Like Ca\(^{2+}\) and InsP\(_3\), the ATP-dependent potentiation of endogenous and recombinant InsP\(_3\)R channels is both complex and isoform dependent (Mak et al., 1999; Mak et al., 2001). ATP can exist in the cell in two forms. One is the “free” form which is ATP not bound to any ions, and the other is the “bound” form which is ATP bound typically to Mg\(^{2+}\). It was found for the type 1 InsP\(_3\)R that free ATP concentrations ([ATP]\(_\text{FREE}\)) in the cytoplasm could increase the channel’s \(P_o\). The mechanism of this potentiation was found to be through the enhancement of the channel’s sensitivity to Ca\(^{2+}\) activation; however, the [ATP]\(_\text{FREE}\) had no effect on the maximal \(P_o\) observed for the channel (Mak et al., 1999; Mak et al., 2001). Likewise, the InsP\(_3\)R3 showed enhanced sensitivity to Ca\(^{2+}\) activation with increasing [ATP]\(_\text{FREE}\), while no effect on the max \(P_o\) was reported (Mak et al., 1999). The effect of [ATP]\(_\text{FREE}\) on the InsP\(_3\)R2 has not been reported.

In addition to regulating InsP\(_3\)R function directly, ATP has been postulated to indirectly regulate the InsP\(_3\)R via kinases. ATP is hydrolyzed by protein kinases for use
in phosphorylation of target proteins. It was shown that specifically the glycolytically
generated ATP fueled the CaMKII-dependent phosphorylation of the InsP₃R in
endothelial cells, and inhibition of glycolysis decreased the ATP available to the kinase
and reduced the inhibitory effect of the kinase on the InsP₃R (Aromolaran et al., 2007).
Thus the source of the free ATP in the environment of the InsP₃R, either from
mitochondrial respiration or cytoplasmic glycolysis, is important in the differential direct
and indirect regulation of the InsP₃R by ATP.

Based on sequence homology, three putative ATP binding sites were identified in
InsP₃Rs as glycine-rich motifs with the sequence G-x-G-x-x-G (Wierenga and Hol,
1983; Maes et al., 1999). Of these three sites, only two of them have been biochemically
proven to bind ATP in the InsP₃R (Maes et al., 1999; Maes et al., 2001). The two sites
are referred to as the ATPA and ATPB sites. The ATPA site is found only in the type 1
InsP₃R and is located at residues 1773-1778. The ATPB site, residues 2016-2021 of the
InsP₃R1, is found in all three isoforms (Maes et al., 1999). Site-specific binding of ATP
to either the A or B site has not yet been correlated with functional effects of ATP on the
InsP₃R channel.

Modulation of the channel’s sensitivity to Ca²⁺ activation by [ATP]FREE has been
postulated to have important physiological consequences. The apparent affinity of the
two proven ATP binding sites (A & B) of the InsP₃R1 and InsP₃R3 has been shown to be
~300 µM, which correlates with the cytoplasmic [ATP]FREE of ~400-600 µM (Mak et al.,
1999; Mak et al., 2001). This allows the activity of InsP₃Rs to be adjusted to the
metabolic state of the cell. The proximity of the ER to the mitochondria has been well-
documented, specifically in regions of high InsP₃R protein density and Ca²⁺ release (Otsu
et al., 1990; Satoh et al., 1990; Rizzuto et al., 1998). This location allows the mitochondria to sense the \( \text{Ca}^{2+} \) released from the \( \text{InsP}_3 \)Rs (Babcock and Hille, 1998; Csordás et al., 1999) and the \( \text{InsP}_3 \)Rs to sense the free ATP released by the mitochondria (Klingenberg, 1980). This two way communication between the ER and mitochondria could be significant in regulated signaling microdomains and potentially play a role in apoptosis.

A final agonist of \( \text{InsP}_3 \)Rs worth mentioning is Adenophostin A (AdA). AdA, a fungal glyconucleotide metabolite, activates \( \text{InsP}_3 \)Rs by binding to the \( \text{InsP}_3 \)-binding site (Takahashi et al., 1994; Glouchankova et al., 2000). In fact, AdA binds with a much higher affinity than \( \text{InsP}_3 \) and is much more efficacious at stimulating \( \text{Ca}^{2+} \) release (Glouchankova et al., 2000; Bird et al., 1999). Of significance experimentally, AdA is metabolically stable, whereas \( \text{InsP}_3 \) is susceptible to conversion to other inositol polyphosphates by kinases and phosphatases (Adkins et al., 2000b). Furthermore, the use of AdA and also many synthetic analogs of \( \text{InsP}_3 \) are invaluable tools in studying \( \text{InsP}_3 \) binding and the gating mechanisms of the \( \text{InsP}_3 \)Rs (Rossi et al., 2009; Borissow et al., 2005; Correa et al., 2001).

The activation of \( \text{InsP}_3 \) receptors results in a release of \( \text{Ca}^{2+} \) from intracellular stores, and they therefore play an important role in the regulation of intracellular \([\text{Ca}^{2+}]\). The sarcoplasmic/endoplasmic reticulum (SR/ER) is the primary \( \text{Ca}^{2+} \) storage organelle in most cell types. The SR/ER membrane \( \text{Ca}^{2+} \)-ATPases (SERCA) accumulate \( \text{Ca}^{2+} \) in the SR/ER lumen, with free \([\text{Ca}^{2+}]\) estimated to be between 100 and 700 \( \mu \text{M} \) (Palmer et al., 2004). The low free \([\text{Ca}^{2+}]\) in the cytosol (50-100 nM) provides a steep chemical gradient for calcium to diffuse from ER stores into the cytoplasm when \( \text{InsP}_3 \) binds to ER.
membrane InsP₃Rs. Calcium in the cytoplasm moves by passive diffusion, and its rate is reduced by calcium binding proteins present there. The result is the formation of calcium microdomains due to the steep concentration gradients that form and dissipate around the mouth of an InsP₃R channel. Calcium concentrations at the exit of the pore can be 100 µM or more, with a dramatic reduction of approximately 100-fold less as one moves even 1-2 µm away from the pore (Naraghi and Neher, 1997; Rios and Stern, 1997; Neher, 1998).

Calcium release events from InsP₃Rs can be grouped into three broad categories. First is the calcium blip, which is the elementary calcium release event from a single InsP₃R channel (Parker et al., 1996). The next level of release is the calcium puff, observed when a cluster of InsP₃Rs become activated and open (Swillens et al., 1999). Finally, the calcium wave is the largest unit of calcium release events from InsP₃Rs observed in cells. The wave propagates via a cycle of calcium release, diffusion, and calcium-induced calcium release (CICR) between InsP₃R channels (Bootman and Berridge, 1996; Cheng et al., 1996; Berridge, 1997; Callamaras et al., 1998). Here the calcium released from a cluster of InsP₃Rs diffuses to a nearby cluster of InsP₃Rs and activates them, thus releasing calcium and beginning the cycle again. Of course InsP₃ must also be present for the channels to activate, albeit at a concentration that is dependent on the isoform of InsP₃R expressed and the amount of calcium sensed by the InsP₃R protein. The Ca²⁺ released can then regulate a multitude of cellular signaling cascades and even feedback to inhibit the activity of the type 1 InsP₃R and terminate Ca²⁺ release.
The elementary calcium release events described above allow for highly localized signals due to the fact that Ca\(^{2+}\) in the local vicinity of the InsP\(_3\)R pore is much higher than in the bulk cytosol. Thus, it is no surprise that Ca\(^{2+}\) released locally via InsP\(_3\) receptors has multiple consequences aside from potentially inducing CICR from neighboring Ca\(^{2+}\) release channels. For example, it can stimulate the activation of key intracellular enzymes, one of which is Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (Wu et al., 2006). Furthermore, an extremely elegant subcellular arrangement of InsP\(_3\)R subtypes with their distinct properties and expression of calcium binding proteins within a given cell type allows those cells to have diverse spatial and temporal control over the InsP\(_3\)-mediated calcium signals that can be utilized. Along with localization and isoform expression levels, the distinct single channel properties of the InsP\(_3\)Rs allow for this type of control of calcium release and termination.

3. Properties of the InsP\(_3\)R channel

Based on site-directed mutagenesis (Boehning et al., 2001) and the results using the construct with the deletion of TMRs 1-4 described previously (page 5; Ramos-Franco et al., 1999), the pore of the InsP\(_3\)R is proposed to be contained in TMR-5 and 6 and their intervening sequence. Studies have shown the pore of the InsP\(_3\)R to have relatively low energy barriers for divalent cation entry when compared to monovalent cation entry. Thus the InsP\(_3\)R exhibits higher divalent cation permeabilities and stronger binding to the divalent cations once they are inside of the pore. This results in observed divalent blocking of the channel (Mak and Foskett, 1998) and also causes decreased monovalent conductance when divalents are present. Although the InsP\(_3\)R is selective for divalent
over monovalents, it shows little selectivity between divalent cations including Ca$^{2+}$, Mg$^{2+}$, and Ba$^{2+}$ (Mak and Foskett, 1998). Despite the preference for divalents over monovalents, the InsP$_3$R has shown higher conductances for monovalent cations implying that selectivity and conductance are distinct properties.

Ramos-Franco et al. (1998a and 1998b; 2000) studied the channel properties of native InsP$_3$R1 and InsP$_3$R2 purified from tissues and reconstituted into proteoliposomes. These two channels were found to each have very similar conductances for Ca$^{2+}$ (InsP$_3$R1 and InsP$_3$R2 both approximately 70 pS) and Cs$^+$ (InsP$_3$R1 and InsP$_3$R2 both approximately 280 pS), suggesting that different InsP$_3$Rs have similar permeation properties. Other groups have measured InsP$_3$R channel conductances using InsP$_3$R protein from various sources (purified from native tissue, overexpressed recombinant protein in mammalian and insect cells), lipid environments (native ER or proteoliposomes), and recording conditions (presence of divalents and monovalents). The clear result from these studies is that the InsP$_3$R channel conducts monovalents better than divalents. Another advantage to using the monovalent Cs$^+$ as opposed to the divalent Ca$^{2+}$ as the charge carrier is that this allows precise control of Ca$^{2+}$ levels around the channel, thus removing any uncontrolled Ca$^{2+}$ inhibition or activation from these measurements. Furthermore, Cs$^+$ blocks K$^+$ channels that may be present in the preparation. Current amplitude was found to be dependent on the driving force, with a linear I-V relationship for both Ca$^{2+}$ and Cs$^+$ over a range of membrane holding potentials from -70 to +60 mV. At 0 mV, a Cs$^+$ current amplitude of ~5-8 pA was recorded (Ramos-Franco et al., 1998b).
Based on homology with RyR and K\(^+\) channels, a putative selectivity filter was determined to be part of the pore-forming S5-S6 linker region. For the rat InsP\(_3\)R1, the residues \(^{2547}\text{GVGD}^{2550}\) have been the focus of studies examining the selectivity filter of the InsP\(_3\)R. Mutating these residues individually to conserved or non-conserved mutations produced either altered selectivity or altered conductances. Thus the InsP\(_3\)R pore has distinct sites that control permeation and selectivity (Boehning et al., 2001; Schug et al., 2007). The activation gate is also a feature of the InsP\(_3\)R that is not well characterized. Similar to the identification of the selectivity filter, much of what is predicted about the location and function of the activation gate of the InsP\(_3\)R channel comes from comparisons and homology to K\(^+\) channels (Kuo et al., 2003; Long et al., 2005). It has been speculated that conformational changes from InsP\(_3\) binding are transduced to the activation gate, enabling ion flow through the pore. It is possible the interaction between the N-terminus and S4-S5 linker (Schug and Joseph, 2006) provides the conduit for the binding of InsP\(_3\) to translate into the movement of the activation gate through conformational changes that allow ions to move through the pore.

As stated above, the InsP\(_3\)R is more permeable to divalent cations; however, it has a higher conductance for monovalent cations. Therefore, it has been estimated that the actual Ca\(^{2+}\) current through an open InsP\(_3\)R in the ER of a cell is approximately 0.1-0.2 pA (Kettlun et al., 2003; reviewed in Foskett et al., 2007) due to the K\(^+\) and Mg\(^{2+}\) present in the cytoplasm.

4. **Phosphorylation of InsP\(_3\)Rs by Protein Kinases**
Along with InsP₃ and Ca²⁺ signals, a third important facet to the regulation of InsP₃Rs is phosphorylation. Protein phosphorylation was first discovered in 1955 when it was observed that both ATP and a “converting enzyme” (now known to be a kinase) were required to convert a protein into a phospho-protein (Fischer and Krebs, 1955). Since then, cycles of phosphorylation by protein kinases and de-phosphorylation by protein phosphatases have been found to be an essential signaling mechanism present in all living organisms. Protein kinases mainly phosphorylate their substrates on one of three hydroxyl-containing amino acids. These amino acids are Serine, Threonine, and Tyrosine, with Serine/Threonine phosphorylation accounting for 98.2% of phosphorylation sites present in the human proteome (Olsen et al., 2006). Multiple kinases, including cAMP-dependent protein kinase (PKA), protein kinase B (PKB/Akt), protein kinase C (PKC), and Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) have been shown to phosphorylate InsP₃R proteins. Furthermore, purified InsP₃R1 has been shown to be phosphorylated by PKA and PKC on serine residues as determined by electrophoresis of phosphorylated amino acids after acid hydrolysis of phosphopeptides (Ferris et al., 1991).

The InsP₃R1 contains two consensus PKA sequences (R-R-x-S/T) at Ser-1589 and Ser-1755; however, these sites are not conserved in either the type 2 or type 3 isoforms. PKA phosphorylation of the type 1 InsP₃R has been the most fully characterized of all the isoform-specific phosphorylation events. Initially, it appeared that PKA phosphorylation of the InsP₃R resulted in an increase in the affinity of InsP₃R for InsP₃ because less InsP₃ was needed to produce increases in the channel’s \( P_o \). However, in an in-depth single channel characterization of the InsP₃R1 that has been
phosphorylated by PKA, it was found the phosphorylation of the channel results in an increase in the $P_o$ of the channel by destabilizing a long-lived closed state of the channel to facilitate bursting activity (Wagner et al., 2008). It is also of note to recognize that the InsP$_3$R1 forms a complex with PKA and distinct protein phosphatases. Pull-down experiments resulted in precipitation of the InsP$_3$R1 along with PKA, protein phosphatase 1 (PP1), and protein phosphatase 2A (PP2A), thus showing a macromolecular signaling complex exists in the rat brain (DeSouza et al., 2002). PKA has been shown to phosphorylate the mouse InsP$_3$R2 at Serine 937. This phosphorylation results in an increase in the $P_o$ of the channel, but the mechanism behind this increase has not yet been elucidated (Betzenhauser et al., 2009).

PKB/Akt is a serine/threonine protein kinase that phosphorylates a number of key substrates to promote cell survival and proliferation. It can phosphorylate the InsP$_3$R1 on Serine 2681, causing a decrease in InsP$_3$-induced calcium release. Physiologically, this pathway is proposed to be important to cell survival and the apoptotic pathway. Under normal growth conditions, PKB/Akt maintains a basal level of InsP$_3$R1 phosphorylation. In the absence of growth factors, decreased PKB/Akt activity results in larger calcium release from unphosphorylated InsP$_3$Rs. It is hypothesized that this calcium can be sensed by closely localized mitochondria causing permeability transition and apoptosis. In the context of cancer where there is increased PKB/Akt activity, this “protects” the cancer cell from apoptotic stimuli allowing the cell to survive and proliferate (Szado et al., 2008). No PKB/Akt phosphorylate of the type 2 or 3 InsP$_3$R has been reported.

PKC has been shown to increase InsP$_3$-induced Ca$^{2+}$ release from tissues such as cerebellum, which express predominantly the type 1 isoform (Matter et al., 1993; Poirier
et al., 2001). The InsP₃R2 has recently been shown to be a substrate for PKC; however, it was found to decrease InsP₃-induced Ca²⁺ release in cells as a result of a diminished apparent affinity of the receptor for InsP₃ (Arguin et al., 2007)). This study was done in a cell line known as AR4-2J that expresses predominantly the type 2 isoform. This provides a negative feedback mechanism for the type 2 InsP₃R whereby calcium released by the receptor can activate PKC which phosphorylates and inhibits InsP₃R2 activity.

CaMKII can phosphorylate the InsP₃R1 on a serine residue (Ferris et al., 1991), and this has been shown to result in a decrease in InsP₃-dependent Ca²⁺ release in HeLa cells (Zhu et al., 1996) and Xenopus oocytes (Matifat et al., 2001). In calf pulmonary artery endothelial (CPAE) cells, CaMKII was found to exert a profound inhibition of Ca²⁺ release from ER stores (Aromolaran and Blatter, 2005; Aromolaran et al., 2007). These cells were reported to express predominantly type 2 InsP₃Rs, and the inhibition of Ca²⁺ release was postulated to be through CaMKII-dependent phosphorylation of the InsP₃R (Aromolaran et al., 2007). Furthermore, it has been shown that CaMKII can associate with and phosphorylate the InsP₃R2 in cardiac ventricular myocytes (Bare et al., 2005). The region of phosphorylation was determined to be in the first 1078 amino acids and resulted in a decrease in channel open probability (Bare et al., 2005); however, the specific site still remains unknown. CaMKII has been shown to preferentially phosphorylate the amino acid motif R-x-x-S/T, with other amino acids upstream and downstream of this motif participating in the affinity of the kinase for this site (White et al., 1998). Sequence search for the minimal consensus CaMKII phosphorylation site of amino acids R-x-x-S/T (White et al., 1998) or the RyR2 CaMKII phosphorylation site S-
x-D motif (Wehrens et al., 2004) in the type-2 receptor reveals 17 potential phosphorylation sites.

5. InsP₃Rs in the heart

The expression of the InsP₃R has been shown to be most abundant in the early embryonic development stages, with levels decreasing with progression to adulthood (Rosenblit et al., 1999). Despite this, the InsP₃R has been shown to have multiple roles in the adult heart including modulation of excitation-contraction coupling (ECC), part of the excitation-transcription coupling pathway (ETC), and a contributor to generation of arrhythmias in both atria (Zima and Blatter, 2004) and ventricles (Proven et al., 2006) of different species. In cardiac myocytes, a number of neurohumoral agents such as ET-1, phenylephrine, and acetylcholine can act via cell-surface receptors to activate the pathway leading to production of InsP₃ and activation of InsP₃Rs. The type 2 InsP₃R is expressed in multiple tissues and has been shown to be the predominant isoform in cardiac myocytes (Perez et al., 1997). In addition, the type 2 and type 3 receptors were found to be expressed at higher levels in the atria as compared to ventricles in rabbit myocytes (Domeier et al., 2007). Although it is the predominant InsP₃R isoform, a close relative of the InsP₃R known as the ryanodine receptor (RyR) is the major Ca²⁺ release channel of the sarcoplasmic reticulum (SR) in excitation-contraction coupling in cardiac myocytes. In atrial tissues, more functional InsP₃Rs may be expressed compared to ventricular myocytes, and populations of them have been shown to co-localize with RyRs in the subsarcolemmal space (Mackenzie et al., 2002). Here, InsP₃-dependent Ca²⁺ release can enhance calcium-induced calcium release (CICR) from neighboring RyRs,
thus causing positive inotropy in the heart (Zima and Blatter, 2004). Recent data from Domeier et al. (2007) has also shown that InsP$_3$-dependent Ca$^{2+}$ release from InsP$_3$Rs presumably located on the SR of the cell has an effect on excitation-contraction coupling in rabbit ventricular myocytes. Activation of InsP$_3$R2 resulted in positive inotropic effects due to facilitation of Ca$^{2+}$ release from RyR clusters on the SR providing a role for InsP$_3$Rs to modulate E-C coupling in ventricular myocytes. However, this increase in Ca$^{2+}$ transients can also lead to arrhythmias in atrial and also ventricular myocytes. InsP$_3$Rs have been shown to play a role in the modulation of ECC and also in the generation of arrhythmias. ET-1 increased diastolic [Ca$^{2+}$], increased the amplitude of electrically evoked transients, and caused spontaneous transients in cat atrial myocytes (Zima and Blatter, 2004). In support of these data, Li et al. (2005) showed that in response to ET-1 application InsP$_3$R2-KO mouse atrial myocytes failed to increase diastolic [Ca$^{2+}$], and to have a positive inotropic effect. In addition, ET-1 also failed to elicit spontaneous Ca$^{2+}$ release events typically seen with ET-1 application.

Aside from increasing cytoplasmic Ca$^{2+}$ transients, ET-1 was also shown to increase Ca$^{2+}$ transients in the nucleus. In fact, the increase in nuclear Ca$^{2+}$ transients was greater than the increase in cytoplasmic transients. Furthermore, ET-1 at low concentrations caused selective increases in nuclear Ca$^{2+}$ transients through InsP$_3$Rs (Kockskämper et al., 2008). A study using isolated nuclei from whole heart showed that InsP$_3$Rs release Ca$^{2+}$ into the nucleoplasm when stimulated with InsP$_3$. InsP$_3$-induced calcium release (IICR) was observed at both the outer and inner face of the nuclei, with nuclear [Ca$^{2+}$] rising to levels higher than those observed in the area immediately surrounding the outer membrane of the nucleus (Zima et al., 2007). The nuclear
envelope was also loaded with the low affinity Ca\(^{2+}\) dye fluo-5N to measure nuclear envelope [Ca\(^{2+}\)] directly, and InsP\(_3\) application reduced the Ca\(^{2+}\) stored in the nuclear envelope. The presence of InsP\(_3\)Rs on the nuclear envelope and its function as a releasable calcium store are not surprising given the fact that the sarcoplasmic reticulum and the nuclear envelope and are one highly interconnected Ca\(^{2+}\) store throughout the cardiac myocyte (Wu and Bers, 2006). These results also suggests that possibly a higher density of the nuclear InsP\(_3\)Rs are located on the inner nuclear membrane for Ca\(^{2+}\) release into the nucleoplasm where it can activate nuclear localized CaMKII resulting in local nuclear Ca\(^{2+}\) signaling (Zima et al., 2007).

To go along with the presence of InsP\(_3\)Rs on the inner and outer nuclear envelope, it was shown that InsP\(_3\) can diffuse long distances through the cytosol and even into the nucleus. Remus et al. (2006) used the FRET (fluorescence resonance energy transfer)-based InsP\(_3\) biosensor, FIRE1 (fluorescent InsP\(_3\)-responsive element), to follow InsP\(_3\) produced at the plasma membrane in cells treated with endothelin-1. With this biosensor, they were able to show that InsP\(_3\) diffused through the cytosol of ventricular myocytes and into the nucleus via nuclear pore complexes. InsP\(_3\) levels rose more rapidly and to a higher level in the cytosol compared to the nucleus, and the experiment convincingly demonstrates the diffusional capabilities of InsP\(_3\) intracellularly. They had also shown that neurohumoral activation in ventricular myocytes resulted in rapid rises of [InsP\(_3\)] in the cytoplasm and nucleus.

The InsP\(_3\)R2 is part of a nuclear signal transduction cascade involving CaMKII and HDAC5 phosphorylation and its resulting nuclear export. This pathway seems to be isolated from the beat-to-beat global fluctuations in [Ca\(^{2+}\)], creating an elegant system
that is dependent on very local control of \([\text{Ca}^{2+}]\) around the pore of the InsP\(_3\)Rs. The hypothesis is that InsP\(_3\) can activate nuclear envelope InsP\(_3\)Rs, releasing \(\text{Ca}^{2+}\) into the nucleoplasm with very high concentrations of \(\text{Ca}^{2+}\) immediately adjacent to the mouth of the InsP\(_3\)R activating CaMKII, which can phosphorylate HDAC5 and cause its translocation out of the nucleus. The chronic, sustained stimulation with InsP\(_3\)-producing agonists causes de-repression of MEF2-dependent transcription allowing altered gene
Figure 3: Immunofluorescence staining of heart failure and wild-type rabbit myocytes

Isolated rabbit myocytes were immunostained with T2NH antibody against the InsP\textsubscript{3}R2. Myocytes were isolated from both healthy (WT) and heart failure (HF) New Zealand white rabbits (supplied by S.M. Pogwidz). Heart failure in the HF rabbits was induced by combined aortic insufficiency and stenosis (Pogwidz, 1995). Both heart failure and WT myocytes show fluorescent signal around the myocyte nuclei (A & B); however, the heart failure myocytes (A) show enhanced nuclear envelope staining for the InsP\textsubscript{3}R2 when compared with the faint signal seen in WT myocytes (B). The myocytes were incubated with the same dilution of primary and secondary antibodies, and the images were acquired at similar exposure intervals.
expression that leads to hypertrophy and heart failure in a process that has been termed excitation-transcription coupling (ETC) (Wu et al., 2006).

InsP$_3$Rs have also been implicated, as previously mentioned, in pathological conditions of the heart. Specifically, their role in development of hypertrophy, heart failure (HF), and atrial fibrillation (AF) has been studied by multiple groups. It has been shown that InsP$_3$Rs are upregulated in the atrial myocytes of human patients with AF (Yamada et al., 2001). Presumably, the increased expression of InsP$_3$Rs along with the increased levels of InsP$_3$-producing agonists ET-1 and also angiotensin II found in subjects with AF, lead to the initiation and progression of atrial fibrillation. The role of InsP$_3$Rs in hypertrophy come from studies showing that in pressure-overload induced hypertrophy PLC activity was increased leading to increased levels of InsP$_3$ (Kawaguchi et al., 1993), and in volume-overload induced hypertrophy the levels and activities of PLC1 and PLC3 were increased again leading to increased levels of InsP$_3$ (Dent et al., 2004). In heart failure, the levels of expression of the InsP$_3$R2 have been shown to be increased (Go et al., 1995; JT Maxwell & GA Mignery, Figure 3). Specifically, CaMKIIδ contributes to the altered expression levels of multiple proteins including the upregulation of InsP$_3$R2 in the ventricles during the development of pressure overload-induced HF (Ling et al., 2009). Thus InsP$_3$Rs and their ligand InsP$_3$ have roles in the initiation and progression of severe cardiac dysfunctions.

6. Interacting Proteins

The InsP$_3$R has been shown to interact with a multitude of proteins and factors, some of which have been described already; however, there are a few more that are
relevant to this study that warrant discussion. One of those proteins is Calmodulin (CaM), which is a $\text{Ca}^{2+}$-binding protein shown to be a co-factor of many ion channels (Saimi and Kung, 2002). The first CaM binding site on the InsP$_3$R1 was found to be $\text{Ca}^{2+}$-dependent and was identified as encompassing residues 1564-1585 (Maeda et al., 1991). A second CaM site that bound both CaM and apoCaM equally was found in the N-terminus suppressor domain, with amino acids 49-81 and 106-128 involved in the interaction (Adkins et al., 2000a; Sienaert et al., 2002). The exact function that CaM has in complex with the InsP$_3$R has been studied, but a consensus is yet to be reached. Also, CaM binding to the other isoforms has not been demonstrated; however, the regions of interaction with the InsP$_3$R1 are conserved in both the InsP$_3$R2 and InsP$_3$R3 proteins. Aside from potentially conferring $\text{Ca}^{2+}$-activation or $\text{Ca}^{2+}$-inhibition to the channel, it is possible that CaM is part of a larger macromolecular complex regulating other facets of InsP$_3$R activity. The location of the CaM binding site on the large cytoplasmic domain of the receptor in cell allows it to interact with many other cytoplasmic proteins and also positions it in a region where it can be activated by InsP$_3$R2-mediated $\text{Ca}^{2+}$ release.

Two other proteins that bind to the N-terminus of the InsP$_3$R are RACK1 and Homer. RACK1 is an adaptor protein identified through a yeast two-hybrid screen to interact with two specific sites in the InsP$_3$R at residues 90-110 and 580-600 (Patterson et al., 2004). In addition to its function as an adaptor protein, RACK1 may serve to stabilize the InsP$_3$-binding domain and increase the channel’s sensitivity to InsP$_3$ (Patterson et al., 2004). The scaffolding protein Homer binds to a proline-rich region of the N-terminus of the InsP$_3$R at residues 49-54 (Fagni et al., 2002). Homer contains a coiled-coil region important for binding other Homer proteins and forming a multimeric
protein complex (Tu et al., 1998; Yuan et al., 2003). A direct effect of Homer on the İnsP₃R has not been demonstrated. As detailed here, the large cytoplasmic domain of the İnsP₃Rs comprised of the ligand-binding domain and the modulatory/coupling domain contains many sites of interaction and regulation by a variety of proteins and factors in addition to its reported intramolecular interaction with the C-terminus.
B. Ca\textsuperscript{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II

1. Structure and Regulation

CaMKII is a multifunctional Ser/Thr protein kinase. The enzyme is composed of homomultimers or heteromultimers of 6-12 subunits, with the subunit isoforms being derived from a family of four closely related genes (\(\alpha, \beta, \delta, \gamma\)) (Hudmon and Schulman, 2002). All isoforms contain an amino-terminal catalytic domain where ATP binds, a central regulatory domain containing an auto-inhibitory and calmodulin binding sites, and a carboxyl-terminal association domain (Figure 4). The association domain is responsible for oligomerization of CaMKII subunits. Calmodulin (CaM) is a Ca\textsuperscript{2+}-binding protein that contains four EF hand motifs, each of which binds a Ca\textsuperscript{2+} ion (Braunewell and Gundelfinger, 1999). CaM has a structure of two symmetrical domains linked by a central hinge region. CaM cooperatively binds four Ca\textsuperscript{2+} ions then undergoes a conformational change that allows it to recognize and bind target proteins. Thus Ca\textsuperscript{2+} regulates the activity of CaM through structural changes in the protein from the Ca\textsuperscript{2+}-bound form to the Ca\textsuperscript{2+}-free form (apo-calmodulin). The complex of Ca\textsuperscript{2+} and calmodulin binds to the regulatory domain of CaMKII, removing its hindrance on the active site. The now open active site can bind to target substrates within the cell. Half maximal activation of CaMKII occurs at [Ca\textsuperscript{2+}] of approximately 500-1000 nM. This Ca\textsuperscript{2+}-dependent activated state can then undergo an auto-phosphorylation event at Threonine-286 (Threonine-287 in some isoforms). This would convert the enzyme to a Ca\textsuperscript{2+}-independent state, allowing it to remain active after intracellular [Ca\textsuperscript{2+}] has
Figure 4: CaMKII domains and oligomerization.

The three main domains of the CaMKII monomer are indicated in the linear layout (top). Middle left shows that CaMKII forms homo- or heteromultimers (6-12 monomers) in wheel-like structures (a second one may sit on top of the one shown, forming a double wheel). Lower, middle, and right panels show activation of CaMKII by Ca\(^{2+}\)-CaM binding and subsequent autophosphorylation at Thr-286 (or Thr-287 in δ\(_B\) isoform). CaM binding is sufficient to activate CaMKII so the active site (ATP) can interact and phosphorylation target proteins, but autophosphorylation makes CaMKII active (20-80\%) even after CaM dissociates. (Figure modified from Maier & Bers, 2002).
decreased, such as during diastole. This auto-phosphorylation has multiple effects on the enzyme and is not a prerequisite for kinase activity (Fukunaga et al., 1992). First, it increases the affinity of the kinase for the calmodulin complex. Autophosphorylation, and even high \([\text{Ca}^{2+}]\), can cause the affinity of CaMKII for CaM to increase \(~700\) fold, thus the kinase retains full activity as long as calmodulin is trapped by CaMKII. Also, due to the position of the auto-phosphorylation site, addition of the phosphate to Thr-286 is sufficient to remove autoinhibition and produce 20-80% kinase activity (reviewed in Maier and Bers, 2002). The activity of CaMKII can be blocked with organic inhibitors KN-62 or KN-93, a non-phosphorylatable peptide inhibitor known as autocamtide-2 related inhibitory peptide (AIP), and also with non-hydrolysable analogs of ATP (Hudmon et al., 2005). Constitutively active CaMKII mutants have previously been made by point mutagenesis of Threonine-286 to Aspartate-286, which mimics autophosphorylation and allows the kinase to maintain activity in the absence of the \(\text{Ca}^{2+}/\text{calmodulin} \) complex (Backs et al., 2006).

2. **Localization and Function**

It has been shown that CaMKII, due to its ability to sense changes in intracellular \([\text{Ca}^{2+}]\), plays a role in decoding the frequencies of \(\text{Ca}^{2+}\) oscillations. Various cell types exhibit changes in intracellular \([\text{Ca}^{2+}]\) as a response to stimuli ranging from electrical to chemical. A key pathway in changing intracellular \([\text{Ca}^{2+}]\) is the phosphoinositide cascade resulting in the production of \(\text{InsP}_3\) as an intracellular signaling molecule. It has long been speculated that information may be encoded in the amplitude, frequency, and duration of these oscillations of intracellular \([\text{Ca}^{2+}]\) (Bootman and Berridge, 1996).
CaMKII has many intracellular phosphorylation targets and therefore would be well suited to connect changes in intracellular \([\text{Ca}^{2+}]\) to changes in cellular functions (DeKoninck and Schulman, 1998). It has been shown that CaMKII phosphorylation of membrane proteins requires that the enzyme be targeted and anchored to the membrane containing these substrates, whereas phosphorylation of cytosolic proteins does not require any CaMKII translocation (Tsui et al., 2004). This allows CaMKII to be tightly regulated in a signaling microdomain comprised of a macromolecular complex of proteins including the kinase, its substrate(s), and protein phosphatases. It has previously been shown in the brain, where CaMKII expression is high, that this CaMKII is involved in frequency dependent changes in synaptic transmission, such as long-term potentiation (LTP) and long-term depression (LTD) (Malinow et al., 1989). Thus repetitive \(\text{Ca}^{2+}\) spikes may result in auto-phosphorylation of the enzyme and prolonged kinase activity. In fact, DeKoninck and Schulman (1998) have shown that this is the case, as the function of CaMKII as a frequency decoder of \(\text{Ca}^{2+}\) oscillations is critically dependent on the \(\text{Ca}^{2+}\) spikes producing auto-phosphorylation of the enzyme. Above the threshold for kinase activation some subunits of the enzyme become auto-phosphorylated, and the response to low-frequency stimulation is increased. Sub-maximal activation of the kinase by single \(\text{Ca}^{2+}\) spikes would not result in auto-phosphorylation, and the enzyme would remain dependent on \(\text{Ca}^{2+}\) for activity. These features of the enzyme allow CaMKII to transition to persistently activated forms depending upon the frequency of \(\text{Ca}^{2+}\) oscillations. In a study by Hudmon et al. (2005), a physical interaction of CaMKII with L-type \(\text{Ca}^{2+}\) channels was shown, and this interaction was proven to be critical for CaMKII’s role as an integrator of \(\text{Ca}^{2+}\) spikes involved in \(\text{Ca}^{2+}\)-dependent facilitation (CDF) of voltage-
gated Ca\(^{2+}\) channels. This interaction of CaMKII and its substrate position the kinase to act as a specific frequency decoder of the calcium spikes. Another key finding from this study is that CaMKII is not dependent on its interaction with the L-type Ca\(^{2+}\) channel to maintain activity. Thus, it is possible that CaMKII can dissociate from the channel and maintain its kinase activity to act on other targets. This study elegantly demonstrated that the interaction of CaMKII with the L-type Ca\(^{2+}\) channel allowed for the enzyme to act as a local sensor monitoring activity of the Ca\(^{2+}\) channel and as a kinase to regulate that activity.

3. CaMKII in the heart

The \(\delta\) and \(\gamma\) isoforms of CaMKII are the predominant isoforms found in the heart (Hudmon et al., 2005). The \(\delta\)-isoform can be classified as CaMKII\(\delta_C\), the cytosolic splice variant, or CaMKII\(\delta_B\), which is the nuclear splice variant since it contains a nuclear localization signal (Kohlhaas et al., 2006). These isoforms have been implicated in the modulation of excitation-contraction coupling (ECC) and in regulating gene expression in cardiac tissue (summarized in Maier and Bers, 2007). It was found that phosphorylation by either the \(\delta_B\) or \(\delta_C\) isoform has distinct effects on Ca\(^{2+}\)-handling proteins in the heart, however, both isoforms similarly regulated hypertrophic gene expression through HDAC phosphorylation (Zhang et al., 2007). CaMKII plays a role in modulating ECC by direct phosphorylation of several key proteins involved in the regulation of Ca\(^{2+}\). These proteins include the RyR (Witcher et al., 1991; Hain et al., 1995), phospholamban (PLB) (Davis et al., 1983; Simmerman et al., 1986) and L-type Ca\(^{2+}\) channels (see Maier and Bers, 2002; Hudmon et al., 2005). Furthermore, endogenous CaMKII\(\delta\) was shown to
directly associate with the RyR2 to modulate its activity in rabbit heart (Currie et al., 2004). The functional consequences of each of these phosphorylation events in the normal heart is a facilitation of the Ca$$^{2+}$$ current through L-type Ca$$^{2+}$$ channels, increased reloading of the SR by relieving PLB-mediated inhibition of SERCA, and an increased open probability of the RyR. This allows the heart to function at higher rates, such as during β-adrenergic stimulation. This acute activation of the β-adrenergic system results in the “fight-or-flight” response causing positive chronoptropy and inotropy. However, in cases of heart failure where the CaMKII is upregulated (Ai et al., 2005) and β-adrenergic stimulation is chronic due to the decreased contractility, CaMKII phosphorylation of RyR causes increased leak during diastole and can lead to delayed after depolarizations (DADs) and triggered arrhythmias. It was shown that inhibition of CaMKII could completely abolish these effects, thus it possible this phenomenon is regulated entirely by CaMKII (Curran et al., 2010). One hypothesis for the activation of CaMKII by β-adrenergic stimulation is that the increased frequency and magnitude of Ca$$^{2+}$$ signals in the cytosol arising from β-adrenergic stimulation can activate the CaMKII enzyme (Grimm and Brown, 2010). Of significance, the nuclear-localized CaMKII may be activated in the same way; however, the source of the activating Ca$$^{2+}$$ has been shown to come from InsP$_3$-induced Ca$$^{2+}$$ release (IICR) from nuclear InsP$_3$Rs (Wu et al., 2006).

As stated above, CaMKII activity and expression were found to be increased in human heart failure (Hoch et al., 1999; Kirchhefer et al., 1999). The function effects of acute and long-term overexpression of CaMKII were examined in the heart to determine its effects on the Ca$$^{2+}$$ signaling causing initiation and progression of heart failure. The overexpression of CaMKII$$^{\delta_C}$$ in the mouse heart caused contractile dysfunction and heart
failure. Ryanodine receptor phosphorylation was increased even before development of heart failure. As typically seen in heart failure, SR Ca\(^{2+}\) content was also decreased, and sodium/calcium exchanger (NXC) activity was increased. Furthermore, phospholamban phosphorylation specifically at the CaMKII site was increased, thus providing evidence for the involvement of CaMKII\(\delta_C\) activation in the pathogenesis of heart failure (Zhang et al., 2002). In addition to the effects of CaMKII\(\delta_C\), CaMKII\(\delta_B\) was found to lead to cardiac hypertrophy and heart failure. CaMKII\(\delta_B\) transgenic mice developed cardiac hypertrophy and changes in gene expression consist with development of a dilated cardiomyopathy. This supports the hypothesis that CaMKII\(\delta_B\) can function within the nucleus to upregulate the expression of genes leading to hypertrophy (Zhang et al., 2002).

It was also recently shown that acute adenoviral-mediated overexpression of CaMKII\(\delta_B\) can cause the translocation of HDAC4 out of the nucleus. This results in the de-repression of hypertrophic genes and thus could contribute to the onset or progression of heart failure (Bossuyt et al., 2008).

### 4. CaMKII Interaction and Modulation of InsP\(_3\)R2

The type 2 inositol 1,4,5-trisphosphate receptor isoform is the predominant isoform expressed in atrial and ventricular myocytes. The majority of InsP\(_3\)R2 is localized to the nuclear envelope in ventricular myocytes, where it has been hypothesized to play a role in calcium-dependent nuclear specific signaling. Using nuclei isolated from rat hearts, Bare et al. (2005) have shown that the InsP\(_3\)R2 is an integral nuclear envelope Ca\(^{2+}\) release channel, implying a specific and unique role for the InsP\(_3\)R2 in regulating nuclear Ca\(^{2+}\) transients. Furthermore, the receptor forms a complex with recombinant
CaMKII \textit{in vitro} and with endogenous CaMKII \textit{in vivo}. It was also shown that the receptor is a substrate for the kinase. Full-length recombinant InsP$_3$R2 immunoprecipitated from COS-1 cells could be phosphorylated by exogenously supplied CaMKII. This phosphorylation could be inhibited by KN-93, a CaMKII inhibitor. The same was shown for the type 2 carboxyl terminal truncated construct that only contained the first 1078 amino acids. Phosphorylation of the receptor by CaMKII in a cellular context was also demonstrated in cardiac nuclei. No exogenous CaMKII was supplied and the phosphorylation state of immunoprecipitated InsP$_3$R2 was evaluated. The native InsP$_3$R2 had $^{32}$P label incorporated, which could be decreased by addition of KN-93, suggesting that this is a physiological relevant phosphorylation process that takes place in the nuclei of myocytes. Furthermore, the expressed recombinant InsP$_3$R2 was phosphorylated by exogenous CaMKII$\delta_B$, resulting in a dramatic decline in open probability of the channel when incorporated into planar lipid bilayers for single channel analysis. The open probability was profoundly reduced, from $\sim$0.43 to 0.04, when pretreated with CaMKII. As a result, there was a 12-fold increase of the channel mean close time. This effect could be abolished by the addition of the CaMKII inhibitor KN-93, suggesting the effect was a result of phosphorylation of the channel (Bare \textit{et al.}, 2005). These studies support the hypothesis that IP$_3$-dependent Ca$^{2+}$ release via the InsP$_3$R2 localized to the nuclear envelope in adult ventricular myocytes may activate CaMKII$\delta_B$ that is in complex with the receptor. Activated CaMKII$\delta_B$ can signal to downstream molecules, such as HDAC5, and also negatively feedback on InsP$_3$R2 function, resulting in a decrease in channel activity.
Wu et al. (2006) investigated the InsP$_3$R2s in ventricular myocytes and the receptor’s functional relationship with CaMKIIδ$_B$ and one of its downstream targets, HDAC5. It had been shown previously that CaMKII can phosphorylate type II histone deacetylases (HDAC 4, 5, 7, 9), which normally act to repress gene expression by favoring condensed DNA (McKinsey et al., 2000). Phosphorylated HDAC is transported from the nucleus, allowing hypertrophic gene expression activation. An InsP$_3$R antagonist, 2-APB, blocked HDAC5 nuclear transport, while adenophostin (InsP$_3$R agonist) and ET-1 (InsP$_3$ production agonist) induced HDAC5 transport from the nucleus. Depletion of nuclear envelope Ca$^{2+}$ stores with thapsigargin (TG) abolished the effect of ET-1 on HDAC5 nuclear export. Notably, the normal beat-to-beat global fluctuations in [Ca$^{2+}$]$_i$ in the myocyte responsible for ECC were not able to activate the CaMKII responsible for the phosphorylation of HDAC5, suggesting a very specific and localized signaling microdomain. Also, the CaMKII inhibitor KN-93 partially blocked HDAC5 nuclear export. Endothelin-1 and adenophostin were shown to induce autophosphorylation of CaMKII in permeabilized myocytes, with both effects being blocked by the InsP$_3$R inhibitor, 2-APB. This provides a role for the InsP$_3$R2 in the activation of CaMKII (Wu et al., 2006). These data discussed above support a hypothesis of local Ca$^{2+}$ release via InsP$_3$R2 activating CaMKIIδ$_B$ coupled to the receptor in a macromolecular signaling complex, allowing the activated CaMKII to act on downstream targets and feedback on the activity of the InsP$_3$R2 in a nuclear signaling cascade leading to expression of hypertrophic genes and heart failure. Thus, the feedback regulation of CaMKII-mediated phosphorylation of the InsP$_3$R2 is an important part of the pathway and may serve as a way to terminate the nuclear IP$_3$-dependent Ca$^{2+}$ signal.
CHAPTER TWO
AIMS AND HYPOTHESIS

The hypothesis to be tested is that activated CaMKII feedback modulates the InsP₃R₂ via direct phosphorylation, and disruption of those sites can abolish phosphorylation and the resulting functional consequences. This study will give us a better functional understanding of how the InsP₃R and CaMKII macromolecular complex is regulated and will be accomplished by the following specific aims.

**Specific Aim 1:** Identify Putative CaMKII Phosphorylation Sites on the InsP₃R₂

This Aim will be accomplished by identifying through $^{32}$P-incorporation which InsP₃R₂ fragments are phosphorylated by CaMKII. Recombinant proteins will be treated with exogenous CaMKII enzyme to determine which ones can be phosphorylated by CaMKII.

**Specific Aim 2:** Mutagenize Putative CaMKII Phosphorylation Sites on the InsP₃R₂
This Aim will be accomplished by mutagenizing potential CaMKII phosphorylation sites identified in Aim 1 and using those constructs in $^{32}$P-incorporation assays to determine if mutation of specific amino acid residues can abolish CaMKII-mediated phosphorylation of the InsP$_3$R2.

**Specific Aim 3:** Test the Hypothesis that Ablation of a CaMKII Phosphorylation Site in Full-length InsP$_3$R2 Affects the Functional Characteristics of the Channel

This Aim will be accomplished by using single channel recordings of recombinant WT, non-phosphorylatable, and phosphomimetic InsP$_3$R2 channels in planar lipid bilayers and evaluating the channel’s open probability and subconductance states in the presence and absence of activated CaMKII.
CHAPTER THREE
MATERIALS AND METHODS

A. REAGENTS

Reagents used were obtained from the following vendors: Ins(1,4,5)P\textsubscript{3} was obtained from Alexis (San Diego, CA). All restriction enzymes, DNA ligase, and DNA phosphatase used for molecular cloning were obtained from New England Biolabs (Ipswich, MA). PP1 and PP2A were from Sigma Aldrich (St. Louis, MO). Dulbecco’s modified eagle medium (DMEM), HyClone TNM-FH insect cell medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin used for tissue culture and cell propagation were obtained from Cellgro/Media tech (Herndon, VA). All other chemicals used were reagent grade.

B. PLASMID CONSTRUCTION

1a. \textit{InsP}_{3}R2: Full length

The construction of the full-length type 2 \textit{InsP}_{3}R protein expression vector (p\textit{InsP}_{3}R2) was described in total previously (Ramos-Franco \textit{et al.}, 2000). Briefly, the expression plasmid was assembled using overlapping cDNA clones originally isolated from a rat brain library (Sudhof \textit{et al.}, 1991). The fully expressed protein includes amino acid residues 1-2701 from the rat type 2 cDNA (accession number X61677).
1b. *InsP$_3$R2: pInsP$_3$R2-Stop1078*

The pIP$_3$R2-Stop1078 construct is a mammalian expression vector of the first 1078 amino acids of the type-2 InsP$_3$ receptor. This sequence is followed by the 12 carboxy-terminal amino acids of the 116K subunit of the proton pump and was previously described (Sudhof *et al.*, 1991). Briefly, pIP$_3$R2-Stop1078 was constructed by cloning a 2.45 kb EcoRI-KpnI fragment followed by the 1.04 kb KpnI-PstI fragment of InsP$_3$R2 into pCMV2 followed by an oligonucleotide encoding the carboxy-terminal proton pump epitope. The fully expressed protein includes amino acid residues 1-1078 from the rat type 2 cDNA (accession number X61677).

1c. *InsP$_3$R1-Stop 1081*

The pInsP$_3$R1-Stop 1081 construct is a mammalian expression vector of the first 1081 amino acids of the InsP$_3$R1. This sequence is followed by the 12 carboxy-terminal amino acids of the 116K subunit of the proton pump and was previously described (Mignery and Sudhof, 1990). Briefly, the oligomerized nucleotides encoding the proton pump tag followed by a stop codon were inserted at a PstI site at 3567 in the pInsP$_3$R1 construct, thus producing the pInsP$_3$R1-Stop 1081 construct.

2a. *InsP$_3$R2 Fragments: Mammalian Expression Vectors*

The pInsP$_3$R2 vector was used as the template in a series of PCR reactions to create three mammalian expression vectors of the InsP$_3$R2 carboxy terminal amino acids 1074-1640, 1635-2118, and 2114-2701 (Remus and Mignery, unpublished). The
fragments were constructed using the following PCR primer pairs (all reverse primers are written 5’-3’):

**Fragment 1074-1640**
(forward) GM338-CCGGAATTCTCCGGGGCCCTGCAG
(reverse) GM339-CCGGAATTCTGGGAACAGTAGCTCCGG

**Fragment 1635-2118**
(forward) GM340-CCGGAATTCCCGGAGCTACTGTTCCTCA
(reverse) GM341-CCGGAATTCGTGGCGGGCCAACTG

**Fragment 2114-2701**
(forward) GM342-CCGGAATTCCAGTTGGCCCGCCAC
(reverse) GM343-CCGGAATTCTCAGTGCGGTGGCATGTG

Each of the PCR products were digested with the restriction enzymes *SalI, XhoI*, and/or *EcoRI* and inserted into a similarly digested pCMV-3Tag-1a vector obtained from Stratagene (La Jolla, CA) creating new protein fusions that contained three copies of the FLAG epitope on the N-terminus of the InsP$_3$R2 protein fragment (Remus and Mignery, unpublished).

2b. *InsP$_3$R2* Fragments: Bacterial Expression Vectors

Regions cloned into the bacterial expression plasmid pET-3a were PCR-amplified using pInsP$_3$R2 as template and specific primers engineered to contain and N-terminal NdeI restriction site and a C-terminal BglII site in addition to a Methionine inserted directly after the NdeI site and a termination codon directly before the the BglII site. The fragments were constructed using the following PCR primer pairs (all reverse primers are written 5’-3’):
Fragment 1-546
(forward) GM356-CCATGGGCCATATGTCTGACAAAAATGTCCAGCTTCC
(reverse) GM362-GGCCAGATCTTTAATAGCGCTGGTCGGGCCCAGGTCC

Fragment 234-1078
(forward) GM361-GGCCATATGAACTGAGTTACTCTCTTCATACG
(reverse) GM357-GGCCGAAGATCTTTACTGAGGCCGGCCCG

Fragment 320-1078
(forward) GM363-GGCCATATGCTATCTTGAGATGCTCAA
(reverse) GM357-GGCCGAAGATCTTTACTGAGGCCGGCCCG

Fragment 1-338
(forward) GM356-CCATGGGCCATATGTCTGACAAAAATGTCCAGCTTCC
(reverse) GM364-GGCCAGATCTTTATGGAAGCTCCCCGTCTCTCAC

Fragment 106-338
(forward) GM368-GGCCATATGGAAAGGAGGGAACATTTTTGGGAGAAATT
TTG
(reverse) GM364-GGCCAGATCTTTATGGAAGCTCCCCGTCTCTCAC

Fragment 134-338
(forward) GM369-GGCCATATGCTGGAAAGGTTTACCTGCCTTACTGG
AGAAG
(reverse) GM364-GGCCAGATCTTTATGGAAGCTCCCCGTCTCTCAC

Fragment 151-338
(forward) GM374-GGCCATATGGATGCTGGAAAGGAGGGTCTCTGG
AGAAG
(reverse) GM364-GGCCAGATCTTTATGGAAGCTCCCCGTCTCTCAC

Fragment 172-338
(forward) GM370-GGCCATATGAGGGGTGATATAATATCGCTGGAGAT
AAAGTC
(reverse) GM364-GGCCAGATCTTTATGGAAGCTCCCCGTCTCTCAC

PCR products were then digested with Ndel/BglII and ligated into Ndel/BamHI digested pET-3a plasmid to form the various bacterial expression plasmids.
C. SITE-DIRECTED MUTAGENESIS

Mutagenesis was performed using Change-IT Multiple Mutation Site-Directed Mutagenesis Kit (USB Corp.). Bacterial expression vectors described above were used as templates for the mutagenic PCR reactions. Primer pairs were designed to bind to unique sites on the template, with the bases coding for the mutation located in the middle of the primer and flanked by 10-15 bases complementary to the template DNA. For each mutagenic reaction, primer pairs had to bind to opposite strands of the DNA template. After the mutagenic PCR reaction, the template DNA was digested with DpnI enzyme for 1 hour at 37°C to reduce background colonies when the reaction was transformed. DH5α E. coli cells (Invitrogen) were transformed with 1µl of the digested PCR reactions. Cells were plated on agar plates with the appropriated antibiotic for growth of transformed cells and incubated overnight at 37°C. Colonies were then selected and propagated for extraction of the transformed DNA plasmids. Plasmids isolated from the bacterial cells were sequenced to confirm that they contained the desired mutations.

D. DNA SEQUENCING

Newly synthesized plasmid DNA constructs were verified by DNA sequence analysis using a commercial facility at University of California Davis (Davis Sequencing) using the Applied Biosystems Big Dye Terminator V3.0 sequencing chemistry.

E. COS-1 CELL TRANSFECTION

COS-1 cells were transiently and singly transfected with the expression plasmids for pInsP3R2, pInsP3R2-S150A, pInsP3R2-S150E, pIP3R2-Stop1078, and with the
mammalian expression plasmids for amino acids 1074-1640, 1635-2118, and 2114-2701 described above using a diethyaminoethyl-dextran method (DEAE) as described previously (Mignery et al., 1990). When needed, sheared salmon sperm DNA (ssDNA) was used to represent mock transfected cells and serve as a negative control. Cells were incubated at 37° C, 5% CO₂ and harvested 48-72 hours post-transfection.

F. HARVESTING OF COS-1 CELLS

Following the expression period, the COS cells were washed with phosphate buffered saline (PBS), harvested by scrapping the cells off the plate in immunoprecipitation (IP) buffer (50 mM HEPES, pH 7.6, 1.0% bovine serum albumin (BSA), 10 mM magnesium acetate, 50 mM NaCl2, 0.5 mM CaCl₂, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg/ml soybean trypsin inhibitor, 10 µM leupeptin, and 10 µM pepstatin) and lysed by 20-40 passages through a 27-gauge needle. The membranes were pelleted by centrifugation using a Beckman fixed angle rotor (TLA-110) at 4° C for 10 minutes at 135,000 x g<sub>max</sub> and the supernatant containing the soluble protein fraction was removed from the microsomes. To solubilize any membrane bound proteins, the membrane fraction was resuspended in IP Buffer with the addition of 1.0% Triton X-100 and incubated on ice with stirring for 1-2 hours followed by removal of insoluble material by centrifugation at 135,000 x g<sub>max</sub> at 4° C.

G. BACTERIAL CELL EXPRESSION

*E. coli* Rosetta 2 (DE3) competent cells (Novagen) were transformed with the various bacterial expression plasmids and grown at 37°C with shaking in liquid culture
until $\text{OD}_{600}$ reached 0.6. Isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was incubated for 3 hours at 37°C with shaking for induction of protein production.

**H. INCLUSION BODY PURIFICATION AND SOLUBILIZATION**

After incubation for protein production, cells were pelleted at 3220 x $g_{\text{max}}$ for 20 min. and resuspended in lysis buffer (8% Sucrose, 0.5% Triton X-100, 10 mM Tris-Cl pH 8, 50 mM EDTA, 1 mM PMSF) with 10 mg/mL Lysozyme (Sigma) and incubated at room temperature with agitation for 20 min. The lysate was then homogenized by hand and 1 mg/mL DNase and 100 mg/mL RNase were added and the solution was incubated at room temperature for 25 min with agitation. The sample was then spun at 12000 x $g_{\text{max}}$ for 10 min., and the pellet was washed with Wash Buffer (0.5M NaCl, 0.5% Triton X-100, 1 mM PMSF). This was repeated twice with the final wash containing 1 M urea. The resulting inclusion body pellet was then solubilized in solubilization buffer (8 M Urea, 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 3 mM 2-Mercaptoethanol, 3 mM DTT) and insoluble material was pelleted by centrifugation at 106,000 x $g_{\text{max}}$ for 10 min. Soluble material was transferred to a dialysis bag (MWCO=12 kDa) and dialyzed against 4 L of dialysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.4, 2 mM 2-Mercaptoethanol) to remove the urea and allow refolding of recombinant proteins for downstream applications. Recovered material was cleared by centrifugation at 106,000 x $g_{\text{max}}$ for 10 min and the lysate containing the purified, refolded proteins was stored at 4°C.
I. IMMUNOPRECIPITATION OF RECOMBINANT PROTEINS

The supernatant from COS-1 cells (containing recombinant proteins from either solubilized membrane fractions or the cytosolic fractions of lysed cells) or the purified, refolded bacterially expressed proteins were divided into equal volume aliquots, and the appropriate primary antibodies were added and incubated overnight at 4°C with agitation. Protein-A Sepharose CL-4B beads (Amersham Biosciences) were added, and incubated for 2 hrs. at 4°C with agitation. Immune complexes were then washed three times with IP buffer before addition of kinase.

J. CAMKII PHOSPHORYLATION OF RECOMBINANT PROTEINS

IP complexes were used as substrates for CaMKII phosphorylation in the in vitro 32P incorporation assays. Immune complexes were incubated at 30°C for 20 min with 500 units of exogenous pre-activated CaMKII (New England Biolabs) or with 10 µM of the specific CaMKII inhibitor, KN-93 (Seikagaku Corp.). The reaction buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 2 mM DTT, 0.1 mM Na2EDTA, 2 mM CaCl2, 1.2 µM Calmodulin, 200 µM ATP) was supplemented with [γ-P32]ATP to a final specific activity of 200 µCi/µmol for visualization of phosphorylated proteins via autoradiography.

K. ANTIBODIES

The InsP3R2-specific antibodies T1NH, T2NH and T2C have been previously described. The T1NH antibody is directed against the sequence CLATGHYLAEEVDPDQDASR (amino acids 308-341). The T2NH and T2C antibodies are directed against the sequences CPDYRDAQNEGKTVRDGELP (amino
acids 320-338) and CNKQRLGFLGSNTPHENHHMPPH (amino acids 2679-2701) of the rat InsP$_3$R2, respectively (Ramos-Franco et al., 1998a and 1998b). These three InsP$_3$R-specific rabbit polyclonal antibodies were affinity purified using immunogenic peptides. Anti-FLAG antibody was purchased from Affinity BioReagents and is a rabbit polyclonal against the peptide DYKDDDDDKC.

L. SDS-PAGE AND WESTERN BLOTTING

SDS-PAGE and Western blotting were performed as previously described (Mignery et al., 1990) using 5%, 7.5%, or 10% SDS-polyacrylamide gels. Western blotting was performed by electro-transfer of the resolved proteins to Osmonics Nitrobind nitrocellulose (Fisher Scientific; Pittsburgh, PA.) at 250 mA for 1 hour at 4°C in Transfer Buffer (192 mM glycine, 25 mM Tris-HCl, and 20% methanol). Following transfer, the nitrocellulose was blocked and blotted using the ReliaBLOT IP/Western Blot Reagent Kit (Bethyl Laboratories; Montgomery, TX). ReliaBLOT Block was reconstituted in TBST (150 mM sodium chloride, 10 mM Tris-HCl, 0.05% Tween-20, pH 8.0) and the nitrocellulose was placed in this solution for 30 minutes at room temperature on a rocking platform. The primary antibodies were diluted in ReliaBLOT Block/TBST at dilutions ranging from 1:3000 to 1:5000 and added to the nitrocellulose and allowed to incubate overnight with shaking. After the primary antibody incubation, the nitrocellulose was washed three times with TBST. ReliaBLOT Horseradish Peroxidase (HRP) Conjugate secondary antibody was diluted 1:3000 in ReliaBLOT Block/TBST and then added to the nitrocellulose to incubate for 45 minutes to 1 hour. The nitrocellulose was washed three times with TBST. Visualization was accomplished using enhanced
chemi-luminescence (ECL) reagents (GE Healthcare). Visualization of $^{32}$P-labeled proteins was done by exposing the dried nitrocellulose to laboratory film (Kodak) for periods of 4-24 hours. Films were developed using an automatic film developer.

**M. SF9 CELL CULTURE AND BACULOVIRUS INFECTION**

Sf9 cells were grown in 75 cm$^2$ tissue culture flasks in culture medium containing TNM-FH Medium (Cellgro) and 10% Fetal bovine serum. Cells were split every 3 days. Liquid cultures of Sf9 cells in TNM-FH medium were also used to increase cell numbers quickly. These cultures were typically between 100-200 mL and incubated at 27 °C with shaking for 24-48 hrs. Cells were plated onto the flasks again prior to baculoviral infection. A baculovirus expressing the full-length rat type 2 InsP$_3$R (RT2, kindly provided by Dr. Iyla Bezprozvanny) was added to the medium of flasks containing Sf9 cells at a multiplicity of infection of 1 (MOI=1) and cells were incubated at 27 °C for 72 hours to allow for expression of recombinant protein.

**N. PREPARATION OF MICROSOMES FOR LIPID BILAYERS**

Microsomes from Sf9 and COS-1 cells were prepared for use in planar lipid bilayers as previously described (Kaznacheyeva *et al.*, 1998). Briefly, COS-1 or Sf9 cells were harvested in homogenization buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA, 5 mM sodium azide, 0.25 mM PMSF, 10 µM leupeptin, 10 µM pepstatin, 100 µg/ml trypsin inhibitor) and lysed with a glass/Teflon Potter-Elvehjem tissue grinder. An equal volume of buffer containing 0.5 M sucrose was added, and the material was re-homogenized and centrifuged for 5 min at 1,200 x g$_{max}$. The supernatant containing the microsomes was
recovered, and KCl and disodium pyrophosphate were added to a final concentration of 0.6 M and 20 mM, respectively. The material was homogenized and mixed for 30 min at 4 °C. Following a final homogenization, the microsomes were centrifuged for 5 min at 200 x \( g_{\text{max}} \), and the supernatant was recovered. Finally, the microsomes were pelleted at 100,000 x \( g_{\text{max}} \) for 10 min, resuspended in storage buffer containing 10% sucrose, and snap-frozen and stored in liquid nitrogen.

**O. PLANAR LIPID BILAYERS**

Single channel recordings of recombinant InsP3R type 2 activities were performed by fusing microsomes into planar lipid bilayers. Bilayers were formed across a 150 µm diameter hole in the wall of a Delrin cup using a 7:3 lipid mixture of phosphatidylethanolamine and phosphatidylcholine (stock concentrations of 50 mg/ml in decane were used, Avanti Polar Lipids, Alabaster, AL). The bilayer separated two pools \((cis)\) and \((trans)\). The microsomes were added to the \(cis\)-side of the bilayer. Standard solution contained 20 mM HEPES-Tris, pH 7.4, 1 mM EGTA, \([Ca^{2+}]_{\text{FREE}} = 250\) nM, and 220 mM CsCH\(_3\)SO\(_3\) in the \(cis\)-chamber (20 mM CsCH\(_3\)SO\(_3\) in the \(trans\)). Free calcium concentration was calculated using MaxChelator software. 2 µM InsP\(_3\) was used in the \(cis\) side. The \(trans\) pool was held at virtual ground. The channels were positively identified by their sensitivity to InsP\(_3\). Unitary currents were recorded using a conventional patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA). The current signal was digitized at 10 kHz with a 32-bit AD/DA converter (Digidata 1322A, Axon Instruments) and filtered at 1 kHz with a low pass eight pole Bessel filter. Data acquisition, unitary current measurement, statistical analysis, and data
processing were performed using commercially available software packages (pClamp V10.2, Axon Instruments and OriginPro 7, Microcal).
CHAPTER FOUR
IDENTIFICATION OF THE CAMKII PHOSPHORYLATION SITE ON THE
TYPE 2 INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR

A. INTRODUCTION

To determine the exact site of phosphorylation of the InsP$_3$R2 by CaMKII, I used exogenously expressed fragments of the InsP$_3$R2 in in vitro phosphorylation assays. Figure 5 shows a diagram of the fragments of the InsP$_3$R2 I used in these assays. Fragments containing a FLAG-tag were expressed in COS-1 cells, while the remaining fragments were expressed in bacterial cells. The recombinant proteins were treated with exogenous CaMKII in the presence of radioactive ATP ($\gamma^{32}$P-ATP). These reactions were resolved by polyacrylamide gel electrophoresis and phosphorylated proteins were determined by autoradiography. The results of these experiments identified a single potential CaMKII site based on the minimal InsP$_3$R2 fragment that was phosphorylated by CaMKII. This phosphorylation site was abolished by site-directed mutagenesis of the Serine residue to the small, non-charged amino acid Alanine, which has no hydroxyl side group for a phosphate group to be attached to. This has been proven to be a reliable and successful method to inhibit phosphorylation of a residue while not disrupting the structure or function of the recombinant protein. Mutation of Serine 150 to Alanine successfully abolished CaMKII-dependent phosphorylation of two different InsP$_3$R2 sub-
fragments that were tested. In addition, this mutation was made in the full-length rat InsP$_3$R2, and it was able to prevent CaMKII-mediated phosphorylation of the recombinant full-length InsP$_3$R2 in vitro. These results unambiguously identify Serine-150 of the rat InsP$_3$R2 as the CaMKII phosphorylation site. Furthermore, these results suggest that this is the only CaMKII phosphorylation site present on the InsP$_3$R2 since mutagenesis of this site completely abolished CaMKII-mediation phosphorylation.
Figure 5: InsP$_3$R2 fragments used in phosphorylation assays

The colored regions of the full-length rat InsP$_3$R2 represent the ligand binding domain (red), and coupling/modulatory domain (green), and the channel domain (blue). All fragments were generated by PCR amplification using full-length InsP$_3$R2 as template. Those containing an N-terminal FLAG-tag (yellow) were created with restriction sites for insertion into the pCMV-3Tag-1A vector. Those not containing the FLAG-tag were created with restriction sites for insertion into the pET-3a vector for bacterial cell expression.
B. RESULTS

1. CaMKII-dependent phosphorylation of pInsP$_3$R2-Stop1078

It has been shown previously in our lab that CaMKII could phosphorylate a fragment spanning amino acids 1-1078 of the InsP$_3$R2 (Bare et al., 2005). This is shown in Figure 6 along with the rest of the fragments spanning the entire InsP$_3$R2 protein. The fragments were expressed in COS-1 cells and recombinant proteins were immunoprecipitated with an amino terminal antibody (T2NH, for pInsP$_3$R2-Stop1078) or anti-FLAG antibody (for 1074-1640, 1635-2118, and 2114-2701). Prior to immunoprecipitation, the 2114-2701 fragment had to be solubilized from the microsomal fraction since it contains the 6 transmembrane regions. Immunoprecipitated complexes were then treated in the presence of radioactive ATP with exogenously supplied pre-activated CaMKII (500 units) or the specific CaMKII inhibitor KN-93 (10 µM), resolved on 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and autoradiographed and Western blotted. As shown in Figure 6, the fragment spanning amino acids 1-1078 was the only one that showed $^{32}$P-incorporation upon treatment with CaMKII. This effect was blocked when KN-93 was used. These data show that the CaMKII phosphorylation site on the InsP$_3$R2 lies in the N-terminal 1078 amino acids and no other CaMKII sites are present downstream of amino acid 1078.
CaMKII phosphorylation assays performed using IPs of InsP₃R2 subfragments expressed in COS-1 cells as substrates reveal that the fragment spanning amino acids 1-1078 is phosphorylated by CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 7.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. With the addition of CaMKII, the 1-1078 construct showed significant incorporation of $^{32}$P, while no signal could be detected with the 1074-1640, 1635-2118, or 2114-2701 constructs (upper panel). Western blotting of the membrane with T2NH (1-1078) or anti-FLAG (1074-1640, 1635-2118, and 2114-2701) antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (lower panel). The results suggest that the CaMKII phosphorylation site on the InsP₃R2 lies in the N-terminal 1078 amino acids and no other CaMKII sites are present downstream of amino acid 1078.
2. CaMKII-dependent phosphorylation of bacterially expressed InsP₃R2 fragments

To further elucidate the site of CaMKII modulation of the InsP₃R2, I used four bacterially expressed fragments spanning amino acids 1-1078 (Figure 5). As shown in Figure 7, the 1-546 and 1-338 constructs showed significant incorporation of $^{32}$P after CaMKII treatment, while no signal could be detected with the 234-1078 and 320-1078 constructs. This CaMKII effect on the 1-546 and 1-338 constructs could be inhibited with KN-93. These results suggest that the CaMKII phosphorylation site on the InsP₃R2 lies within amino acids 1-234.

The last four fragments (shown in Figure 5) were created as N-terminal deletions in order to preserve the T2NH antibody epitope (amino acids 320-338) for immunoprecipitating the proteins. As shown in Figure 8, the 106-338 and 134-338 constructs showed significant incorporation of $^{32}$P, along with the 1-338 fragment as shown previously. No significant signal could be detected with the 172-338 construct. These results suggest that the CaMKII phosphorylation site on the InsP₃R2 lies within amino acids 134-171. In addition, a fragment spanning amino acids 151-338 could not be phosphorylated by CaMKII. These results suggest the CaMKII phosphorylation site is located within amino acids 134-150 of the InsP₃R2.
Figure 7: CaMKII-dependent phosphorylation of bacterially expressed InsP$_3$R2 fragments 1-338 and 1-546.

CaMKII phosphorylation assays performed using IPs of bacterially expressed InsP$_3$R2 subfragments as substrates reveal that fragments spanning amino acids 1-546 and 1-338 are phosphorylated by CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 12.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. With the addition of CaMKII, the 1-546 and 1-338 constructs showed significant incorporation of $^{32}$P, while no signal could be detected with the 234-1078 and 320-1078 constructs (upper panel). Western blotting of the membrane with T2NH antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (lower panel). The results suggest that the CaMKII phosphorylation site on the InsP$_3$R2 lies within amino acids 1-234.
CaMKII phosphorylation assays performed using IPs of bacterially expressed InsP$_3$R2 subfragments as substrates reveal that fragments spanning amino acids 106-338 and 134-338 are phosphorylated by CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 12.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. With the addition of CaMKII, the 106-338 and 134-338 constructs showed significant incorporation of $^{32}$P, along with the 1-338 fragment as shown previously. No significant signal could be detected with the 151-338 or the 172-338 constructs (upper panel). Western blotting of the membrane with T2NH antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (lower panel). The results suggest that the CaMKII phosphorylation site on the InsP$_3$R2 lies within amino acids 134-150.
3. CaMKII can Phosphorylate InsP$_3$R2 at Serine-150

The results from Figure 8 indicate that the CaMKII phosphorylation site on the InsP$_3$R2 lies within amino acids 134-150. Within this span of amino acids, there is one potential CaMKII site located at Serine-150. This Serine is part of an S-x-D motif in the InsP$_3$R2. This motif was shown to be the CaMKII-dependent phosphorylation site on the RyR2 (Wehrens et al., 2004). Site-directed mutagenesis was performed to mutate the Serine at residue 150 to Alanine to abolish phosphorylation in two of the fragments previously shown to be phosphorylated by CaMKII. Figure 9 shows that this resulted an observable decrease in the $^{32}$P-incorporation of the fragments containing the S150A mutation when treated with active CaMKII. 

The S150A mutation was also made in the full-length InsP$_3$R2 protein for use in an in vitro kinase assay in Figure 10. Upon treatment with CaMKII, the InsP$_3$R2-S150A showed no $^{32}$P-incorporation when compared to the InsP$_3$R2-WT protein that could be phosphorylated by exogenously supplied CaMKII. These results presented above clearly identify Serine-150 as the CaMKII phosphorylation site on the InsP$_3$R2. Furthermore, it can be concluded that this is the only CaMKII phosphorylation site present on the InsP$_3$R2 since mutagenesis of this site completely abolished CaMKII-mediation phosphorylation of the full-length protein.
CaMKII phosphorylation assays performed using IP’ed bacterially expressed InsP3R2 fragments as substrates (upper panel) show that mutation of Ser150→ Alanine could abolish CaMKII-mediated phosphorylation of Fragments 1-338-S150A and 134-338-S150A. Fragments 1-338 and 134-338 not containing the mutation showed significant incorporation of $^{32}$P when treated with CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 12.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions (lower panel). The results show that CaMKII can phosphorylate InsP3R2 fragments on Ser150 and mutation of this site abolishes all CaMKII-mediated phosphorylation of the fragments.
Figure 10: CaMKII can phosphorylate the full-length InsP$_3$R2 on Serine-150

CaMKII phosphorylation assays performed using full-length InsP$_3$R2 expressed in COS-1 cells as substrates show that mutation of Ser150→Alanine could abolish CaMKII-mediated phosphorylation of full-length InsP$_3$R2-S150A (upper panel). Full-length InsP$_3$R2 not containing the mutation showed significant incorporation of $^{32}$P when treated with CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Western blotting of the membrane with T2NH antibody confirmed the presence of similar protein amounts within samples used in the phosphorylation reactions (lower panel). The results show that Ser150 is the CaMKII phosphorylation site on the InsP$_3$R2.
C. DISCUSSION

Phosphorylation of the InsP3R2 by CaMKII represents an important physiological mechanism by which CaMKII can modulate the activity of the InsP3R2. This study is the first to identify the precise site of CaMKII phosphorylation on the InsP3R2. The results presented above show that CaMKII can phosphorylate the InsP3R2 on Ser150. Figure 10 shows that mutation of this residue can completely prevent CaMKII-mediated phosphorylation of the InsP3R2, indicating that this is the only site that CaMKII phosphorylates on the InsP3R2. This particular Serine residue is part of an S-x-D motif that was also found to be a target for CaMKII phosphorylation in the RyR2 (Wehrens et al., 2004). The conservation of this region in the other isoforms of the InsP3R2 and other CaMKII substrates is shown in Figure 11. Homology of the region CaMKII phosphorylation site in the InsP3R2 with the RyR2 and can be seen with the actual phosphorylation site (S-x-D) along with two alanine residues directly following the S-x-D motif. Furthermore, the Valine directly before Ser-150 is also conserved in the RyR2 and the InsP3R1 and InsP3R3. Interestingly, Serine-150 is conserved as a Threonine in the Type 1 and Type 3 InsP3Rs. No CaMKII-dependent phosphorylation of the InsP3R3 has been demonstrated; however, I have shown that CaMKII can phosphorylate the IP3R1 on the first 1081 amino acids (Figure 12). This supports the hypothesis that Thr-150 of the InsP3R1 is a potential CaMKII phosphorylation site. This provides a potential mechanism of differential regulation between isoforms given that one is a Serine and the other is a Threonine. CaMKII has not been observed to show any preferential phosphorylation of either residue. The Ca2+-dependence of the type 1 InsP3R is such that
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<td>bInsP3R3</td>
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**Figure 11: Homology of CaMKII phosphorylation sites**

CaMKII phosphorylation site alignment with the three isoforms of the InsP3R and from various species (mouse, rat, human, bovine) show that Serine-150 of the type 2 receptor is conserved as a Threonine in the type 1 and type 3 receptors. The region surrounding Ser150/Thr150 is also highly homologous in the three isoforms and among species. The CaMKII phosphorylation sites for human RyR2 and HDAC4 are also shown to illustrate the homology the InsP3R2 Serine-150 site shares with these known CaMKII phosphorylation sites on other cardiac proteins.
CaMKII phosphorylation assays performed using IPs of InsP$_3$R2 subfragments expressed in COS-1 cells as substrates reveal that the fragment spanning amino acids 1-1081 of the InsP$_3$R1 is phosphorylated by CaMKII. Reactions were carried out with the addition of exogenous CaMKII (500 units) or the specific CaMKII-inhibitor KN-93 (10 μM), resolved on 7.5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. With the addition of CaMKII, the 1-1081 and 1-1078 constructs showed significant incorporation of $^{32}$P, while this signal could be decreased with KN-93 treatment for both the 1-1081 and 1-1078 constructs (upper panel). Western blotting of the membrane with T1NH (1-1081) or T2NH (1-1078) antibody confirmed the success and uniformity of immunoprecipitations used in the phosphorylation reactions (lower panel). The results suggest that a CaMKII phosphorylation site on the InsP$_3$R1 lies in the N-terminal 1081 amino acids.

Figure 12: CaMKII-dependent phosphorylation of InsP$_3$R1 fragment 1-1081

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high calcium (>1 µM) can begin to inhibit the activity of the receptor, up to 10 µM where the receptor is nearly completely inhibited (Ramos-Franco et al., 1998b). This poses the question of what effect CaMKII-mediated phosphorylation has on the properties of the InsP₃R₁ channel. Looking at the amino acids surrounded Ser150/Thr150 in the type 2 and type 1 InsP₃Rs, it is reasonable to hypothesize that CaMKII phosphorylation of the type 1 receptor could have an even greater effect on the function of the channel due to the presence of the negatively charged amino acid Glutamate just 3 residues away from the phosphorylated Threonine, whereas this residue is the small uncharged amino acid Alanine in the InsP₃R₂. Upon phosphorylation, the electrostatic repulsion between negatively charged phosphate on Threonine-150 and the acidic amino acid Glutamate-153 of the InsP₃R₁ could potentially be the greatest of all the isoforms and thus produce the greatest functional effects on the activity of the channel. Elucidation of the mechanism of CaMKII-mediated inhibition of channel function of the InsP₃R₂ will help to shed light on what, if any, mechanisms contribute to CaMKII regulation of the InsP₃R₁ and the InsP₃R₃.

In addition to the local amino acid residues and phosphorylation motifs shown in Figure 10, the location of Serine-150 in the larger crystal structure of the suppressor domain of the InsP₃R₂ is shown in Figure 13A. Based on the resolved crystal structure for the protein domain comprising the suppressor domain of the mouse InsP₃R₁ (Bosanac et al., 2005), the location of Serine-150 (or Throneine-150) is shown in Figure 13B. Serine-150 is located on β-strand 7, just downstream of a CaM binding site. Furthermore, this residue seems to be accessible based on the crystal structure. It is located in close proximity to key residues involved in the interaction between the
suppressor domain and the binding core. The location and the accessibility of this site along with the observation that CaMKII phosphorylation inhibits InsP$_3$R2 channel activity (Bare et al., 2005), leads into the hypothesis that the mechanism behind CaMKII-mediated inhibition of the InsP$_3$R2 is that phosphorylation of this residue sterically hinders InsP$_3$ binding to the binding core or disrupts the suppressor domain/binding core interaction, both of which are critical for gating of the channel. Based on the location of Serine-150, it is possible that the negatively charged phosphate group attached to Serine-150 can cause a decrease in the affinity of the binding core for InsP$_3$ due to the repulsion between the phosphate on Serine-150 and the phosphate groups of InsP$_3$. The residues of the binding core proposed to be important for InsP$_3$ binding are not surprisingly basic, positively charged amino acids creating pockets of positive charge for the three negatively charged phosphates of InsP$_3$ to interact with. The addition of the phosphate to Serine-150 could perturb one or more of these positively charged pockets, thus decreasing the affinity of the receptor for InsP$_3$. Additionally, this negative charge that results from CaMKII-dependent phosphorylation of Serine-150 of the InsP$_3$R2 could affect the intramolecular interactions of the LBD, thus creating a structural arrangement of the LBD that is unfavorable for transducing ligand binding into channel gating. Further discussion on the mechanism of inhibition is found in Chapter Six in light of the results showing the functional effects of the phosphomimetic mutation and molecular modeling of the effect this mutation has on the ligand binding domain.
(A) Surface representation of key residues in the suppressor domain involved in inter- and intra-molecular interactions. Regions of interaction of the suppressor domain and the InsP3-binding core are shown along with regions of interaction with other proteins (figure modified from Bosanac et al., 2004). (B) Ribbon structure of the suppressor domain in similar orientation as in (A). Residues involved in suppressor domain/binding core interaction are highlighted in yellow. The highlighted and circled residue shows the location of Serine-150. Arrows represent β-strands, while the cylinders represent α-helices. From the structure, it looks as though Serine-150 is not only accessible on the surface of the structure, but it is also located in close proximity to the residues involved in the suppressor domain/binding core interaction.
CHAPTER FIVE

FUNCTIONAL CHARACTERISTICS OF TYPE 2 INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR S150A AND S150E MUTANTS

A. INTRODUCTION

To determine what effect phosphorylation of the InsP$_3$R2 by CaMKII had on the single channel properties of the receptor, I used exogenously expressed phosphomimetic and non-phosphorylatable mutants of the InsP$_3$R2 channel in planar lipid bilayer studies. Previous studies have shown that mutation of a phosphorylated residue to a negatively charged amino acid such as Glutamate or Aspartate can mimic the effect of phosphorylation of that residue (Wehrens et al., 2004). In the way that mutation of a phosphorylatable residue to Alanine leaves the kinase with no hydroxyl group to attach a phosphate group to, mutation of the residue to Glutamate or Aspartate creates a residue with a negatively charged side group at that position to mimic phosphorylation. Both Glutamate and Aspartate are negatively charged at physiological pH ranges due to pK$_a$’s of 4.07 and 3.9, respectively. In addition, the molecular weight of a phosphorylated Serine residue is approximately 166.98 Da (87.08 Da for Serine + 79.90 Da for phosphate group), and replication of this change in molecular weight and bulk upon phosphorylation is better represented with the 129.12 Da Glutamate compared to the 115.09 Da Aspartate.
Planar lipid bilayers represent an excellent system in which to study the single channel function of a native or recombinant ion channel. Channels incorporate into a lipid bilayer formed across a small hole separating two chambers. The concentration of ions and other molecules such as agonist can be tightly controlled on each side of the bilayer. In the following results, recordings were done at free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_\text{FREE}\)] of 250 nM or greater along with 2 µM InsP\(_3\) or greater on the cis (cytosolic side) of the bilayer. These concentrations of agonists have been shown to produce maximal activation of the InsP\(_3\)R2 in similar recordings (Ramos-Franco et al., 1998a and 1998b). Theoretically, only an InsP\(_3\)R2 that incorporates with its ligand binding domain facing the cis side would be able to bind the InsP\(_3\) there and open, as opposed to channels that may incorporate with the LBD facing the trans side where no InsP\(_3\) is present. This mimics the InsP\(_3\)R2 topography in the cellular environment, with the cis side being equivalent to the cytoplasmic compartment, while the trans being the lumen of the ER. WT and mutant channels were expressed in COS-1 cells, and microsomes were prepared from these cells for use in the bilayer recordings. Cesium (Cs\(^+\)) is used as the charge carrier in all recordings. In addition to preserving the tight control of [Ca\(^{2+}\)] on the cis and trans side of the bilayer, Cs\(^+\) also inhibits any endogenous potassium (K\(^+\)) channels that may be present contaminating the microsomal preparation. The hypothesis was that the phosphomimetic channel would have a very low \(P_o\) like that seen with the receptor when treated with exogenous CaMKII, while the non-phosporylatable channel would show no effect with CaMKII treatment. Single channel recordings of the S150E mutant channel were also performed at various [Ca\(^{2+}\)] and [InsP\(_3\)] in order to gain some further mechanistic insight as to the effect of CaMKII-mediated phosphorylation of the InsP\(_3\)R2.
The results presented below confirm that Serine-150 of the InsP₃R2 is the modulatory site of CaMKII-dependent phosphorylation. Abolishment of this site prevented the CaMKII-mediated effects on the function of the InsP₃R2. Furthermore, the phosphomimetic mutation produced channels with a very low $P_\alpha$ and also showed some interesting features of their Ca$^{2+}$-sensitivity that may be physiologically relevant and contribute to elucidating the mechanism of CaMKII-mediated regulation of the InsP₃R2.
B. RESULTS

1. Modulation of InsP₃R2 activity by CaMKII is reversible with phosphatase treatment

Previous data from our lab has shown that the InsP₃R2 and CaMKII form a macromolecular complex in cardiac ventricular myocytes and that CaMKII can modulate InsP₃R2 channel activity (Bare et al., 2005; Wu et al., 2006). Specifically, InsP₃R2 overexpressing microsomes were phosphorylated in vitro by pre-treatment with active CaMKII, and this resulted in a dramatic decrease in the open probability of the channel when incorporated into planar lipid bilayers (Bare et al., 2005). To further investigate the role of CaMKII in mediating InsP₃R2 activity, planar lipid bilayers were used for single channel analysis of the InsP₃R2. Insect Sf9 cells were infected with a baculovirus coding for the InsP₃R2 (RT2). Microsomes from the insect cells infected with RT2 baculovirus were prepared and used in the planar lipid bilayer single channel recording experiments shown in Figure 14. In Figure 14A, the InsP₃R2 channel is recorded under normal condition and displayed a $P_o$ of approximately 0.37. Active exogenous CaMKII was added to the cis side of the chamber. This treatment resulted in a decrease in the $P_o$ to 0.09. The addition of protein phosphatases (PP1 and PP2A) and KN-93 were able to relieve CaMKII-mediated regulation of the channel’s activity and the $P_o$ returned to approximately starting level (0.33). The control experiment was performed by adding all components present in the CaMKII activation mix except the enzyme itself to the bath solution and showing these components had no effect on InsP₃R2 activity (Figure 14B). Figure 14C shows summary bar graphs of the planar lipid bilayer studies. CaMKII
Figure 14: Modulation of recombinant InsP3R2 activity by CaMKII is reversible with phosphatase treatment.

Representative recordings of InsP3R2 single channels from InsP3R2-baculovirus infected Sf9 cell microsomes. (A) A channel $P_o$ of 0.36 was recorded under normal conditions. To the bath solution, 1500 units of active CaMKII enzyme were then added and recording continued after 20 min. This treatment resulted in a decrease in the $P_o$ to 0.09. The addition of protein phosphatases (10 units PP1 and 50 ng PP2A) and KN-93 (30 μM) were able to relieve CaMKII-mediated regulation of the channel’s activity and the $P_o$ returned to approximately starting level (0.33) after 20 min with treatment. (B) A channel $P_o$ of 0.38 was recorded under normal conditions. To the bath solution, CaMKII reaction buffer alone was added and recording continued after 20 min. This treatment resulted in no change in the recorded open probability of the channel. Both A & B were taken as representative windows having open probabilities ($P_o$) reflecting the 10 min recordings used for the actual determination of $P_o$. Channel openings are shown as upward deflections from the zero current level. (C) Summary bar graphs of the planar lipid bilayer studies. CaMKII treatment resulted in a dramatic decrease in the $P_o$ of the channel. Reversal of this effect by treatment with protein phosphatases and a CaMKII inhibitor is shown. The conditions for this study were 220/20 mM CsCH3SO3 (cis/trans), $[Ca^{2+}]_{FREE} =250$ nM, 2 μM InsP3. Channels were recorded at 0 mV holding potential.
treatment resulted in a dramatic decrease in the $P_o$ of the channel. Reversal of this effect by treatment with protein phosphatases and a CaMKII inhibitor is shown (Figures 14A & C). These data add support to the hypothesis that it is the CaMKII-mediated phosphorylation of the channel that is responsible for the decrease in $P_o$ seen upon CaMKII addition. In addition to implicating direct phosphorylation of the InsP$_3$R2 by CaMKII as the mechanism of regulation, these results also show that the site of CaMKII-dependent phosphorylation resides on the large cytoplasmic portion of the InsP$_3$R2. Since CaMKII added to the *cis* side of the bilayer could produce the inhibitory effect on the InsP$_3$R2, it can be concluded that the phosphorylation site for CaMKII is exposed to the *cis* solution when a channel correctly orients itself in the lipid bilayer. With InsP$_3$ present on only the *cis* side of the bilayer, I know that any channel activity that is observed is due to an InsP$_3$R2 that has incorporated with the LBD facing the *cis* side. In correlation with my previous results shown in Chapter 4, this is where Serine-150 is located in the quaternary structure of the tetrameric protein.

2. **Serine-150 is a site of CaMKII-dependent modulation of the InsP$_3$R2 channel**

In addition to the InsP$_3$R2-S150A non-phosphorylatable mutation used in Figure 10, the InsP$_3$R2-S150E phosphomimetic mutation was made for use in planar lipid bilayer studies by using site-directed mutagenesis to change Serine-150 to a Glutamate (S150E). WT and the two mutant (S150A and S150E) constructs were transiently transfected into COS-1 cells for expression of the proteins. Microsomal fractions from the three samples were prepared for use in channel recordings from planar lipid bilayers.
In **Figure 15A**, a sample recording from WT microsomes shows that upon CaMKII treatment, the open probability of the channel is significantly decreased. Conversely, the channel trace shown in **Figure 15B** from InsP$_3$R2-S150A microsomes did not show any decrease in open probability when active CaMKII enzyme was added to the bath solution, indicating that channel was resistant to the effect of CaMKII-dependent phosphorylation. Finally, **Figure 15C** is a representative channel trace from InsP$_3$R2-S150E microsomes showing that this channel protein exhibits a constitutively low open probability thus mimicking an InsP$_3$R2 that has been phosphorylated by CaMKII. This supports the hypothesis I proposed earlier that a phosphomimetic mutation of the modulatory CaMKII phosphorylation site on the InsP$_3$R2 would create a channel with an open probability similar to that of an InsP$_3$R2-WT channel treated with CaMKII. Indeed this is the case, and it also confirms that replacing Ser-150 with a Glutamate did not alter the ability of the receptor to form a functional tetrameric channel. **Figure 15D** is a summary bar graph showing the open probability of the three InsP$_3$R2 channels and the effect CaMKII has on each of them.
Figure 15: Representative single channel recordings of WT, S150A and S150E InsP₃R2s

(A) For WT-InsP₃R2 single channels, a channel $P_o$ of 0.57 was recorded. To the bath solution, 1500 units of active CaMKII enzyme were then added and recording continued. This treatment resulted in a decrease in the $P_o$ to 0.04 after 20 min. (B) For S150A channels, a channel $P_o$ of 0.58 was recorded. To the bath solution, 1500 units of active CaMKII enzyme were then added and recording continued. This treatment resulted in no change in the recorded $P_o$ of the channel after 20 min. (C) For S150E channels, a channel $P_o$ of 0.02 was recorded. A, B & C were taken as representative windows having $P_o$’s reflecting the 5 min recordings used for the actual determination of $P_o$. Channel openings are shown as upward deflections from the zero current level. (D) Summary bar graphs show CaMKII treatment resulted in a dramatic decrease in the $P_o$ of the WT channel. This effect could be inhibited by mutation of S150 to Alanine (S150A). The S150E show a constitutively low Po, similar to the WT channel treated with CaMKII. The conditions for this study were 220/20 mM CsCH₃SO₃ (cis/trans), $[\text{Ca}^{2+}]_{\text{FREE}}=250$ nM, 2 μM InsP₃. Channels were recorded at 0 mV holding potential.
3. A subconductance state prevails in the InsP$_3$R2-S150E channel

Further analysis of the InsP$_3$R2-S150E channel revealed the appearance of a subconductance state (Figure 16). This open state was $\sim$2.5-3 pA in amplitude, which is approximately half of the normal opening current amplitude seen in the WT channel. The traces in Figure 16 show this subconductance state. The records are expanded (see scale bars) to illustrate the small open state seen in the InsP$_3$R2-S150E channels. The asterisk denotes a full opening ($\sim$5.0 pA) interspersed within the subconductance openings. Upon further review of the InsP$_3$R2-WT channels recorded from microsomes from COS-1 cells transfected with the InsP$_3$R2-WT construct, it was revealed that this subconductance state is also observed in these traces (see Figure 17), with a similar incidence of this state compared to the main opening. In Figure 17, amplitude histograms of an InsP$_3$R2-WT channel (A) and an InsP$_3$R2-S150E (B) channel are shown for comparison. The histograms represent small portion of the two channel’s full traces in order to illustrate the presence of the subconductance state in the WT channel as well as the S150E. No statistical analysis of this is provided because of a lack of a sufficient number of channels due to the difficulty obtaining these traces for S150E channels.

4. High cytoplasmic [Ca$^{2+}$]$_{\text{FREE}}$ activation of the InsP$_3$R2-S150E channel

In an effort to try and “override” the low open probability of the InsP$_3$R2-S150E with a large [InsP$_3$], an interesting characteristic of the InsP$_3$R2-S150E was found. As shown in Figure 18A, an InsP$_3$R2-S150E channel in the presence of 800 nM [Ca$^{2+}$]$_{\text{FREE}}$ and 8 $\mu$M InsP$_3$ exhibited high activity not characteristic of this channel. The 800 nM
Figure 16: InsP$_3$R2-S150E channel shows a subconductance state

Representative single channel traces of InsP$_3$R2-S150E channels. The sample records shown are 375 ms in duration and were taken as representative windows of the complete trace. Channel openings are shown as upward deflections from the zero current level. Note the decreased amplitude scale bar to illustrate the decreased amplitude of this observed subconductance state. A full opening is indicated (*). The conditions for this study were 220/20 mM CsCH$_3$SO$_3$ (cis/trans), [Ca$^{2+}$]$_{FREE}$ = 250 nM, 2 μM InsP$_3$. Channels were recorded at 0 mV holding potential.
Figure 17: Amplitude histograms of InsP$_3$R2-WT and InsP$_3$R2-S150E channels

(A) Amplitude histogram of a portion of an InsP$_3$R2-WT channel shows the presence of an approximately 2.5-3 pA subconductance state along with the full open state (~5 pA). (B) The InsP$_3$R2-S150E channel shows a small population of opening events at the 2.5 pA level, with the majority of events at the 0 pA level. Red traces are Gaussian fits of the histograms.
[Ca\(^{2+}\)]_{FREE} \text{ was present in order to increase the molarity of the cis chamber and promote fusion of the microsomes with the lipid bilayer. Recording of this channel was begun (Figure 18A) and after a few minutes, 1 mM EGTA was added to bring the [Ca\(^{2+}\)]_{FREE} to the normal recording value of 250 nM. Within one minute of this addition, a change in the current amplitudes of the channel could be observed (Figure 18B). The channel seemed to shift to a subconductance state of 2.5 pA with more closings present than prior to EGTA addition. This is illustrated in the amplitude histogram of this recording shown in Figure 19A & B. After 5 min post-EGTA addition, the channel recording resembled that of a typical InsP3R2-S150E channel (Figure 18C and 19C). The channel was in a state of very low activity and even the presence of the increased [InsP\(_3\)] did not have an effect on the channel’s activity. In the presence of 800 nM Ca, the S150E channel was highly active with the stoichiometry of main conductance state and the subconductance approximately 1:0.6. This is similar to the observed distribution for WT channels. Addition of EGTA resulted in an immediate closure of the main open state, with the subconductance openings remaining; however, this open state was also reduced after 5 min at 250 nM Ca\(^{2+}\). These results may also suggest that the Ca\(^{2+}\) sensitivity of the S150E mutants are altered.
Figure 18: InsP$_3$R2-S150E channel shows increased activity in high $[\text{Ca}^{2+}]_{\text{FREE}}$

Representative single channel traces of WT, S150A, and S150E InsP$_3$R2 in various $[\text{Ca}^{2+}]_{\text{FREE}}$. (A) Representative trace of an InsP$_3$R2-S150E channel in 800 nM $[\text{Ca}^{2+}]_{\text{FREE}}$. The same channel is shown 1 min (B) and 5 min (C) after the addition of 1 mM EGTA to adjust $[\text{Ca}^{2+}]_{\text{FREE}}$ to 250 nM. With the decrease of the $[\text{Ca}^{2+}]_{\text{FREE}}$ from 800 nM to 250 nM, the channels appears to transition from a state with relatively few closings (0 pA) and many full (5 pA) and subconductance (2.5 pA) openings (A), to an intermediate state with more closings and a prevalence of the subconductance state (B), and eventually to a state where the channel is essentially inactive (C). The conditions for the single channel studies were 220/20 mM CsCH$_3$SO$_3$ (cis/trans) and 8 µM InsP$_3$. Open (O), closed (C), and substate (S) shown.
Figure 19: Amplitude histograms of an InsP₃R2-S150E channel in response to changes in $[\text{Ca}^{2+}]_{\text{FREE}}$

(A) Amplitude histogram of an InsP₃R2-S150E channel in 800 nM $[\text{Ca}^{2+}]_{\text{FREE}}$. The same channel 1 min (B) and 5 min (C) after the addition of 1 mM EGTA to adjust $[\text{Ca}^{2+}]_{\text{FREE}}$ to 250 nM. With the decrease of the nM $[\text{Ca}^{2+}]_{\text{FREE}}$ from 800 nM to 250 nM, the channels appears to transition from a state with relatively few closings (0 pA) and many full (5 pA) and subconductance (2.5 pA) openings (A), to an intermediate state with more closings and a prevalence of the subconductance state (B), and eventually to a state where the channel is essentially inactive (C). A, B and C correspond to the same traces in Figure 18. Red traces are Gaussian fits of histograms.
C. DISCUSSION

1. Summary of Results

InsP$_3$R2s are intracellular Ca$^{2+}$-release channels that play a role in the regulation of Ca$^{2+}$-signaling in the cardiac myocyte. Their role in the regulation of ET-1 induced positive inotropy in both atrial and ventricular myocytes has been recently well-characterized, along with their part in a nuclear signal transduction cascade involving CaMKII-mediated activation of gene expression. Thus, CaMKII-dependent phosphorylation of the InsP$_3$R2 is an important method of physiological regulation of the channel in a cellular environment. This is the first report of the exact site of CaMKII-mediated phosphorylation of the InsP$_3$R2 along with the functional effects of this post-translational modification on the channel’s activity. In addition, important function implications into the mechanism of the inhibition of activity of the channel can be conjectured from my results of single channel analysis of the InsP$_3$R2-S150E channel.

The data presented above clearly show that S150E of the InsP$_3$R2 is the site of modulation of the channel’s activity by CaMKII-dependent phosphorylation. Phosphorylation leads to inhibition of the InsP$_3$R2 and this functional effect can be inhibited by mutation of S150 to Alanine in the InsP$_3$R2. Furthermore, a phosphomimetic InsP$_3$R2 channel (InsP$_3$R2-S150) exhibits a constitutively low open probability much like that seen with a WT channel phosphorylated by CaMKII. My data also indicate that this phosphorylation event can actually shift the Ca$^{2+}$ activation curve (Figure 2B) of the InsP$_3$R2 dramatically to higher [Ca$^{2+}$]$_{FREE}$ (~800 nM).
2. Functional Consequences of CaMKII-mediated phosphorylation of the InsP$_3$R2

Type 2 InsP$_3$R-mediated signals have a sigmoidal Ca$^{2+}$-dependence meaning termination of this signal is not intrinsically controlled by a Ca$^{2+}$-dependent inactivation mechanism. Of significance in cardiac tissues where type 2 receptors are present, this sigmoidal Ca$^{2+}$-dependence makes the InsP$_3$R2 resistant to the RyR-mediated Ca$^{2+}$ signals driving the cardiac contractile cycle and allows InsP$_3$-dependent intracellular signaling cascades to operate independent of the global Ca$^{2+}$ fluxes associated with contraction. Potential mechanisms of termination of the InsP$_3$R2 response include total depletion of the Ca$^{2+}$ store, removal of the InsP$_3$ signal, or regulation through a modulatory protein (i.e. CaM, CaMKII, CaBP, PKA, etc.). My data show that CaMKII can directly phosphorylate the InsP$_3$R2 at Serine 150, and phosphorylation results in the inhibition of channel activity and thus termination of InsP$_3$-mediated SR and nuclear Ca$^{2+}$ release (Figure 14A and Figure 15A).

The InsP$_3$R2 is localized to both the SR and the nuclear envelope in cardiac myocytes (Mackenzie et al., 2002; Bare et al., 2005). CaMKII-mediated phosphorylation of InsP$_3$R2 most likely has different physiological effects based on which population of receptors is being phosphorylated. Data show that knock-out of InsP$_3$R2 in mouse myocytes abolished the positive inotropic and arrhythmogenic effects of ET-1 in cardiac myocytes (Li et al., 2005) and the ET-1 induced HDAC5 nuclear translocation in ventricular myocytes (Wu et al., 2006). This HDAC5 pathway seems to be isolated from the beat-to-beat global fluctuations in [Ca$^{2+}$]$_{i}$, creating an elegant system that is dependent on very local control of [Ca$^{2+}$] around the pore of the InsP$_3$R. The hypothesis
is that InsP₃ can activate nuclear envelope InsP₃Rs, releasing Ca²⁺ into the nucleoplasm with very high concentrations of Ca²⁺ immediately adjacent to the mouth of the InsP₃R activating CaMKII, which can phosphorylate HDAC5 and cause its translocation out of the nucleus (Wu et al., 2006). Furthermore, it has been proposed that the activated CaMKII can feedback inhibit the InsP₃R to close the channel and terminate the InsP₃R-mediated release of Ca²⁺ from the nuclear envelope into the nucleoplasm. My results presented here strengthen this hypothesis and show that CaMKII feedback phosphorylates the InsP₃R2 at Serine 150, and this phosphorylation results in a decrease of the channel’s open probability. This is an important part of signaling pathway leading to hypertrophy and heart failure. In HF this could have a potentiating effect as a feedback loop as the increased gene expression through InsP₃R2-dependent Ca²⁺-activation of CaMKII leads to increased InsP₃R2 levels and thus further Ca²⁺ in the nucleus thus reinforcing this pathway.

The next question is what functional effects CaMKII-dependent phosphorylation of SR-localized InsP₃R2s has in the cellular context of the cardiac myocyte. InsP₃R2s co-localize with RyRs in both atrial and ventricular cells and can modulate the activity of the RyR (Mackenzie et al., 2002; Zima and Blatter, 2004; Domeier et al., 2007). IICR can potentiate CICR from RyRs leading to enhanced systolic SR Ca²⁺ release and positive inotropy. One possibility is that CaMKII-mediated phosphorylation of this population of InsP₃R2s would restrain or limit the potentiating effects of IICR on CICR. This could serve as a mechanism to prevent the arrhythmogenic effects that occur upon ET-1 application. In addition, it could have a significant effect on diastolic SR Ca²⁺ leak by decreasing spontaneous openings of the RyR due to decreased propensity for CICR.
This effect of phosphorylation can also be anti-arrhythmogenic during diastole preventing activation of the electrogenic Na\(^{+}/Ca^{2+}\) exchanger (NCX) and generation of DADs. In addition to modulation of CICR from RyRs to prevent arrhythmias, phosphorylation of the InsP\(_3\)R2 by CaMKII in the cytosol could provide a mechanism to prevent arrhythmias during \(\beta\)-adrenergic and ET-1 stimulation. In instances of ET-1 stimulation, InsP\(_3\) levels in the cytosol will be significantly higher due to activation of the PLC-InsP\(_3\) pathway. This would prime the InsP\(_3\)R2 to become more sensitive to Ca\(^{2+}\) activation and more likely to cause spontaneous arrhythmogenic SR Ca\(^{2+}\)-release events. Due to the Ca\(^{2+}\)-sensitivity property of InsP\(_3\)R2s, the increased Ca\(^{2+}\) (along with InsP\(_3\)) in this microdomain could cause spontaneous activation of InsP\(_3\)R2s, which in turn activate RyR Ca\(^{2+}\)-release causing DADs or pro-arrhythmic action potential aberrations. The CaMKII localized in this microdomain would be activated by the increased [Ca\(^{2+}\)] and phosphorylate and inhibit the InsP\(_3\)R2. Therefore, due to the relatively low expression levels of InsP\(_3\)R2s compared to RyRs (Moschella and Marks, 1993), SR Ca\(^{2+}\) release from InsP\(_3\)R2s alone doesn’t affect the beat-to-beat global Ca\(^{2+}\) transient during action potential stimulation in the cell. However, it can influence RyR-mediated SR Ca\(^{2+}\) release which does affect the global SR Ca\(^{2+}\) release. Thus, it seems the effect of CaMKII-mediated phosphorylation of the SR-localized InsP\(_3\)R2s would have an indirect effect on global SR Ca\(^{2+}\) release through modulation of the RyR.

Atrial myocytes from the InsP\(_3\)R2-KO mouse exhibited decreased spark frequency when treated with ET-1 compared to WT treated with ET-1 (Li et al., 2005). This is presumably due to the loss of the potentiating effect IICR has on the RyR-mediated SR Ca\(^{2+}\) release. In contrast, in cardiac myocytes from the InsP\(_3\)R2-KO mouse
the β-adrenergic agonist isoproterenol still was able to increase spark frequency and Ca$^{2+}$ transient amplitude, showing that the transgenic animal was able to maintain an inotropic reserve. Furthermore, this indicates that β-adrenergic modulation of SR Ca$^{2+}$ release works through pathways other than the PLC-InsP$_3$ pathway (Li et al., 2005). Therefore, I propose that phosphorylation of the InsP$_3$R2 by CaMKII may have a protective effect during stimulation of the PLC-InsP$_3$ pathway in cardiac myocytes in preventing or reducing the arrhythmogenic effects of ET-1.

In heart failure, the InsP$_3$R2 is upregulated (Figure 3; see also Go et al., 1995). Functionally, the majority of experimental evidence indicates that in cardiac myocytes the modest inotropic effect of the InsP$_3$R2s also comes with the considerable ability to disrupt ECC at normal SR Ca$^{2+}$ load (Mackenzie et al., 2002). Despite this, the upregulation of the InsP$_3$R2 seen in HF may be an attempt to preserve and enhance the inotropic response of ET-1 in this condition. Aside from this positive effect, I propose another possibility for the physiological significance of the increased density of InsP$_3$R2s in the pathological condition of HF. It is that the InsP$_3$R2s have a role in apoptosis of myocytes seen in cases of HF. It was shown that CaMKII deletion in myocytes prevented apoptosis (Ling et al., 2009). Could InsP$_3$R2-mediated Ca$^{2+}$ release be the link between CaMKII and apoptosis in that the deletion of CaMKII prevents it from activation the expression of genes, specifically the upregulation of the InsP$_3$R2? The expression of many other proteins can be modulated by CaMKII-mediated phosphorylation of nuclear proteins that control transcription (Ling et al., 2009), so perhaps the increased InsP$_3$R2 protein level is one of many factors contributing the apoptosis of cardiac myocytes seen in HF. It has been shown that InsP$_3$R-mediated Ca$^{2+}$ release plays a role in apoptosis, and
the InsP$_3$Rs localize in close proximity to the mitochondria (Rizzuto et al., 1998; Hanson et al., 2008; Rong et al., 2008). In rat ventricular myocytes, it has also been shown that Ca$^{2+}$ signals can be passed between the two organelles (Sharma et al., 2000). The hypothesis that IICR can potentiate CICR here from RyRs is also a possibility, as functional RyRs were shown at SR/mitochondrial junctions in rat ventricular myocytes (Lukyanenko et al., 2007). Perhaps the InsP$_3$R protein density is increased in areas of SR/mitochondrial junctions to create a signaling domain in which Ca$^{2+}$ release from InsP$_3$Rs can cause CICR from RyRs or independently be sensed by mitochondria and induce the apoptotic pathway contributing to the pathology of HF.

In addition to the increased InsP$_3$R2 protein levels, the levels of the RyR are decreased in HF. Does the increased InsP$_3$R2 protein level contribute to arrhythmogenesis and the mishandling of Ca$^{2+}$ regulation also seen in HF, or do the InsP$_3$R2s create an environment where this increased expression in the SR can help potentiate CICR from the decreased population of RyRs? As shown in Figure 3, the nuclear envelope InsP$_3$R2s are clearly up-regulated in the HF rabbit myocytes, but areas of staining extending from the nuclear envelope are also seen in HF. This could be a dense population of InsP$_3$R2’s that co-localize with RyRs in myocytes and contribute to the propagation of the Ca$^{2+}$ wave for contraction. The might represent a compensatory mechanism during heart failure, thus allowing greater facilitation of CICR by the calcium released through the InsP$_3$R2. Despite the fact that the InsP$_3$R2 is up-regulated and the RyR is down-regulated in HF, I do not support a notion that the InsP$_3$R2s take the place of the RyR as the main Ca$^{2+}$ release channel of the cardiac myocyte. We have created antibodies to the phosphorylated and non-phosphorylated forms of Serine-150 of the rat
InsP₃R2 to detect the total InsP₃R2 and the CaMKII phosphorylated InsP₃R2 in WT and HF myocytes. These experiments will give us further insights into CaMKII-mediated regulation of the InsP₃R2 during normal and pathological conditions.

One final possibility for the physiological significance of CaMKII-mediated phosphorylation of the InsP₃R2, is that phosphorylation “tunes” the InsP₃R2 to be responsive in conditions of high [Ca²⁺]. The results in Figures 18 and 19 are preliminary (n=1), but it is worth speculating on their potential. The phosphomimetic InsP₃R2 channel could be activated by increased cytosolic [Ca²⁺] (~800 nM) and high [InsP₃] (8 µM). It is unclear what effect [InsP₃] has on this activation, but the [Ca²⁺] may be more critical since the channel activity could be abolished by returning the [Ca²⁺] to normal. Thus, it is possible that CaMKII-mediated phosphorylation of the InsP₃R2 leads to channel inhibition by shifting its Ca²⁺-sensitivity to higher [Ca²⁺], which causes the channel to become responsive only during periods of increased [Ca²⁺], potentially independent of increased [IP₃]. Many possibilities on the physiological significance of this property can be postulated in various instances of increased [Ca²⁺] in the myocyte. For example, this could allow the positive inotropic effects of InsP₃R2 activation to persist in the face of high [Ca²⁺], due to increased heart rates. Another example is during cases of HF and potentially takes place on a similar or shorter timescale to the example above. Perhaps the high [Ca²⁺] during systole creates an environment that activates the InsP₃R2 and allows it to continue having the positive inotropic effect seen in normal myocytes that would be beneficial to sustaining heart function during HF. On the other hand, there is also the possibility that this would cause even more pronounced arrhythmogenic effects based on when the InsP₃R2 actually activates during the Ca²⁺.
cycle. Concerted activation with the RyR may help to prolong or potentially increase the Ca\(^{2+}\) transient amplitude and increase the force of contraction, whereas activation of the InsP\(_3\)R2s at other points during Ca\(^{2+}\) transient could cause EADs or DADs. The question remains whether this concerted activation would help increase the Ca\(^{2+}\) transient amplitude in the condition of decreased SR Ca\(^{2+}\) content as seen in HF. Furthermore, given the timescale necessary to experimentally produce the CaMKII mediated effects on the InsP\(_3\)R2 in vitro (minutes to tens of minutes), it is also unclear if the above examples would still be viable possibilities of the physiological significance of CaMKII-mediated phosphorylation of the InsP\(_3\)R2. However, these hypotheses have yet to be tested in a cellular environment. Considering the data that implicate CaMKII in the induction and progression of hypertrophy and heart failure, it seems unlikely that it would have a compensatory effect in HF. However, the results in Figures 18 and 19 indicate that it could be a possibility. Another important point in the hypothetical examples discussed above is what Ca\(^{2+}\) signal is activating the CaMKII? Is it the increased [Ca\(^{2+}\)] in the microdomain of the InsP\(_3\)R2/CaMKII signaling complex as has been demonstrated for the nuclear cascade, or is it the stimulation of the β-adrenergic pathway of the myocyte? A complete understanding of this complex regulatory pathway and its effects on the myocyte as a whole requires a comprehensive study of the InsP\(_3\)R2, RyR, CaMKII, the separate and overlapping effects of the ET-1 and β-adrenergic pathway stimulation, and the various changes in [Ca\(^{2+}\)]\(_i\) and SR Ca\(^{2+}\) to see the larger picture of this regulation in WT and HF myocytes.
3. Mechanism of Inhibition

IP$_3$ binds to a distinct region of the IP$_3$R located at the amino terminal of each subunit known as the ligand binding domain (LBD). This region can be divided into the IP$_3$-binding core (IBC) and a second region that reduces affinity of binding core for IP$_3$ and thus has been called the suppressor domain (SD). The suppressor domain is required for channel gating as it was shown that removal of suppressor domain increased affinity for IP$_3$ but did not form a functional channel (Uchida et al., 2003). The crystal structures for the IBC (Bosanac, 2002) and the SD (Bosanac, 2005) have both been solved. Based on the location of S150/T150 in exposed beta strand 7, it is in close proximity to regions of potential interaction between the IBC and SD of the LBD (Bosanac et al., 2005). Therefore, I postulate that modification of this site could sterically hinder IP$_3$ from binding to the IBC or that phosphorylation disrupts the interaction of the SD and IBC thus preventing ligand induced activation. **Figure 20** is a surface electrostatic potential representation of the SD with and without the phosphomimetic mutation. This representation shows a large change in the charged regions of the surface of the SD upon replacement of Serine with Glutamate at residue 150 to mimic a phosphorylated Serine-150. Specifically, a decrease in the positive charge pocket exposed on the surface of the SD is shown. This change in the surface electrostatic potential could have a few effects on the function and structure of the LBD. One, is that the increases in negative potential repel the negatively charged IP$_3$, thus decreasing the affinity of the LBD for IP$_3$. A second possibility is that the change in surface electrostatic potential causes a change in the tertiary structure of the LBD. This could be either a stronger or weaker
Surface electrostatic potential representations of the InsP$_3$R2 suppressor domain

Surface charge representations of the InsP$_3$R2-WT (A) and the InsP$_3$R2-S150E (B) suppressor domains. Addition of the S150E mutation in (B) causes the negatively charged pocket in near the center of the representation to become larger and more intense. It also causes a decrease in the positively charged pocket located below it in the representation. A scale bar is shown below the figures corresponding to the intensity of electrostatic potential and is in units kT/e. Red represents negative electrostatic potential, while blue is positive electrostatic potential. This figure was generated with the molecular viewer program Pymol.
intramolecular interaction based on what regions and charges interact, and it could be due to the greatly increased pocket of negative electrostatic potential or the moderate decrease the closely localized positive pocket. Either way, this change in surface electrostatic potential of the LBD with the addition of a negatively charged residue could have drastic structural effects of the LBD and also functional effects on the LBD’s ability to translate IP₃ binding into channel opening. Despite the experimental results and the insights from molecular modeling, the question still remains whether this contributes to a decrease in the affinity of the LBD for InsP₃ or if it interferes with the intramolecular interaction of the SD/IBC necessary for proper channel function; however, this has been the focus of my continual research.

In support of this hypothesis that phosphorylation disrupts the interaction of the SD and IBC, analysis of interactions of the SD and IBC of IP₃R1 established that either or both of E153 and A154 from loop 7 interact directly with the IBC. These sites are extremely close to the CaMKII phosphorylation site of the InsP₃R2 at S150. Furthermore, a series of partial agonists of the IP₃R were synthesized and used to define key steps in IP₃R activation (Rossi et al., 2009). The partial agonists opened the channel to the same open state (current amplitude, single channel conductance, and mean channel open time all the same for agonists and partial agonists); however, the partial agonists opened the InsP₃R less effectively. Utilizing these partial agonists and their ΔG for binding, it was shown that the major conformation changes evoked by IP₃ occur within the N-terminus and pass to the pore entirely via the suppressor domain (Rossi et al., 2009), most likely through its interaction with the S4-S5 linker (Schug and Joseph, 2006). This explanation could also account for the prevalence of the subconductance state
observed as the InsP₃R2-S150E channel is inhibited by decreasing the [Ca²⁺]_{FREE} (Figure 18 and 19). Perhaps the SD/IBC interaction is perturbed in such a way by phosphorylation of the Serine-150 residue that partial gating of the channel results, thus producing the prevalence of the 2.5 pA state observed (Figures 18B and 19B). This subconductance state was also observed in the InsP₃R2-WT channels; however, it is possible that this smaller open state must be reached before the full open state (5 pA) is reached, and CaMKII-mediated phosphorylation prevents transition from the partial to full open state of the InsP₃R2 (Figure 18B and 19B). The energy of InsP₃ binding to the IBC cannot be fully transferred into gating of the channel due to the aberrant interaction of the SD and the IBC, thus producing the subconductance state observed in planar lipid bilayers with the InsP₃R2-S150E channel. The additional Ca²⁺ necessary to produce channel openings (Figure 18A and 19A) may perhaps screen this negative charge present due to the phosphorylation of Serine-150 (see Figure 20) and restore the proper interaction of the SD and IBC. This would be an explanation as to why I see high channel activity in the presence of high [Ca²⁺]_{FREE} (Figures 18A and 19A). Building on the results of Figures 18 and 19, it would be interesting to see what effect any cation has on the reversal of CaMKII-mediation inhibition of the InsP₃R2. Based on my proposal above that the Ca²⁺ serves to screen the negative charge of a phosphorylated Serine-150, any positively charged ion could serve this role of screening the negative charge that results from CaMKII-mediated phosphorylation. If this was the case, perhaps it would be observed experimentally that divalents such as Ca²⁺ or Mg²⁺ are more efficient than monovalents at screening of the negative charge and causing reversal of the inhibition. Furthermore, the negatively charged Glutamate residue at position 2100 has already been
implicated in the Ca\(^{2+}\) sensitivity of the InsP\(_3\)Rs (Chapter 1.A.2, Miyakawa et al., 2001), showing that Ca\(^{2+}\) attraction to negatively charged regions of the InsP\(_3\)R can have an effect on the function of the channel. In summary, I propose a mechanism for inhibition of channel gating by which CaMKII-mediated phosphorylation of S150 causes a disruption of the suppressor domain-binding core interaction, thus uncoupling ligand binding from channel opening.

The FIRE-2 biosensor is an excellent tool with which to test part of this hypothesis. Like FIRE-1, the FIRE-2 biosensor is an InsP\(_3\)-sensor that responds to increasing [InsP\(_3\)] by exhibiting an increase in the FRET between the CFP and YFP proteins that flank the LBD of the InsP\(_3\)R2. Essentially, the change in FRET measured can be plotted along with the [InsP\(_3\)] at which the measurement was taken to create a dose-response curve of the biosensor for InsP\(_3\). From this, I can determine the EC\(_{50}\) for InsP\(_3\) and FRET\(_\text{max}\) for the biosensor, which will give insights into the affinity of the sensor for InsP\(_3\) (EC\(_{50}\)) and how the S150E phosphomimetic mutation may affect the conformation of the sensor (FRET\(_\text{max}\)). Thus, I have a tool with which to measure these properties for FIRE-2 and the phosphomimetic mutant FIRE-2-S150E. Furthermore, FIRE-2 is critical for this study in that it separates InsP\(_3\) binding from ligand-induced gating of the channel leading to InsP\(_3\)-induced Ca\(^{2+}\) release typically used for functional studies. This allows me to separate ligand binding from channel gating and specifically study the effect the S150E mutation has on the LBD’s affinity for InsP\(_3\).
4. The interaction of CaMKII and InsP$_3$R2

Finally, the interaction between CaMKII and the InsP$_3$R2 may be affected by phosphorylation. It has been shown that CaMKII can interact with the first 1078 amino acids of the InsP$_3$R2 in vitro. Furthermore, an in vivo interaction was also shown between CaMKII and the InsP$_3$R2 in rat myocyte nuclei (Bare et al., 2005). My data show that the region of interaction also contains the site of CaMKII-mediated phosphorylation on the InsP$_3$R2. The effect of CaMKII-mediated phosphorylation of the InsP$_3$R2 on the interaction of these two proteins, however, has yet to be demonstrated. Perhaps phosphorylation allows the kinase to dissociate from the receptor and act on downstream targets. It would be worthwhile to determine what proteins and factors also contribute to this interaction in the cell. Previously, the InsP$_3$R1, PKA, PP1 and PP2A have been shown to form a macromolecular complex in the rat brain (DeSouza et al., 2002). Does a similar complex exist in the heart for CaMKII and the InsP$_3$R2? There is a CaM binding site nearby Serine-150, just upstream at amino acids 106-128 of the type 1 that is conserved in the type 2 InsP$_3$R receptor (Sienaert et al., 2002). This strengthens the hypothesis that the InsP$_3$R2 and CaMKII form a macromolecular complex with CaM potentially serving as the link between the two proteins. CaM positions the CaMKII spatially to activate during the Ca$^{2+}$ signals from the InsP$_3$R and then phosphorylate the nearby Serine-150 to inhibit the activity of the InsP$_3$R2. This also implicates CaM as a Ca$^{2+}$-sensor for the InsP$_3$R2, fulfilling the role through activation of CaMII and its subsequent phosphorylation and inhibition of the channel. A second candidate for the interaction of CaMKII with InsP$_3$R2 is αKAP. αKAP may spatially position CaMKII to modulate the InsP$_3$R as it does with CaMKII, phospholamban and SERCA2a (Singh et
al., 2009). Through mutagenesis, it was shown that SERCA2a and CaMKII each interact with distinct regions of αKAP, thus forming a complex of proteins that can regulate each other’s activity. It is through this interaction that αKAP spatially positions CaMKII to phosphorylate PLB and modulate its interaction and regulation of SERCA2a (Singh et al., 2009). Perhaps αKAP plays a similar role in the regulation of the InsP3R2 by CaMKII by forming a similar complex of proteins in the SR/ER or nuclear envelope.
CHAPTER SIX

SUMMARY

One mechanism in which calcium is mobilized from intracellular stores is via the activation of inositol 1,4,5-trisphosphate receptors (InsP₃Rs) by the second messenger, inositol 1,4,5-trisphosphate (InsP₃). InsP₃Rs are a family of highly conserved intracellular Ca²⁺ release channels. InsP₃Rs can be of three isoforms (type 1-3), and are structurally very similar to each other (Mignery et al., 1992) with multiple isoforms often expressed within a single cell. Activation of InsP₃Rs leads to release of calcium into the cytoplasm of the cell, where it can activate a multitude of signal transduction cascades and a variety of local and global cellular events. One such enzyme activated by a rise in intracellular [Ca²⁺] is Ca²⁺-calmodulin-dependent protein kinase II (CaMKII). CaMKII is a multifunctional Serine/Threonine protein kinase involved in many signaling pathways in various cell types. Recent results from our lab have shown that InsP₃Rs in the heart (InsP₃R2) are primarily targeted to the nuclear envelope in ventricular myocytes. Here it forms a macromolecular complex with one of the cardiac isoforms of CaMKII, CaMKIIδB. Upon stimulation of InsP₃ production, Ca²⁺ released through the InsP₃R2 activates CaMKIIδB, allowing it to act on downstream targets, such as HDAC4 & 5 (Little et al., 2007; Wu et al., 2006). Additionally, CaMKII feedback modulates InsP₃R2
function by direct phosphorylation and results in a significant decrease in the channel’s open probability (Bare et al., 2005; Figure 14). The results of this study and others suggest that the activity of InsP$_3$Rs can be inhibited by CaMKII-mediated phosphorylation. Furthermore, the N-terminal 1078 amino acids of the InsP$_3$R2 have been shown to interact with, as well as be phosphorylated by CaMKII (Bare et al., 2005). However, it has not yet been established what amino acid of InsP$_3$Rs is phosphorylated.

The goal of this project was to determine what amino acid residue(s) of the InsP$_3$R2 is phosphorylated by CaMKII and, upon phosphorylation, has an effect on the properties of the channel. This will give us a better functional understanding of how InsP$_3$R and CaMKII regulate each other and perhaps insight into the mechanism of inhibition.

To test the hypothesis that activated CaMKII feedback modulates the InsP$_3$R2 via direct phosphorylation and disruption of those sites can abolish phosphorylation and the resulting functional consequences, I proposed three Specific Aims by which to accomplish this objective. In Aim 1, I used exogenously expressed fragments that I made of the InsP$_3$R2 to show that CaMKII can phosphorylate the InsP$_3$R2 at Serine-150.

In Aim 2, I used site-directed mutagenesis to prevent Serine-150 phosphorylation by mutation to Alanine. Mutation of Serine-150 to Alanine successfully abolished CaMKII-dependent phosphorylation of two different InsP$_3$R2 sub-fragments that were tested. In addition, this mutation was made in the full-length rat InsP$_3$R2, and it was able to prevent CaMKII-mediated phosphorylation of the recombinant full-length InsP$_3$R2 in vitro. These results unambiguously identify Serine-150 of the rat InsP$_3$R2 as the CaMKII phosphorylation site. Furthermore, these results suggest that this is the only CaMKII-
dependent phosphorylation site present on the InsP$_3$R2 since mutagenesis of this site completely abolished CaMKII-mediation phosphorylation.

Finally in Aim 3, I confirmed that Serine-150 of the InsP$_3$R2 is the modulatory site of CaMKII-dependent phosphorylation. Abolishment of this site prevented the CaMKII-mediated effects on the InsP$_3$R2 in planar lipid bilayers. Treatment of a non-phosphorylatable (S150A) InsP$_3$R2 protein with CaMKII had no effect on the open probability of the channel, while a phosphomimetic (S150E) channel protein displayed a constitutively low open probability mimicking a CaMKII phosphorylated InsP$_3$R2. Furthermore, the phosphomimetic mutation also showed some interesting features that may be physiologically relevant and contribute to elucidating the mechanism of CaMKII-mediated regulation of the InsP$_3$R2.

The completion of my Specific Aims and the successful testing of my hypothesis has provided further avenues for study. We have created antibodies against phosphorylated and non-phosphorylated Serine-150 in the InsP$_3$R2 that will be useful in determining the ratios of phosphorylated to non-phosphorylated InsP$_3$R2 in tissues. Of particular interest would be the amounts of phosphorylated and total InsP$_3$R2 in HF myocytes, where the InsP$_3$R2 has been shown to be upregulated. Perhaps immunostaining with these antibodies may even identify specific populations of InsP$_3$R2 within the myocyte that are phosphorylated. Furthermore, testing the InsP$_3$R2-WT and InsP$_3$R2-S150E Ca$^{2+}$-release abilities in a cellular environment in response to InsP$_3$ or an agonist that activates the PLC-InsP$_3$ pathway would also be excellent supporting data to the in vitro planar lipid bilayer results I present here.
In addition to this first look at the single channel properties of the \( \text{InsP}_3\text{R2-} \text{S}150\text{E} \) protein shown here, an in-depth characterization of this channel and its properties may provide further insights to exactly what physiological effect CaMKII-mediated phosphorylation of the \( \text{InsP}_3\text{R2} \) has in the healthy and diseased heart.
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VITA

Joshua Thomas Maxwell was born in Lower Burrell, Pennsylvania on January 3rd, 1982 to Jeffrey and Cathy Maxwell. After completing high school at Burrell High School, he was awarded a scholarship to attend Allegheny College in Meadville, Pennsylvania from 2000-2004. He obtained a Bachelor of Science degree from Allegheny in May, 2004, graduating with a Major in Biology and a Minor in Philosophy. While at Allegheny, Joshua worked in the laboratory of Dr. Margaret K. Nelson performing molecular genetic characterization of the developmental mutant \( fbxA \) in \textit{Dictyostelium discoideum}. Joshua completed his senior comprehensive project in the laboratory of Dr. Glen G. Wurst studying the transcriptional activation properties of the NHD motif of the \textit{Drosophila melanogaster} segment polarity gene, \textit{pygopus}. After graduation from Allegheny, Joshua entered the PhD program at Loyola University in Chicago in the Department of Cellular and Molecular Physiology. He joined the lab of Gregory Mignery in 2005 and spent the next five years studying the structural and functional aspects of the inositol 1,4,5 trisphosphate receptor (InsP\(_3\)R). Josh’s initial projects were aimed at created tools to measure and perturb the InsP\(_3\) signaling pathway in cells. In addition, he collaborated with Dr. Irina Serysheva at Baylor College of Medicine on a project aimed at determining a high resolution crystal structure of the InsP\(_3\)R1. Josh’s dissertation project was to determine of site of Ca\(^{2+}\)/calmodulin-dependent protein kinase II phosphorylation on the InsP\(_3\)R2. Josh has accepted a
postdoctoral position in the laboratory of Dr. Lothar A. Blatter at Rush University Medical Center.