

Gatekeeper Connexin43 Phosphorylation Events Regulate Cardiac Gap Junction Coupling During Stress

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Abstract

Rapid and well-orchestrated action potential propagation through the myocardium is essential to each heartbeat. Gap junctions comprising primarily Cx43 reside within the intercalated discs connecting cardiomyocytes, effecting not only direct intercellular electrical coupling, but the localization of other junctional structures and ion channels. Alterations in Cx43 expression occur in essentially all forms of heart disease and is therefore a topic of intense study. Posttranslational modification of Cx43 is understood to impact trafficking, conduction, and stability. Altered Cx43 phosphorylation is well described during pathological remodeling of gap junctions in response to cellular stress. Research has revealed how phosphorylation of specific residues elicit specific effects on Cx43, but the complexity of this process has left much unknown. In particular, the role phosphorylation of a triplet of double serine residues, Ser365, Ser368, and Ser373, plays in GJ function and Cx43/14-3-3 interaction has been called into question. Using an *ex vivo* whole heart ischemia model we find a decrease in pS368 in mice lacking the ability to phosphorylate S365 and S373 while under stress. *In vitro* transfection of human induced pluripotent stem cell-derived cardiomyocytes when stressed with PMA were also carried out. These data allow us to piece together the exquisite interplay of gatekeeper phosphorylation events upstream of channel closure, altered protein-protein interactions, and gap junction internalization and degradation. It is hoped that our increasing understanding of this important area of gap junction biology will facilitate better understanding of arrhythmogenesis, and potential therapeutic strategies to restore or preserve normal electrical coupling in diseased hearts.

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General Audience Abstract

The heart, an electrically active organ, relies on the propagation of an electrical signal throughout its entirety in order to produce a healthy heartbeat. In order to do so, the heart uses specialized muscle cells known as cardiomyocytes which can not only contract but pass along chemical signals to the cardiomyocyte next in line to signal it to contract as well. The passage of signals occurs through protein units called gap junctions and are made predominantly of Cx43 proteins in the heart. Gap junctions look and function like tubes that travel from the inside space of one cell to the other and allow a flow of small molecules to occur; these small molecules, namely ions, are part of the signal needed to initiate contraction in the adjacent cell. Cx43, like many proteins in our bodies, is slightly altered after it is produced through a process known as posttranslational modification. This allows the cell to alter the localization and function of the protein and tailor it for the needs of the cell. Rather than changing the backbone composition of the protein, small chemical groups are attached, and this imparts a change to how the protein interacts with other proteins or its environment. In particular, one form of modification is known as phosphorylation where a phosphate group is attached to the protein at specific locations along its chain. Cx43 too can be phosphorylated, and while under pathological stress, such as a lack of oxygen or infection, cardiomyocytes increase the amount of phosphorylated Cx43 at a site known to cause pathological changes to the function of Cx43. These changes include how well the gap junctions can transmit signals or associate with other proteins and, in the heart, can predispose the development of arrhythmias or unhealthy heartbeats. However, not all phosphorylation is bad and phosphorylation at other locations also occurs during normal healthy functions of the cardiomyocyte can affect how other sites along Cx43 are phosphorylated. The process of one phosphorylated site affecting another is known as the gatekeeper effect and add a new layer to our understanding of how cells use phosphorylated Cx43 to fine tune its effects. Using cells that do not produce their own Cx43 and subsequently giving them the instructions to produce specific forms of mutant Cx43 that can and cannot be phosphorylated at specific sites, we can understand with greater detail of how cardiomyocytes respond to stress and how some of those responses can be pathological. This will allow future research into the creation of therapies that prevent negative Cx43 phosphorylation after illness, potentially avoiding the development of dangerous arrhythmias.

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Dedication

I dedicate this work to my family and all those who have helped me along the way. Thank you, Jenna, Brad, Shannon, Reilly, and Tyler. This paper would be dedicated to my dog Lulu, if not for the fact that she never once helped me run a western in lab. Pathetic.

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Attribution

Chapter 1 is co-authored by Rachel Padget and Jamie Smyth. Rachel assisted me in the bench top work, while Jamie assisted me in the planning and execution of this project. This thesis was written by me with editorial input from Jamie.

Abbreviations

ID - Intercalated disc

Cx - Connexin

GJ - Gap junction

GJIC - Gap junction intercellular communication

UTR - Untranslated region

TSS - Transcription start site

HDAC - Histone deacetylation

IRES - Internal ribosome entry site

DMD - Duchenne muscular dystrophy

cAMP - Cyclic AMP

PKA - Protein Kinase A

PKB - Protein Kinase B - also referred to as Akt

PKC - Protein Kinase C

CK1 - Casein Kinase 1

MAPK - Mitogen-Activated Protein Kinase

Amino acid residue nomenclature

S365 - Serine 365

pS365 - Phosphorylated Serine 365

S365A - Substituted Serine 365 to Alanine

Introduction

Intercellular communication and the heart

Rapid and orderly contraction of the working myocardium in the heart is required to produce sufficient arterial pressure needed to perfuse the various organ systems present throughout the body [1, 2]. Functioning as a syncytium, cardiomyocytes, the common contractile muscle cell present within the cardiac tissue, must contract in unison and do so through the highly coordinated relaying of an electrical signal. Aberration of this signal risks the development of potentially fatal arrhythmias [1, 3, 4]. Specialized proteins localized to the cellular membranes of the cardiomyocytes allow for such signal propagation and are the cornerstone of cardiac electrophysiology; they include connexins, ion channels, adhesive, and anchoring proteins[5-8]. The union of two cardiomyocytes occurs at the intercalated disc (ID) and is the primary structure involved in intercellular communication within the heart[1-7].

Connexins

Connexins (Cx) are a family of transmembrane proteins found extensively throughout various cell types of vertebrate organisms[9]. The primary function of Cx proteins involves the formation of hexameric connexons acting as a bidirectional size-exclusive (<1 kDa) cellular membrane pores [9]. Also termed hemichannels, these pores allow the facile flow of extracellular molecules into the cytosol to participate in communicatory pathways. When present on two adjacent cells, including adjoined cardiomyocytes, these connexon hemichannels may further coalesce into a gap junction (GJ) facilitating the flow of ions and small molecules between the two connected cytoplasms – a process known as gap junction intercellular communication, GJIC[9, 10]. The low resistance nature of this connection allows for rapid communication and has been implicated for use in innate immunity, embryonic development, neural coupling, and cancer, in addition to the electrical coupling of cardiomyocytes[11-17]. By allowing participating ions to flow from an electrically active cardiomyocyte to the next in sequence through a GJ, action potentials are rapidly propagated through the working myocardium [1, 2, 8].

Various structurally related connexins can be found throughout the heart, each with different incidences but all serving a similar purpose[9, 18]. As such, Cx45 (gene *GJCI*) and Cx40 (gene *GJA5*) are enriched within the cardiac conduction system, while Cx43 (gene *GJAI*) is found in greatest concentrations in the working myocardium of the atria and ventricles; the size of the working tissue as compared to the conduction system makes Cx43 the most ubiquitously expressed Cx protein within the heart[19-22]. Due to the presence of various connexin species, hemichannels can be either formed of all entirely the same connexin (homomeric) or a collection (heteromeric); likewise, gap junctions can either be a collection of two identical hemichannels (homotypic) or two hemichannels with unique compositions (heterotypic)[23]. The ubiquity of Cx43, coupled with its presence in the working myocardium, has led Cx43 to be well studied in its effects on tissue function and etiology of various cardiac pathologies.

Connexin43 regulation

Gap junctions are highly dynamic with a half-life of only 1 to 5 hours[24]. This rapid turnover allows for quick cellular adaptation to newly presented stresses and conditions but can also lead to pathological remodeling as seen in the etiologies of some diseases[25-29]. As such, the presence of mRNA UTRs, truncated isoforms, oligomerization within the endoplasmic reticulum, localized trafficking from the Golgi bodies, and posttranslational modifications, makes the regulation of Cx43 a highly controlled and extremely intricate.

Transcription

The genetic structure of *GJAI* is quite simple and consists of two exons (E1 and E2) spaced on either side of a single intron[30]. The protein coding sequence is wholly located within E2, with the remaining upstream mRNA comprising the 5' UTR[30]. Present approximately 150 nucleotides on either side of the transcription start site (TSS), and active in each studied cell type, are four highly conserved Sp-binding domains, namely Sp1 and Sp3, in addition to one AP1-binding site[30-33]. These transcription factor binding sites appear to be necessary for global *GJAI* transcription, and KO studies show a marked decrease in both mRNA and protein expression in various cell types[30-33]. Cardiomyocyte specific *GJAI* expression has been linked to Nkx-2.5, a common homeobox protein pinned to the proper development of the heart[30, 34-36]. Also known as CSX, aberrations in the function or expression in this transcription factor are associated with congenital heart disease, including pathological conduction and gross anatomical defects, as well as acquired hypertrophy following cardiac insult[30, 34]. Interestingly, the expression pattern Nkx-2.5 imparts on *GJAI* follows both that of an activator and a repressor, each in specific situations, and its full genomic interaction has yet to be completely described[30, 34-36]. In addition, the T-box transcription factors Tbx2 and Tbx3 have been identified as important *GJAI* repressors and can bind to Nkx-2.5[30, 37-39]. Important during development, Tbx3 represses the chiefly ventricular Cx43 in favor of Cx45 in the SA node and AV bundle[38]. Additional interaction between Tbx3 and Sox4 has been described in zebrafish cardiac tissue[40].

Important for the later described posttranslational modifications, protein kinase C (PKC) represents an important regulatory pathway for *GJAI*[41]. Research on myometrial cells highlighted an increase in *GJAI* promoter activity upon exposure to phorbol 12-myristate 13-acetate (PMA), a known PKC activator. The pathway is thought to include the involvement of the AP1-binding domain. In cardiomyocytes specifically, increased *GJAI* mRNA expression has been linked to the Wnt-signaling pathway, in opposition to a decrease seen in activation of the JNK pathway[42-44]. Interestingly, AP1, a known transcription activator in *GJAI*, is additionally a target of JNK – highlighting the need for further research into the specifics of cardiomyocyte *GJAI* expression and regulation[44].

Regulation of *GJAI* expression, especially in decreased expression hallmarked in some forms of cancer, has also been attributed to the actions of histone deacetylation (HDAC)[45]. In prostate cancer, research has highlighted the relationship between increased expression of *GJAI* following treatment with HDAC inhibitor TSA[45]. These results have been recapitulated in

other cancer cell lines with a variety of HDAC inhibitors and represents a potentially clinically important pathway in the treatment of Cx43 repressing tumors[46-48].

Translation

Cx43 mRNA undergoes canonical cap-dependent translation in order to form the full-length polypeptide. In addition, the 5' UTR harbors an IRES and *GJAI* mRNA can also undergo internal ribosome entry, a cap-independent manner of initiating translation[49]. Commonly found in genes that are highly expressed during cellular stress or apoptosis, an internal ribosome entry site (IRES) is hypothesized ensure Cx43 expression during times of inhibited cap-dependent translation; a hint to the important function connexins play in cellular function[50-52]. Other hypotheses include that ability to “jump-start” translation of existing mRNA in response to novel cellular signals, without the burden of canonical transcription/translation pathways[50-53].

Less common in eukaryotic mRNA, the *GJAI* mRNA is polycistronic and allows for the internal, or alternative, translation of several N-truncated peptide isoforms, the most robustly expressed of which is termed GJA1-20k [54]. Initiating at one of several AUG sequences within E2, these shortened isoforms are a product of *GJAI* mRNA alternative ‘internal’ translation initiation. GJA1-20k appears to be the most expressed isoform in the heart[54, 55]. The study of the interaction between full length Cx43 and GJA1-20k is a rapidly evolving topic with ever increasing investigational interest. First reported in 2013, GJA1-20k was quickly identified as an important piece in the global regulation of Cx43[54]. Our current understanding highlights two major domains of GJA1-20k function are assisted Cx43 localization and mitochondrial fission.

GJA1-20k is known to interact with actin and tubulin, major proteins involved in the vesicular transport of Cx43[56, 57]. Using *in vivo* gene delivery of exogenous GJA1-20k, infarcted mouse hearts are resistant to GJ degradation with greater GJA1-20k expression[56]. Additionally, GJA1-20k localizes to the Golgi apparatus where it is theorized to assist in the hexamer oligomerization of full length Cx43[58]. Cells which lack GJA1-20k are known to have Cx43 retention to the Golgi apparatus, and subsequently smaller GJ plaques, highlighting the importance of 20k in the intermediate step of Cx43 trafficking[58]. The stability of Cx43 is likewise affected by GJA1-20k and GJA1-20k $-/+$ mice have a 50% reduction in protein half-life, most likely due to the increased rate of degradation seen in cytosolic Cx43 when compared to membrane bound Cx43[59]. Cardiac pathogenesis involving GJA1-20k is abundant and well reported, including the protein’s involvement in ventricular arrhythmias, cardiac hypertrophy, arrhythmogenic cardiomyopathy, I/R injury, Duchenne muscular dystrophy, and ischemia[59-63].

Interestingly, GJA1-20k is directly involved in mitochondrial regulation and localization. First reported in 2017, GJA1-20k colocalizes with mitochondria and interacts both with the mitochondria and microtubules to assist in proper localization of the organelle[64]. Ablation of the microtubule-binding domain from GJA1-20k does not interfere with the protein’s association with mitochondria, but hampers mitochondrial transport[64]. Additionally, increased GJA1-20k expression promotes mitochondrial fission, a process which leads to cardioprotection[65]. Mitochondrial fission takes place canonically during periods of high metabolic demand, such as exercise, and increased mitochondrial copy number reduces ROS production [65, 66].

Trafficking

Cx43 oligomerization occurs in the endoplasmic reticulum. It is here that single Cx43 protomers oligomerize into connexon hemichannels and thus forming one half of the gap junction. Like many membrane-bound proteins, trafficking of Cx43 subsequently takes place at the Golgi apparatus, where the connexons are embedded into transport vesicles and prepared for delivery to the cellular membranes[67]. Trafficking regulation of Cx43 involves “Targeted Delivery” of the hemichannels to specific subdomains following microtubule and actin pathways[67-69]. This model of transport allows direct supply to Cx43 to the intercalated discs, replenishing the loss of protein following rapid turnover. Trafficking of Cx43 along microtubules requires binding of the EB1 protein, present on the + end of the microtubule, to the intercalated disc-bound beta-catenin/N-cadherin complex[67, 69-71]. Such an interaction anchors the microtubule to the ID, allowing for proper localization of the Cx43[69-71]. Disruptions to the EB1-microtubule association has been found in diseased hearts and suggests a major role in the regulation of Cx43[68].

Cx43 vesicles are also trafficked via F-actin[72-75]. F-actin, present both along microtubules and the ID, is thought to either act as a storage site of Cx43 hemichannels or to assist in proper localization of both the microtubules and the associated Cx43 hemichannels[75, 76].

As mentioned previously, GJA1-20k appears to play a major role in the proper anterograde trafficking of Cx43 to the intercalated disc, highlighted by a decrease in membrane bound Cx43 when coupled with mutant GJA1-20k[54]. Recent research has linked the inhibition of the mTOR signaling pathway to increased GJA1-20k production and subsequent ID localized Cx43[54]. The exact mechanism behind this “chaperoning” of Cx43 by GJA1-20k is still unknown, but recent work suggests it may be in part due to interaction with F-actin[76, 77].

Dysregulated trafficking of Cx43 can result in non-ID bound hemichannels present along the lateral sides of a cardiomyocyte[78, 79]. These pathological changes are often precipitated by cardiac stress, including ischemia, and are thought to occur due to altered ZO-1/Cx43 interaction[80-82]. Lateralized hemichannels do not efficiently participate in intercellular gap junction communication and contributes to conduction changes seen in the heart following insult[80, 82].

Posttranslational modifications

Cx43 is extensively regulated through posttranslational modification, both within the Golgi apparatus and while present at the cellular membranes. This section will cover the roles played by the following modifying pathways: phosphorylation, ubiquitination, sumoylation, nitrosylation, as well as other lesser described pathways.

Ubiquitination can target proteins for degradation and is an important step in the life cycle of Cx43[83-87]. Cx43 may first be ubiquitinated following synthesis and subsequent quality control within the endoplasmic reticulum[87, 88]. Misfolded proteins, estimated to represent as much as 40% of all nascent Cx43 and Cx32, are quickly polyubiquitinated and

destined for endoplasmic reticulum associated degradation[88]. Once present at the membrane, Cx43 bound for endocytosis and breakdown are ubiquitinated by Nedd4, allowing the Cx43 to follow various internalization pathways and ultimately either recycled or degraded[85, 89, 90]. However, recent data from Dunn et al. highlighted that ubiquitination may be unnecessary for GJ internalization and degradation. They found that Cx43 without lysine, and thus the inability to bind ubiquitin, was degraded at a similar rate to WT Cx43[90]. These data call into question the true mechanism of Cx43 internalization[90].

Sumoylation (Small Ubiquitin-like modifier proteins – SUMO), a process akin to ubiquitination was recently described to increase membrane bound Cx43 and to increase GJ cellular communication[91]. However, it appears that sumoylation increased Triton X-100 soluble GJ, implying an increase in internalization. These conflicting data imply that the mechanism and effect of sumoylation on Cx43 has yet to be elucidated[91].

Nitrosylation, or the addition of NO to cysteine residues, has been linked to increased cell membrane permeability during periods of stress[92-94]. Prior research shows increased dye uptake when hemichannels are nitrosylated and that this effect is ablated upon administration of reducing agents[92, 93]. Recently, the association between cardiac remodeling and patients with Duchenne muscular dystrophy (DMD) has been shown to be linked to aberrant Cx43 nitrosylation[94]. Mice affected by a DMD model appear to have greater Cx43 hemichannel nitrosylation, especially during periods of cellular stress, and that these changes increase the risk of fatal arrhythmias[94].

Unlike the related protein family of pannexins, connexin proteins do not undergo glycosylation[95-97]. Prior studies have demonstrated however, increased membrane bound Cx43 and GJIC was correlated with inhibition of glycosylation; although, this effect is believed to be the result of cAMP signaling rather than Cx43 glycosylation[98-100].

At least 21 residues within the carboxyl-terminus of Cx43 are understood to undergo phosphorylation[99, 101-107]. Research into the effects of Cx43 phosphorylation is an intensely studied field and it is known that phosphorylation can take place prior to trafficking to the cell surface[101]. However, phosphorylation and dephosphorylation events at the cell surface are considered most critical in regulation of gap junction function in response to varied environmental conditions and stressors[101-103, 105-113].

The phosphorylation of Cx43 S244 and S314 have been shown to occur via CaMKII, and the inhibition of this kinase is associated with improved intercellular communication[113, 114]. Increased phosphorylation at these sites appears to follow chronic remodeling seen in diseased hearts[113]. Y247 and Y265 are phosphorylated via Src and are associated with a decrease in GJIC via increased Cx43 internalization[115, 116]. Interestingly, recent results appear to suggest that the phosphorylation of Y247 and Y265 may not be directly responsible for reduced GJIC, but may influence phosphorylation at other Cx43 inhibiting sites[117]. S255, S262, S279, and S282 are phosphorylated by means of MAPK and interfere with the formation of GJ plaques[112]. Additionally, phosphorylation of these four residues is necessary for Cx43/Cyclin E interaction. Removal of these sites through substitution to alanine has been shown to interfere

with pathogenic smooth muscle cell proliferation in atherosclerosis models[118]. PKC ϵ phosphorylates S262 which has been associated with increased cardiomyocyte proliferation in vitro[111, 119]. This effect appears to be mediated through inhibited Cx43 expression and is important in cell survival in hypoxic conditions[111]. The exact kinase responsible for S296, S297, and S306 are a matter of debate. However, each appears to be phosphorylated under baseline conditions and become dephosphorylated under stress; preservation of their phosphorylation is known to improve cell survival while under stress[110, 120]. Substitution studies highlight that S296A and S297A do not appear to significantly affect conduction, but S306A impairs cardiac conduction[110]. S325, S328, and S330 are phosphorylated by CK1 and play a key role in the formation of stable gap junctions in the intercalated disc. Data suggest that these three serine residues are only phosphorylated after localization to the intercalated disc and substitution with alanine shows decreased intercellular communication. These residues are dephosphorylated under stress, including hemodynamic overload, and precedes a loss of ID bound Cx43 and a slowing of conduction velocity[102, 109].

Akt-mediated phosphorylation of S373 creates a 14-3-3 mode-1 binding motif and leads to increased Cx43 endocytosis. Data show that substitution to S373A leads to greater membrane stability of Cx43, and additionally decreased phosphorylation at S368 and S255. Knockdown of 14-3-3 τ , the 14-3-3 isoform known to complex with Cx43, leads to increased gap junction plaque size, consistent with the aforementioned results[106, 107]. In addition to S262, PKC ϵ phosphorylates Cx43 at S368, a key residue in pathological remodeling and function of gap junctions in the heart following insult. Data suggest that phosphorylated S368 decreases the open channel probability of the gap junction pore and leads to impaired conduction[101, 102, 108]. S365 is phosphorylated by PKA and is present in significant amounts in basal Cx43. This phosphorylation is subsequently lost upon insult to the cardiomyocyte[104]. It is worth noting that Cx43 expression and GJ formation is positively associated with cAMP, a known activator of PKA, beyond the effects seen by phosphorylation. These data imply the multifaceted role that many signaling molecules play in their regulation and control of Cx43[99, 105].

Gatekeeper/Cascade residues

A triplet of double serine residues exists on the distal portion of the Cx43 carboxyl-terminus: S364/365, S368/369, and S372/373. Central to the gatekeeper effect is the phosphorylation of S368, an action that is known to decrease cardiac conduction[101, 104, 106]. The phosphorylation of S365, a PKA mediated event, has been shown to block the phosphorylation of S368 through conformational changes to the structure of the carboxyl-terminus[104]. As pS365 is present at baseline in a healthy cardiomyocyte, this site must first be dephosphorylated prior to phosphorylation of S368 via PKC[104]. In addition, phosphorylation of S373 has been shown to precede pS368 and influence the phosphorylation at this residue[106, 107]. Likewise, pS373 leads to increased endocytosis through 14-3-3/Cx43 binding[106, 107]. Therefore, the question remains of which residue, S365 or S373 is dominant in its control of S368 and what role does 14-3-3 play in this mechanism?

Conclusion

Cx43 is dynamically regulated at various levels throughout its life cycle, resulting in many avenues for cellular regulation of gap junction function. From synthesis to degradation, Cx43 spends between one and five hours present within the cell, and even less time within the cell membrane, and thus can be used to rapidly adjust to changing cellular needs, environmental conditions, and stressors. Posttranslational modification, including phosphorylation, enables dynamic cellular control of Cx43 lifecycle, localization, and function. Pathological remodeling of gap junctions following cellular insult results in diminished cellular communication and predisposes the heart to fatal arrhythmias. Varied in their effects on the cell and disease processes, phosphorylation sites represent a viable drug target to modify how a cell response to stress and potentially alleviate pathological substrates present.

**Chapter 1: Gatekeeper Connexin43 Phosphorylation Events
Regulate Cardiac Gap Junction Coupling During Stress**

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Abstract

Rapid and well-orchestrated action potential propagation through the working myocardium is essential to each heartbeat, and disruptions in this process underlie deadly arrhythmias. Aberrations in Cx43 protein expression and/or localization occur in essentially all forms of heart disease and includes altered posttranslational modification of Cx43 – particularly phosphorylation. Prior research has revealed how phosphorylation and dephosphorylation of specific residues elicit specific effects on Cx43, including the phosphorylation of a triplet of double serine residues, Ser365, Ser368, and Ser373, being known to impart downstream effects on other phospho-sites in this region. Acting as a “gatekeeper” or cascade, pS365 has been associated with decreased pS368, whereas pS373 appears to positively influence levels of pS368. Using an *ex vivo* whole heart ischemia model we find a decrease in pS368 in mice lacking the ability to phosphorylate S365 and S373 while under stress. However, surface level EKGs do not detect any abnormalities in these mice. Immunofluorescence highlights greatly increased membrane bound Cx43 in S368A mice and moderate increases in S365A/S373A mice. *In vitro* transfection of Cx43 KO human induced pluripotent stem cell-derived cardiomyocytes revealed that cobalt chloride is a poor model of ischemic cardiac changes. Finally, we find alterations in both Cx43 expression and levels of pS368 in differentially transfected forms of Cx43. These data allow us to piece together the exquisite interplay of gatekeeper phosphorylation events upstream of channel closure, altered protein-protein interactions, and gap junction internalization and degradation. It is hoped that our increasing understanding of this important area of gap junction biology will facilitate better understanding of arrhythmogenesis, and potential therapeutic strategies to restore or preserve normal electrical coupling in diseased hearts.

Introduction

Primarily localized to the intercalated disc (ID), the structure responsible for electrically coupling cardiomyocytes, Cx43 is responsible for the formation of gap junction (GJ) channels who take part in GJ intercellular communication (GJIC)[1-3, 9, 10]. GJIC, along with the effects of Cx43 hemichannels, ion channels, scaffolding molecules, acts to allow the propagation of an electrical signal between cardiomyocytes[4-7]. The rapid handling and transmission of this signal is needed to facilitate the proper contraction of the heart and aberrations can dispose the occurrence of cardiac pathologies[1-3].

Cx43 is rapidly turned over within the cell and has a half-life between 1 and 5 hours[6, 10, 12, 24]. This pattern of rapid expression and subsequent endocytosis makes the acute localization and modification of Cx43 an important regulatory pathway in the development of disease; remodeling of Cx43 is seen within minutes of ischemia in the heart and can present a foundation for the development of more severe pathologies[3, 26-28]. Part of this remodeling includes altered phosphorylation of various serine and tyrosine residues located on the cytosolic carboxyl-terminus of Cx43 and is associated with changes to gap junction formation, localization, function, and stability[8, 24, 65, 99, 101, 102, 104-117, 119, 120]. Phosphorylated by a multitude of kinases responding to an even greater number of signaling pathways, the control of Cx43 phosphorylation represents a potential therapeutic drug target and greater understanding of the interplay between Cx43 form and function will inform those developments [8, 24, 57, 93, 95-112].

Located on the distal portion of the carboxyl-terminus are a triplet of double serine residues, S364/S365, S368/S369, and S372/S373 of which S365, S368, and S373 appears more controlling to the fate of Cx43[99, 101, 104-108]. Altered phosphorylation at these sites has been implicated in numerous disease states, including the remodeling found after ischemia insult, chronic heart failure, and predisposes patients to the development of dangerous arrhythmias[101, 104, 106, 108]. Interestingly, and adding another layer of control to the Cx43 proteome, these residues appear to not exist in a vacuum and exude a level of influence on the other residues within this triplet[104, 106, 107].

Central to this cascade are the effects of pS368 on gap junction function[101, 106-108]. Phosphorylated by Protein Kinase C epsilon (PKC ϵ), pS368 is present at low levels in healthy cardiomyocytes[108]. However, as highlighted in previous data involving both chronic and acute damage to the myocardium, S368 becomes increasingly phosphorylated in response to cellular stress[102, 107]. This increased phosphorylation is associated with decreased dye permeability and gap junction closure, but not lateralization. Interestingly, it appears that inducing greater pS368 prior to ischemic stress imparts a protective effect on the heart and may be due to decreased spread of pathological signaling[108].

Prior research indicates that S365 is phosphorylated at baseline within the heart and is primarily the result of PKA activity[104, 108]. Upon the introduction of cellular stress, S365 becomes dephosphorylated in conjunction with the subsequent increased phosphorylation of S368 and implies the existence of a “cascade” effect[104]. To explore this phenomenon, researchers employed phosphomimetic glutamic acid in place of S365 to mimic a constantly

phosphorylated residue[104]. The results of this study showed that the cellular response to phosphorylate S368 under stress was hampered as evidenced by increased intercellular communication[104].

Phosphorylation of S373 occurs predominantly by means of Protein Kinase B (also referred to as Akt) and is not found at significant levels at baseline within the heart[106, 107]. However, phosphorylation is greatly increased under stress and, in part, creates a putative 14-3-3 binding domain within the carboxyl-terminus of Cx43[106, 107]. A transport protein involved in the degradation of membrane bound Cx43, GJ localization to the intercalated disc can be increased through the knockdown of 14-3-3 theta. Therefore, it has been shown that preventing 14-3-3/Cx43 interaction through the use of S373A stabilizes Cx43 to the membrane. In addition, it appears that phosphorylation of S368 is hindered in cells that cannot phosphorylate S373, allowing it to act as an upstream gatekeeper likewise[106, 107].

The existence of two “gatekeeper” phosphorylated serine residues acting on S368 implies of existence of a Cx43 regulatory mechanism beyond that of simple kinase and phosphatase interactions. Presently, there is a dearth of understanding regarding how these residues work in unison to affect Cx43 during periods of cellular stress – particularly in research employing double S365A/S373A mutants. In this paper, we take advantage of two *in vivo/ex vivo* phosphomutant mouse models – S368A (termed PKC mice) and S365A/S373A (termed Akt mice) – to study Cx43 posttranslational modification cascades.

Methods

Animal husbandry

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University and conducted in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Adult mouse cardiomyocyte isolation and culture

Mixed sex adult C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) aged 9 to 12 weeks were administered .5 mL 5 mg/mL porcine intestinal Heparin sodium salt (Sigma, St. Louis, MO, USA) in PBS 5 minutes prior to dissection. The mice were then anesthetized with isoflurane and sacrificed by means of cervical dislocation. Placed in a surgical environment, a thoracotomy was used to excise the heart and associated vasculature, which were rinsed in warm perfusion buffer (of 120.4 NaCl, 12.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄-7H₂O, 4.6 NaHCO₃, 30 Taurine, 5.5 Glucose, 10 2,3-Butanedione 2-monoxime, and 10 HEPES brought to pH 7.4 with HCl). The aorta was cannulated within 4 minutes of chest excision and Langendorff perfused with non-recirculating 37.0 °C perfusion buffer for 2 minutes; proper perfusion was confirmed following visible blood clearance of the coronary arteries and the adoption of a pale hue by the whole organ. Hearts were then perfused by 37.0 °C 57 mL 2.25 mg/mL collagenase type II in perfusion buffer (Worthington Biochemical Corporation, Lakewood, NJ, USA), supplemented with 3 uL 100 mM CaCl₂ just prior to exhausting the perfusing buffer. Prior to

decannulation, associated lung, vascular, and adipose tissues were excised. The hearts were decannulated and placed in a 60 mm dish with 3 mL collagenase buffer, atria were removed, and remaining ventricular tissues was gently shredded with forceps; the tissue was then pipetted up and down for two minutes using a disposable pipette. 2 mL Stop buffer was added to dish and pipetting continued for 2 more minutes. Cell suspension was transferred to a 50 mL conical tube and any remains in the 60 mm plate were washed into the conical tube using 5 mL stop buffer. The conical tube is spun in a tabletop ultracentrifuge at 30 g for 3 minutes at RT, supernatant removed, and resuspended in Stop buffer 2. This step was repeated for Stop buffers 3 and 4 before resuspending the cells in 7.5 mL cardiomyocyte media and plated in a 10 cm dish precoated with 100 µg/mL laminin (Gibco, Waltham, MA, USA), and placed in a humidified 5% CO₂ incubator at 37 °C for 1 to 3 hours. The growth media was gently aspirated off to remove all non-adherent cells and cell debris before growth media was replaced. Cells were then promptly returned to the incubator for maintenance. Growth media was replenished every other day. Cardiac myocyte media (CMM) (ScienCell, Carlsbad, CA, USA) with provided cardiac myocyte growth supplement, 5% FBS, 50 mM 2,3-Butanedione monoxime (Acros Organics, Fair Lawn, NJ, USA), and MyocoZap Plus-PR (Lonza, Basel, Switzerland) was changed on cardiac myocytes at 2 hr post-plating.

Neonatal mouse ventricular myocyte isolation and culture

Neonatal mouse ventricular myocytes (NMVCM) were isolated from 1 to 3 day old C57BL/6j mice as previously described (Smyth et al., 2012). Briefly, pups were decapitated, and hearts removed and washed in ice cold Hank's balanced salt solution without calcium or magnesium (HBSS; Thermo Fisher Scientific). Atria were removed and quartered ventricles placed in 5 mL HBSS with 1.5 mg/ml collagenase II (Worthington Biochemical) warmed to 37°C. Tissue was stirred for 5 min at 37°C and gently triturated 20 times with a transfer pipette prior to a 2 min rest allowing tissue to settle. Supernatant was removed and added to 5 mL ice-cold FBS (Thermo Fisher Scientific), and 5 mL warm HBSS with collagenase added to remaining tissue. This process was repeated three times, after which the cell/FBS suspension was centrifuged for 5 min at 300 x g. The supernatant was discarded and cell pellet resuspended in 10 mL F12/DMEM 50/50 (Thermo Fisher Scientific) supplemented with 5% FBS, insulin-transferrin sodium selenite media supplement (Thermo Fisher Scientific), and Mycozap-PR (Lonza). Cells were pipetted through a 70 µM cell strainer (BD Biosciences) and pre-plated for 30 min at 37°C on 100 mm dishes (Genesee Scientific). Cell suspension was removed, and pre-plating repeated on a fresh 100 mm dish to further enrich cardiomyocytes.

Cell Culture, not otherwise specified

HaCaT-Cx43 ^{-/-} and A549-Cx43 ^{-/-} were maintained and passaged in FMEM, high glucose, with L-Glutamine supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) non-essential amino acids (Life Technologies, Carlsbad, CA, USA), and MycoZap Plus-CL (Lonza, Basel, Switzerland).

hiPSC-CM differentiation, expansion, and maturation

iPSC (Cell Applications, San Diego, CA, USA) were differentiated into cardiomyocytes following per publication with modifications. Briefly, iPSCs were seeded into matrigel coated

12well plates in E8 medium with daily medium changes for 3 days. On day 0, medium was changed to CDM3, defined by publication with 3 μ M CHIR-99021 (Selleckchem) for 2 days. On day 2, medium was changed to CDM3 with 2 μ M Wnt-C59 (Cayman). On day 4, medium was replaced with CDM3. On day 6, medium was changed to RPMI1640 + B27 (- insulin) and refreshed on day 8. On day 10, medium was changed to RPMI1640 without glucose + B27 (- insulin). On day 12, cardiomyocytes were expanded as in [121]. Briefly, cells were lifted with TrypLE Select Enzyme 10X, washed with PBS 20% FBS and centrifuged at 200g for 4 minutes, resuspended in RPMI 1640 + B27 1X with 10% Knock Out Serum Replacement (GIBCO) and Thiazovivin 1.0 μ M (Selleckchem) and transferred into 10 cm dishes. 24 hours later medium was replaced with RPMI 1640 + B27 1X supplemented with 2.0 μ M CHIR-99021. Cells were further expanded for 4 passages prior to cryopreservation. iPSC-CMs were verified by the presence of cardiac troponin-T, N-cadherin and contraction. Prior to experiments iPSC-CMs were matured in a defined maturation medium composed of oxidative substrates and low glucose for 3 weeks according to.

***Ex vivo* whole heart ischemia**

Briefly, mixed sex adult C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) aged 9 to 12 weeks were euthanized with excess isoflurane before a subsequent cervical dislocation. Access to the thoracic cavity was achieved through an incision of the diaphragm and a cardioectomy was performed. The heart was rinsed briefly in warm (37°C) perfusion buffer (described above) before being submerged in warm (37°C) perfusion buffer and rested for the appropriate period of time to mimic no-flow ischemia. Organ samples were then snap frozen for biochemical assay or embedded in OCT (Thermo Fisher Scientific) for future sectioning.

Western blotting

Mouse hearts were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), 1% sodium deoxycholate, 2 mM NaF, 200 μ M Na₃VO₄, 0.1 % sodium dodecyl sulfate, 5 mM n-ethylmaleimide) supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA, USA). Protein was homogenized and then clarified by sonication and centrifugation and concentration determined by DC protein assay (Bio-Rad, Hercules, CA, USA). 4X Bolt LDS sample buffer supplemented with 400 mM DTT was added to samples then heated to 70 °C for 10 min and subjected to SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels and MES (Thermo Fisher, Waltham, MA, USA) running buffer according to manufacturer's instructions. Proteins were transferred to PVDF (Bio-Rad, Hercules, CA, USA) membrane and fixed in methanol and dried. PVDF membranes were reactivated in methanol followed by blocking in 5% nonfat milk (Carnation, Los Angeles, CA, USA) or 5% bovine serum albumin (Fisher Scientific, Waltham, MA, USA) in TNT buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris pH 8.0) for 1 h at room temperature. Primary antibody staining was performed at 4 °C overnight using the following antibodies: rabbit anti-Cx43 (1:3000; Sigma-Aldrich, St. Louis, MO, USA), mouse anti-GAPDH (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-phospho-Cx43 (Ser368) [D6W8P] (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Cx43 (Phospho-Ser373) (1:1000; Singalway Antibody, College Park, MD, USA). Membranes were washed six times with 1X TNT before secondary antibody labeling. Membranes were labeled for

1 h at room temperature with secondary antibodies conjugated with Alexa Fluor 555 or 647 (1:5000; Thermo Fisher, Waltham, MA, USA) or HRP (1:5000; Abcam, Cambridge, UK). Membranes were stripped for 30 minutes in ReBlot Plus Strong Antibody Stripping Solution before reprobing (Millipore Sigma, Burlington, MA, USA). Membranes were imaged by a Chemidoc MP imaging system (Bio-Rad, Hercules, CA, USA).

Surface EKG

Mixed sex adult C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) aged 9 to 12 weeks were recorded using the ECGenie system (Mouse Specifics, Framingham, MA, USA) according to manufacturer's recommendations. Data were recorded until a 5 second uninterrupted span of high-quality data was collected. Data qualities were determined by easily visible R waves with low/negligible background noise. Analysis was performed via the provided EzCG software (Mouse Specifics, Framingham, MA, USA).

Statistics

All quantification was performed on experiments and repeated at least three times (see figure legends for specific replicate values). Data are presented as mean \pm SEM. Statistical analysis was conducted with GraphPad Prims 8.0.2 (GraphPad Software, Inc. La Jolla, CA). Data were analyzed for significance using Student's t-test or one-way ANOVA correcting for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Phosphomutant mice have altered pS368 expression during no-flow ischemia

To determine the effect on Cx43 phosphorylation caused by loss of the ability to phosphorylate the two pertinent gatekeepers of S368, we employed a phosphomutant mouse model with global S365A/S373A (Akt mice) and S368A (PKC mice). Phosphomutant C57BL/6 mice, their predicted pertinent sequences shown and verified, were subjected to cardiac *ex vivo* ischemia for a period between 0 and 15 minutes to simulate whole organ no-flow ischemia. Western blotting of ventricular tissue lysates showed that while WT mice increase levels of pS368 during ischemia, Akt mice have the opposite effect. No phosphorylation at S368 was seen in the PKC mice (**Figure 1.1**). Interestingly, pS368 is present in measurable quantities in both WT and Akt mice suggesting that this site is not purely pathological and plays a role in basal Cx43 function (**Figure 1.1**). These data are supported by recent publications highlighting the need for pS373 prior to phosphorylation of S368 during stress.

No gross cardiac electrophysiological differences are seen between Akt and WT mice at baseline

To investigate whether mouse genotype was associated with measurable whole organ electrophysiological changes, we employed the use of a non-invasive surface level ECG technique (**Figure 1.2**). EKG readings of two phosphomutant mice, Akt and WT, we find no differences in EKG shape or metrics, including RR, PR, QT, QTc, QRS, and ST length during periods of non-stress for the mice (**Figure 1.2**).

Phosphomutant mice show altered Cx43 localization before and after ischemia

We next used tissue sections taken from the *ex vivo* ischemic organs to probe for differential Cx43 localization and expression across the genotypes. In all mice, Cx43 expression increased initially during ischemia, before dropping off by the 15-minute mark (**Figure 1.3**). These data are in agreeance with recent reports by the Lampe lab. The levels of membrane bound Cx43 were highest in the PKC mice across all time points, whereas Akt mice were trending upward as compared to WT (**Figure 1.3**). In agreeance with our western blotting (**Figure 1.3**), we see a similar increase in ID pS368 in WT mice at 15 minutes as compared to 0 mins, but a complete drop off in Akt mice from 15 minutes to base line (**Figure 1.3**)

Cobalt (II) Chloride elicits a different cellular response than ischemia *in vitro*

To acute stress the cells and elicit PKC activation, we utilized cobalt (II) chloride in solution with exposure up to 15 minutes, matching our *ex vivo* work. We compared the ratios of pS368 to total Cx43 western blotting in 293T Cx43 *-/-* cells exposed to 15 minutes of 250 uM cobalt chloride compared to PMA as a positive control for PKC activation (**Figure 1.5**). Due to the lack of significant pS368 response in the cobalt chloride treated cells, we then increased the dose as high as 1200 uM for 15 minutes (**Figure 1.5**). Again, no difference was seen between the untreated and treated 293T cells at all doses (**Figure 1.5**). While cobalt chloride has been use as a hypoxic model in previously published reports, it does not appear to activate similar pathways in 293T cells and a more reliable activator of PKC appears to be PMA.

Phosphomutant expressing cells have altered Cx43 in response to PMA

Using hiPSC-CM-Cx43*-/-* transiently transfected with hCx43 phosphomutant isoforms, we probed for differences regarding pS368 occurrence following exposure to PMA (**Figure 1.4**). While the data are not complete at time of publication, it appears that there are significant decreases in expression and stabilization of Cx43 in the phosphomutant isoforms compared to WT (**Figure 1.5**). However, at this time it is inconclusive whether or not these isoforms affect pS368 in the manner that we were expecting (**Figure 1.5**).

Discussion

Cx43 remodeling and alterations to its posttranslational modification is known to follow damage to the myocardium and can lay the foundation for cardiac arrhythmias. Phosphorylation, occurring both in the Golgi apparatus and at the plasma membrane/intercalated disc, is associated with changes in the localization and function of Cx43. Recently, there has been increased attention towards how individual residues located on the carboxyl-terminus affect the phosphorylation of adjacent sites; in particular, S365, S368, and S373. In accordance with previously reported data, we find that mice lacking the ability to phosphorylation S365 and S373 through point mutations to alanine have reduced capability to phosphorylation S368. This effect is most profound during periods of ischemia, wherein these double mutant mice lose the ability to phosphorylate S368 all together after 15 minutes of no-flow ischemia. Likewise, our data suggest that all genotypes of mice increase Cx43 expression transiently during ischemia, peaking after 5 minutes but dropping off by 15 minutes. These data are in agreeance with our imaging studies where we found increased membrane bound Cx43 staining at 5 minutes of ischemia before dropping off by 15. In a similar vein to the tissue lysate, there appears to be a complete

loss of pS368 staining in our Akt mice by the 15-minute mark, with no discernable localization to the intercalated disc. These data are supported through the use of *in vitro* transiently transfected Cx43 $-/-$ human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs-Cx43 $-/-$), and HaCat Cx43 $-/-$. Transfected HaCat Cx43 $-/-$ transfected to express S365A/S373A Cx43 do not phosphorylate S368 in comparable amounts to WT Cx43 expressing cells. Interestingly, in hiPSC-CMs S365A and S373A separately decrease pS368 in basal conditions. We saw no difference in the EKGs of these mice, however, suggesting no gross electrical differences existed between the genotypes. These data were limited by our collection techniques, surface level EKGs, and a better understanding could be harnessed with more sensitive implanted devices and telemetry. Finally, we show that with regard to pS368, cobalt (II) chloride is a poor drug of choice to mimic acute hypoxic changes in cardiomyocytes and does not appear to be dose responsive. This led us to primarily use PMA as a PKC activator and a inducer of pS368.

The effect of pS365 and pS373 on S368 is often studied in isolation of each other, without the use of multisite mutagenesis. Our *ex vivo* data appear to suggest a dominant effect exhibited by S373 following a similar pattern in previous work [68, 107]. Even though the mice possess S365A, a known inducer of pS368, they systematically decrease pS368 during periods of acute ischemia in counter of the WT mice[104]. While we were not able to detect EKG morphology changes in our phosphomutant models, other groups have been able to detect whole organ electrical aberrations in phosphomutant models. Xue et al. report that S282A, a MAPK phosphorylated site, is arrhythmogenic in rats and induces ST-elevation on EKG readouts [122]. Likewise, pS368 is known to precede QRS duration in models of autoimmune myocarditis[123]. Our data suggesting an acute increase in Cx43 expression following stress may be an effect of PKC on the GJA1 promoter, regardless of phosphorylation[42-44].

The use of cobalt chloride as a hypoxic memetic drug is well known and useful in the activation of various hypoxia induced pathways, including HIF-1 α /2 α [124]. Useful in the study of hypoxia without the challenge of a controlled atmosphere, cobalt chloride is especially needed for acute cellular hypoxia on the scale of minutes. However, it does not appear that PKC mediated phosphorylation of Cx43 is induced on such a short time scale using cobalt (II) chloride. Prior research indicates that cobalt chloride mediation down-regulation occurs following 8 hours exposure of 100 μ M, but our data suggest that even 1200 μ M does not affect Cx43 at 15 minutes of exposure[125]. On the contrary, PMA is able to induce the phosphorylation of S368 within 15 minutes of exposure. PMA has long been used to decrease GJIC, an effect caused in part by induced pS368 expression, and acts as a PKC activator[109]. It was for these reasons, that we modified our protocols to include the use of PMA in place of cobalt chloride for our *in vitro* work.

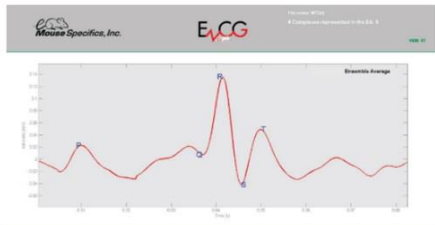
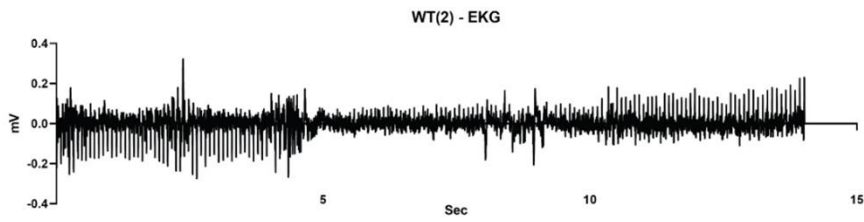
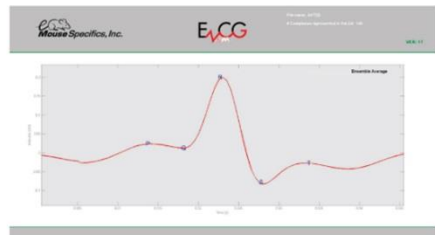
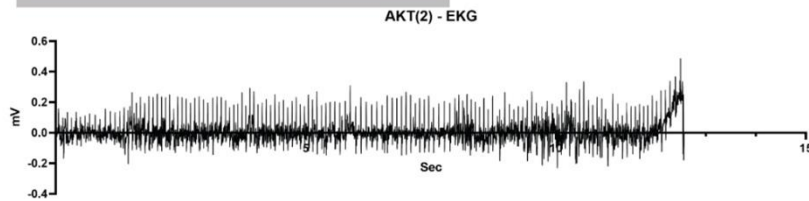
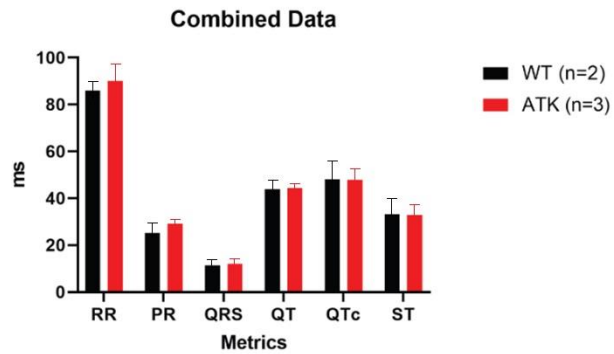
The study of Cx43 phosphorylation will always be translationally impactful in our collective fight against the leading killer in the United States: cardiovascular disease. Cx43 protein dynamics are severely altered through the modification various residues along it's carboxyl terminus – for better or for worse in the patient. However, the study of Cx43 phosphorylation for the most part has viewed each residue in isolation, removing phosphorylation at a single serine and studying the cellular effects. Our data, and data of other

groups, counter this notion and studies the effect that phosphorylated residues have on the status of those around them. Targeting pS368 appears to be most clinically relevant in preventing pathological cardiac remodeling and our data on S365/S373 add a piece to that puzzle. The interplay between these sites, where dephosphorylation at both concurrently reduces pS368, represents a potential drug target to ameliorate cardiac damage following ischemia. Future work in this direction should include the study of the role phosphatases play in this coordinated effect as well as investigate the cardiac electrophysiology of these mice in greater detail.

Conclusion

The importance of Cx43 post-translational modification does not lie in specific residues existing in a vacuum. Cx43 becomes differentially phosphorylated during episodes of intense cellular stress, often studied as ischemia, and it is these pathological changes that guide research towards a potential therapy to counteract and return Cx43 PTM to its baseline morphology. Additionally, the importance of protein interaction with Cx43 cannot be understated with regard to GJ function and serine phosphorylation. As is the case with S373, the binding 14-3-3 mode 1 binding motif is not appreciable until the residue is phosphorylated. Likewise, is the case of ZO-1 and Cx43 association, whether sterically hindered by 14-3-3 or due to conformation changes, pS373 completely removes the interaction between ZO-1 and Cx43 leading to increased gap junction plaque size and stability. Interactions like these highlight the ability of Cx43 to constantly have its regulation evolved to fit the specific needs of the cell, although sometimes these changes are pathological to the tissue as a whole.

At their core, GJs serve as conduits through which ions and molecules smaller than 1kD may passively diffuse across. Serving as important means of communication between electrically connected cells, such as the cardiomyocytes in the heart, GJs allow facile whole tissue responses within a timely manner. Highlighted by the propensity of mutant Cx43 animals to develop cardiac arrhythmias, the tight regulation of Cx43 is needed for impulse propagation throughout the syncytium. A surface level hypothesis might assume that with increased cellular communication benefits to cardiac tissue will be seen. However, as our collective understanding of Cx43 grows and interactions are better defined, it has become abundantly clear that Cx43 normal versus pathological regulation is less about hyper/hypo-phosphorylation and instead the complex interplay between proteins, scaffolds, ions, residue phosphorylation, and toxic ischemia byproducts. By focusing on S373, AKT, and the interplay with S365 and S38, we are adding our bit to the puzzle of Cx43 regulation and role in cardiac tissue.

A**B****C****Figure 1.2**

Surface level conscious EKG of Akt and WT mice. Representative EKG traces and runs for both WT A) and Akt B) animals. Quantification of various metrics pertaining to the QRST complex represented in, n=3 C).

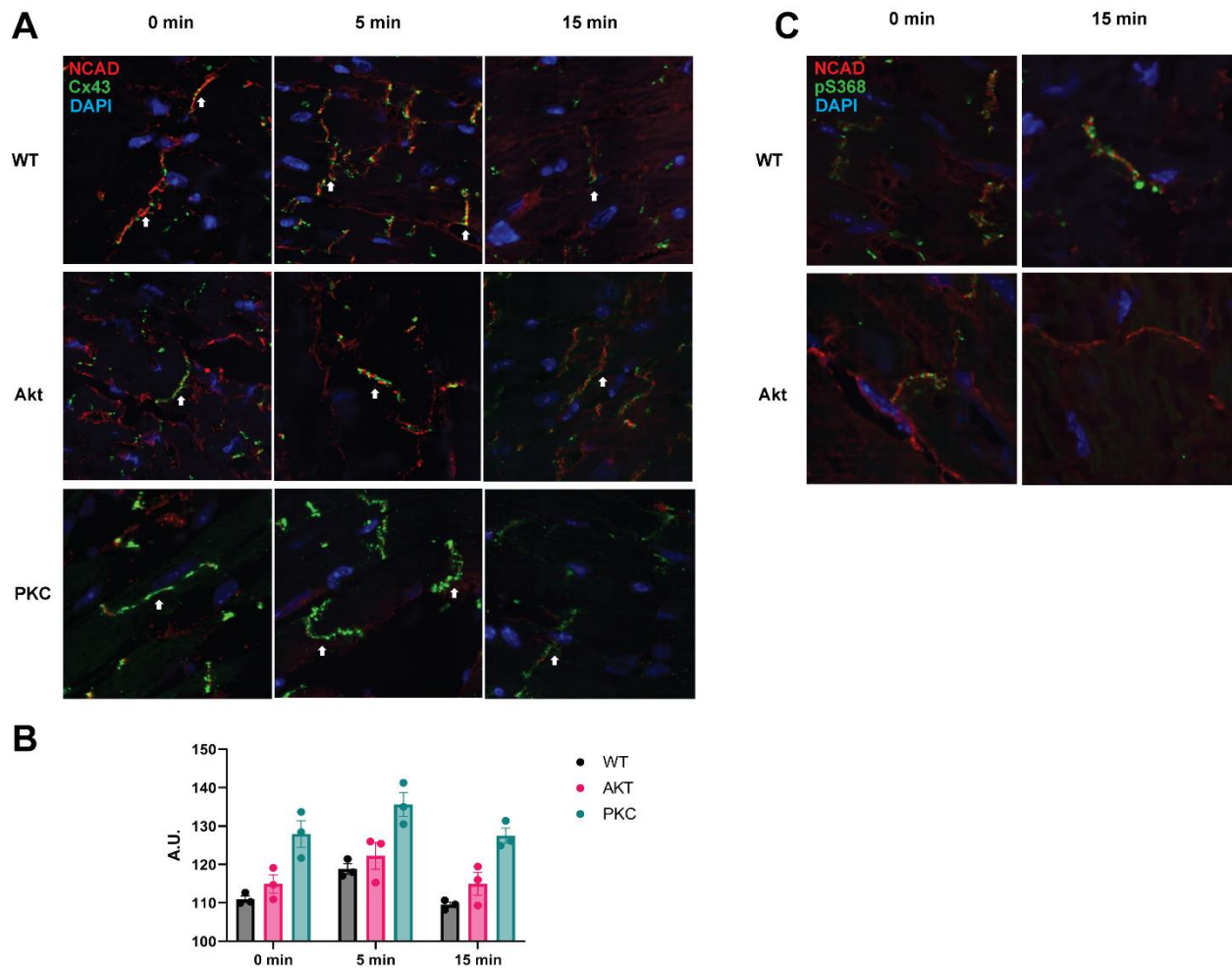


Figure 1.3

Localization of Cx43 and pS368 through immunofluorescence. Representative immunofluorescence data from WT, Akt, and PKC mice following up to 15 minutes of *ex vivo* ischemia with staining for NCAD (red), Cx43 (green), and DAPI (blue) **A**). Quantification of intercalated disc localized Cx43 from **A** represented in **B**), n=3. Representative immunofluorescence data from WT and Akt mice following 15 minutes of ischemia with staining for NCAD (red), pS368-Cx43 (green), and DAPI (blue).

A

				365			368				373						
WT	5'	...	CGA	CCT	TCC	AGC	AGA	GCC	AGC	AGC	CGC	GCC	AGC	AGC	AGA	...	3'
		...	R	P	S	S	R	A	S	S	R	A	S	S	R	...	
S365A	5'	...	CGA	CCT	TCC	GCT	AGA	GCC	AGC	AGC	CGC	GCC	AGC	AGC	AGA	...	3'
		...	R	P	S	A	R	A	S	S	R	A	S	S	R	...	
S368A	5'	...	CGA	CCT	TCC	AGC	AGA	GCC	GCT	AGC	CGC	GCC	AGC	AGC	AGA	...	3'
		...	R	P	S	S	R	A	A	S	R	A	S	S	R	...	
S373A	5'	...	CGA	CCT	TCC	AGC	AGA	GCC	AGC	AGC	CGC	GCC	AGC	GCT	AGA	...	3'
		...	R	P	S	S	R	A	S	S	R	A	S	A	R	...	
Combo	5'	...	CGA	CCT	TCC	GCT	AGA	GCC	AGC	AGC	CGC	GCC	AGC	GCT	AGA	...	3'
		...	R	P	S	A	R	A	S	S	R	A	S	A	R	...	



Figure 1.4

Creation of Cx43 phosphomutant isoforms in pcDNA 3.2/V5-DEST plasmid. A) Predicted plasmid sequences. B) Confirmed plasmid sequences.

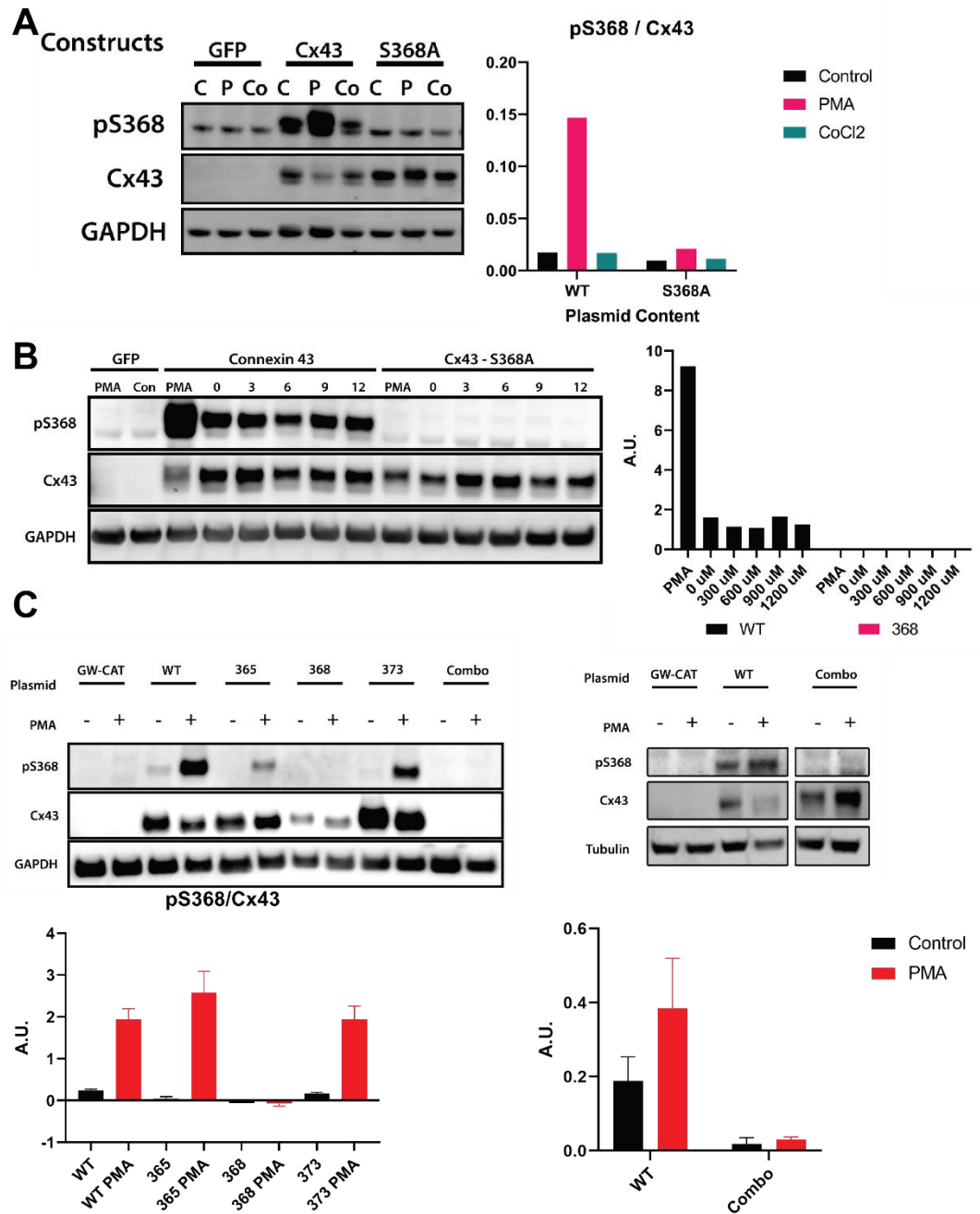


Figure 1.5

In vitro transient transfection of Cx43 phosphomutant isoforms. **A**). Transient expression of either WT Cx43 or Cx43 S368A in HEK293T cells with cobalt chloride challenge. Quantification to the right, n=1. **B**) Cobalt chloride dose response challenge of transient Cx43 WT or S368A expressing 293T cells. Quantification to the right, n=1. **C**) hiPSC-CM Cx43^{-/-} expressing Cx43 phosphomutant isoform. Quantified to the right, n=3.

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