Phosphatase-Resistant Gap Junctions Inhibit Pathological Remodeling and Prevent Arrhythmias

Benjamin F. Remo,* Jiaxiang Qu,* Frank M. Volpicelli, Steven Giovannone, Daniel Shin, Joshua Lader, Fang-yu Liu, Jie Zhang, Danielle S. Lent, Gregory E. Morley, Glenn I. Fishman

Rationale: Posttranslational phosphorylation of connexin43 (Cx43) has been proposed as a key regulatory event in normal cardiac gap junction expression and pathological gap junction remodeling. Nonetheless, the role of Cx43 phosphorylation in the context of the intact organism is poorly understood.

Objective: To establish whether specific Cx43 phosphorylation events influence gap junction expression and pathological remodeling.

Methods and Results: We generated Cx43 germline knock-in mice in which serines 325/328/330 were replaced with phosphomimetic glutamic acids (S3E) or nonphosphorylatable alanines (S3A). The S3E mice were resistant to acute and chronic pathological gap junction remodeling and displayed diminished susceptibility to the induction of ventricular arrhythmias. Conversely, the S3A mice showed deleterious effects on cardiac gap junction formation and function, developed electric remodeling, and were highly susceptible to inducible arrhythmias.

Conclusions: These data demonstrate a mechanistic link between posttranslational phosphorylation of Cx43 and gap junction formation, remodeling, and arrhythmic susceptibility. (Circ Res. 2011;108:1459-1466.)

Key Words: gap junction ■ casein kinase ■ arrhythmias ■ optical mapping ■ mouse ■ connexin

Gap junctional communication is essential for normal cardiac impulse propagation. A growing body of literature provides compelling evidence that supports the concept that dysregulation of cell-cell coupling in the heart contributes to a proarrhythmic substrate.1 The proximate pathological stimuli that result in aberrant cardiac gap junction expression and function are diverse. They include common forms of acquired heart disease, including hypertrophy and ischemic heart disease,1 as well as rare genetic causes such as the somatic mutations in connexin 40 associated with sporadic cases of atrial fibrillation2 or the inherited mutations in connexin 43 (Cx43) responsible for oculodentodigital dysplasia.3

The cardiac connexins (ie, connexins 43, 40, and 45) are all phosphoproteins. Posttranslational phosphorylation is thought to affect multiple aspects of the connexin life cycle, including transport to the plasma and junctional membrane, oligomerization into connexons (hemichannels), gap junction assembly, gating of assembled gap junction channels, and degradative processes that involve lysosomal and proteasomal pathways.4,5 Gap junction remodeling (GJR) is likely to reflect perturbations at any number of these steps in the life cycle of connexins.

Cx43, the major ventricular gap junction protein, undergoes posttranslational phosphorylation in its carboxy-terminus at as many as a dozen amino acids. Significant progress has been made in the identification of specific kinases and phosphatases that act on Cx43, as well as their specific target sites. Protein kinase A–dependent phosphorylation of Cx43 results in increased trafficking to the plasma membrane6–7; phosphorylation at casein kinase 1δ (CK1δ) sites on Cx43 plays a significant role in gap junction assembly8–10; Cx43 interaction with Src kinase has the dual effect of destabilizing Cx43 interaction with the scaffolding protein ZO-1, which leads to Cx43 internalization/degradation,11–13 and downregulating gap junctional cell-cell communication14–16; mitogen-activated protein kinases inhibit intercellular communication via reduced channel open probability17–19; and protein kinase C–dependent phosphorylation of Cx43 influences unitary conductance of assembled channels,20–22 and may be further involved in cell cycle progression.23 The protein phosphatases PP1 and PP2A colocalize with Cx43 and have been found to be upregulated in heart failure models, which suggests a role in GJR.24,25 Many of these data were gleaned from experiments that used pharmacological approaches and from in vitro studies of site-specific connexin mutants. Although these studies have been informative, the relevance of these approaches to in vivo physiology and pathophysiology is uncertain.

CK1δ phosphorylation of Cx43 occurs at serines 325/328/330. Cell culture studies suggest that these sites play an integral role in gap junction assembly and the formation of

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the slower-migrating phosphoisoforms of Cx43 observed when subjected to SDS-PAGE. Interestingly, recent data indicate that phosphorylation at these sites is markedly reduced in response to cardiac ischemia, and the loss of phosphorylation is associated with relocalization of the protein from gap junctions to the lateral borders of myocytes. Moreover, our laboratory demonstrated that not only acute stressors such as ischemia but also the imposition of chronic pressure-overload hypertrophy resulted in early and progressive dephosphorylation of Cx43 at these same residues, in association with GJR and slowing of impulse propagation. These changes were blunted by mineralocorticoid receptor blockade, a therapy that has been shown to diminish sudden cardiac deaths in patients with heart failure.

In the present study, we tested the hypothesis that modulation of posttranslational phosphorylation of Cx43 at this triplet of serines would influence GJR and alter arrhythmic susceptibility. We generated 2 new strains of genetically engineered knock-in mice. Compared with wild-type (WT) mice, cardiac gap junctions in mice that harbored phosphatase-resistant phosphomorphic glutamic acids at residues 325, 328, and 330 (S3E) were resistant to pathological remodeling and to the induction of ventricular arrhythmias. In contrast, introduction of nonphosphorylatable alanines into the carboxyl-terminus (S3A) interfered with gap junction formation and function and promoted a proarrhythmic phenotype. The present data provide the first in vivo evidence to date demonstrating a mechanistic link between Cx43 phosphorylation status, electrophysiological substrate, and arrhythmic susceptibility.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Generation of Cx43 Phosphomutant Constructs and Mutant Mice

Site-directed mutagenesis was performed to introduce the S3E and S3A (residues 325, 328, and 330) into the same gene-targeting vector previously used to conditionally inactivate Cx43 in the heart and other lineages. Cx43 phosphomutant targeting vectors were introduced into the 129/Sv-derived embryonic stem cell line R1 and correctly targeted embryonic stem cell clones were injected into C57BL/6 blastocysts. Highly chimeric male mice were crossed with WT CD1 females to generate F1 Cx43<sup>S3A/WT</sup> and Cx43<sup>S3E/WT</sup> heterozygous mutant mice, which were then bred to generate homozygous Cx43<sup>S3A/S3A</sup> and Cx43<sup>S3E/S3E</sup> mice. Both heterozygous and homozygous intercrosses were maintained, and for all experiments, WT littermates were used as controls.

Echocardiography

Left ventricular dimensions and function were assessed by echocardiography as described previously with an ATL 5000CV Ultrasound System (Philips Medical, Bothell, WA). A Langendorff global ischemia model was performed as described previously. A 2F octapolar intracardiac catheter was used to perform electrophysiology studies. A CMOS video camera (Ultima-L; SciMedia, Inc, Costa Mesa, CA) was used to record epicardial conduction velocity (CV) and optical action potential duration (APD) and APD dispersion measurements were obtained from the left ventricular surface at a basic cycle length of 100 ms.

Electrophysiology Studies

Electrophysiological studies were performed as described previously with a CMOS video camera (Ultima-L; SciMedia, Inc, Costa Mesa, CA). Epicardial conduction velocity (CV) and optical action potential duration (APD) and APD dispersion measurements were obtained from the left ventricular surface at a basic cycle length of 100 ms.

Heart Isolation and Optical Mapping

High-resolution optical mapping experiments were performed as described previously with a CMOS video camera (Ultima-L; SciMedia, Inc, Costa Mesa, CA). Epicardial conduction velocity (CV) and optical action potential duration (APD) and APD dispersion measurements were obtained from the left ventricular surface at a basic cycle length of 100 ms.

Transverse Aortic Constriction

Transverse aortic constriction (TAC) was performed on male mice at 3 to 4 months of age as described previously.

No-Flow Ischemia Protocol

A Langendorff global ischemia model was performed as described previously.

Statistical Analysis

Data are presented as mean±SEM and were analyzed by Student t test. For multiple groups, 1-way ANOVA was performed with post hoc Newman-Keuls test. Arrhythmia inducibility was analyzed by Fisher exact test. P<0.05 was considered statistically significant.

Results

S3A and S3E Mice Develop Normally

To evaluate the effects of Cx43 phosphorylation on gap junction expression and function in vivo, we generated site-specific knock-in mice that expressed either the S3A or S3E mutations using a strategy identical to that previously used to generate Cx43 oculodentodigital dysplasia mutant mice. Homozygous S3A and S3E mice were grossly indistinguishable from WT controls at birth and throughout development into adulthood (>3 months). Histological examination of S3A and S3E adult hearts appeared entirely normal, with no evidence of fibrosis, hypertrophy, or myofibrillar disarray (data not shown). There was no statistically significant difference between the 3 genotypes with respect to contractile performance, left ventricular chamber sizes, wall thicknesses, or heart weight–to–body weight ratio (Online Table I).

Altered Cx43 Phosphorylation and Gap Junction Formation in S3A and S3E Mice

Immunoblotting studies were performed to determine whether the site-specific mutations were associated with changes in Cx43...
electrophoretic fractionation. Prior reports have shown that Cx43 resolves into multiple electrophoretic isoforms when subjected to SDS-PAGE, including a fast-migrating nonphosphorylated band termed P0 and slower-migrating phosphorylated bands referred to as P1, P2, and higher.4 Consistent with this notion, immunoblotting of highly resolved total protein lysates from mice of each genotype revealed distinctive banding patterns consistent with altered phosphorylation. WT hearts with polyclonal panCx43 antibodies revealed a faint P0 band and more prominent P1, P2, and P3 bands. In contrast, homozygous S3A mice demonstrated faint amounts of P0 and an accumulation of the P1 isoform, whereas total protein lysates from the hearts of homozygous S3E mice displayed a near absence of all Cx43 isoforms faster than P2 (Figure 1A, upper panel). Heterozygous mice showed intermediate phenotypes. We also determined the banding pattern of Triton X-100–insoluble protein extracts, which are enriched in junctional membrane proteins.37 This fractionation resulted in the preferential recovery of more slowly migrating forms of Cx43 for all genotypes (Figure 1A, lower panel).

To quantitatively determine whether the site-specific mutants affected the steady state accumulation of Cx43, we performed Western blot analysis of whole-cell lysates and Triton X-100–insoluble pellet fractions prepared from the hearts of a cohort of WT, homozygous S3A, and S3E mutant mice. Compared with hearts expressing WT Cx43, the amount of total Cx43-S3A protein found in whole-cell lysates, as well as amounts within the Triton X-100–insoluble fraction, was significantly reduced to less than 50% of control levels, which is indicative of less accumulation of Cx43 into gap junctions (Figure 1B). In contrast, the abundance of the phosphomimetic S3E mutant protein was indistinguishable from WT levels in both whole-cell lysates and Triton X-100–insoluble fractions (Figure 1B).

To directly visualize gap junctional plaques, we next performed immunofluorescent staining of hearts from each of the 3 strains of mice. Hearts of S3A mice showed significantly less total Cx43 colocalized with N-cadherin at intercalated discs than WT controls or S3E mutant mice (Figures 2A and 2B). Taken together, these findings are consistent with prior in vitro data that suggested that phosphorylation of Cx43 at serine residues 325/328/330 leads to P2 formation, and they also suggest that inhibition of this phosphorylation event in vivo interferes with normal Cx43 trafficking or stability.9

S3E Mice Are Resistant to Structural GJR

We previously demonstrated that chronic pressure-overload hypertrophy secondary to TAC results in dephosphorylation of Cx43 at serines 325/328/330, as well as structural GJR and slowing of impulse propagation.10 To determine whether modification of these sites affected the heart’s response to chronic pathological stress, WT and homozygous S3A and S3E mutant mice were subjected to TAC and followed for 4 weeks. Mice of all 3 genotypes exhibited comparable structural remodeling, with deterioration of contractile function after imposition of pressure overload (Online Table I). As anticipated from our previous work,10 analysis of whole-cell lysates from WT hearts subjected to TAC showed molecular evidence of GJR, with an obvious shift toward higher-mobility/hypophosphorylated forms of Cx43 compared with sham-operated mice (Figure 3A). In contrast, the more rapid electrophoretic mobility of Cx43-S3A

Figure 1. Electrophoretic mobility of Cx43 mutants. A, High-resolution Western blot analysis of whole-cell lysates (WCL) or Triton X-100–insoluble pellets (pellet) prepared from ventricles of mice with the indicated genotypes, probed with polyclonal panCx43 antisera. WT Cx43 lysate treated with calf intestine phosphatase (CIP) migrates at P0 and is shown for comparison with various major phosphorylated forms of Cx43 (P1, P2, P3). B, Western blot analysis of whole-cell lysates (top) or Triton X-100–insoluble pellets (bottom). n=3 for each genotype. Quantification is shown to the right. *P<0.05 vs WT.

Figure 2. Cx43-S3E phosphomimetic mutation prevents pathological gap junction remodeling. A, Representative immunofluorescent staining with panCx43 (Cx43, green) and N-cadherin (N-cad, red) antibodies at baseline and 4 weeks after TAC. Scale bar, 10 μm. Magnified views of individual gap junction plaques for each genotype after TAC are shown below. B, Quantitative analysis of Cx43 and N-cadherin colocalization at baseline (B) and after TAC (B). *P<0.05 vs S3E, Post-TAC; +P<0.05 vs WT and S3E.
and the retarded mobility of Cx43-S3E seen at baseline were largely unaffected by imposition of TAC, consistent with the phosphatase-resistant design of both mutant proteins. However, analysis of Triton X-100–insoluble fractions, enriched in gap junctions, revealed a significant reduction in abundance of Cx43 in WT hearts and particularly in S3A mutant hearts after TAC, whereas the S3E hearts were resistant to molecular GJR (Figure 3B). In agreement with these data, we found by confocal immunofluorescence that although TAC led to significant GJR in WT and S3A mice, the S3E mice were resistant to pathological remodeling, with preservation of Cx43 at the intercalated discs, largely colocalizing with N-cadherin (Figure 2A, compare upper and lower panels; quantification in Figure 2B).

To further examine the relevance of posttranslational phosphorylation of Cx43 at serines 325/328/330, we subjected isolated hearts from WT, S3A, and S3E mice to acute ischemia, as described previously. Confirming prior studies, there was a significant increase in the amount of P0 Cx43 in WT hearts after ischemia. Postischemic S3A hearts also showed an accumulation in the nonphosphorylated Cx43 band; in contrast, the S3E postischemic hearts displayed significantly less dephosphorylation (Figure 4).

**S3E Mice Are Resistant to Conduction Slowing**

To determine whether these effects on GJR were associated with functional sequelae, we performed optical mapping to quantify CV in WT and mutant mice, both at baseline and after TAC. As shown in Figure 5, optical mapping of WT and S3A hearts revealed significantly diminished CVmax and CVmin after TAC, but S3E CV was preserved, consistent with the lack of structural GJR.

**Connexin Mutations Differentially Influence Electric Remodeling**

Recent data suggest that the intercalated disc may behave as a functional unit, and changes in gap junction expression may influence other ionic currents. Therefore, we also measured action potential parameters in isolated perfused hearts. Interestingly, hearts from S3A mice showed a significant increase in APD dispersion compared with WT and S3E mutants (Figures 5A–D). Moreover, the restitution properties of the S3A hearts also differed from the other 2 genotypes; the relationship between coupling interval and APD was steeper in these mice, with a trend toward shorter APD50 values at short S1S2 coupling intervals and greater APD50 values at longer coupling intervals (Figure 5E).
S3E Mice Are Less Susceptible to Induction of Ventricular Arrhythmias

A key goal of strategies to modify GJR is amelioration of arrhythmic activity in the intact organism. Accordingly, using 2 different provocative methods, we tested whether the changes in electrophysiological properties identified above were associated with alterations in arrhythmic propensity. Baseline WT and mutant mice were subjected to programmed electrical stimulation with premature extrastimuli. With this protocol, 40% of S3A mice and 33% of WT mice developed ventricular tachyarrhythmias, but none of the S3E mice were inducible. With the more aggressive burst-pacing protocol, 70% of S3A mice and 42% of WT mice developed ventricular tachycardia, but only 18% of S3E mice were inducible, as shown in Figure 6.

Discussion

Sudden cardiac death due to ventricular tachyarrhythmias results in as many as 462,000 deaths annually in the United States alone. Prior studies suggest that GJR plays an important role in the pathophysiology of these lethal cardiac arrhythmias. Cx43 is the major cardiac gap junction protein, and we have previously shown that the elimination of Cx43-dependent intercellular coupling between cardiomyocytes results in a slowing of ventricular CV and uniform sudden arrhythmic death in mice. These results illustrate the importance of Cx43 to normal cardiac function but do not address the underlying mechanism and functional consequences of pathological GJR that occurs in response to myriad myopathic stimuli associated with acquired forms of heart disease.

The present study was performed to further explore the significance of posttranslational phosphorylation of Cx43 at serines 325/328/330 and the role of this regulatory event in impulse propagation and arrhythmogenicity. Our focus on these particular residues derives from in vitro studies demonstrating the importance of these sites for gap junction formation, as well as our recent observation that dephosphorylation at these residues is an early response to pressure-overload hypertrophy, a clinically important pathological stimulus associated with increased mortality and sudden cardiac death. We established strains of mutant knock-in mice to determine the relevance of these sites in vivo. The results of the present analysis indicated that pseudophosphorylation of Cx43 at serines 325/328/330 (S3E mutant mice) led to gap junctions that appeared resistant to the pathological remodeling associated with both acute (no-flow ischemia) and chronic (TAC) pathological stimuli. Conversely, phosphorylation-deficient S3A mutant mice displayed aberrant gap junction expression even at baseline. Importantly, although WT mice had a similar incidence of ventricular tachycardia induction by programmed electrical stimulation, as reported previously, the S3E mice were highly resistant to the induction of life-threatening ventricular arrhythmias, and the S3A mice displayed increased arrhythmic susceptibility.

The results of the present study are consistent with prior in vitro studies using specific CK1 inhibitors that caused decreased gap junction formation, as well as in vivo work using phosphospecific antibodies that found decreased expression of Cx43 phosphorylated at residues 325/328/330 in gap junctions after ischemia and TAC. The present data expand on these

Figure 5. Functional analysis of isolated-perfused hearts. Representative activation maps (A) and quantification of CV at baseline and after TAC (B). *P<0.05 vs baseline for each genotype; †P<0.05 vs TAC WT and S3A. Max indicates maximum; Min, minimum. C, Representative APD50 maps. The shadow in each image represents a nonpacing positioning. D, Quantification of average values of APD50 dispersion at an S1S2 coupling interval of 95 ms. E, Average values of APD50, taken at a range of coupling intervals. *P<0.05. n=6 hearts for each genotype.
findings by showing a link between phosphorylation of Cx43, GJR, and susceptibility to arrhythmia induction in vivo.

Although S3A mutants were nonphosphorylated at serines 325/328/330, P2 and slower bands were present on immunoblotting of the Triton X-100–insoluble fraction. This may indicate that CK1\textsubscript{H9254}-dependent phosphorylation is electrophoretically silent but facilitates an additional phosphorylation event, which in turn is responsible for the formation of “P2.” In this scenario, the second phosphorylation event responsible for P2 formation may be less efficient in the S3A mutants but not completely abrogated. Alternatively, it may simply be the case that with so many potential phosphorylatable residues within the carboxy-terminus of this gap junctional protein, the various “P” forms represent heterogeneous populations of Cx43 phosphoisoforms. Indeed, it has been shown that certain Cx43 phosphoisoforms can even migrate at P0.8,9,42

Interestingly, the Cx43 phosphorylation-site mutations appear to differentially influence action potential properties. Mice expressing the S3A mutation displayed significantly longer APD\textsubscript{50} values and an increase in the dispersion of repolarization. Moreover, hearts from the S3A mice showed steeper restitution curves. Taken together, these properties may play an unexpectedly significant role in conferring the proarrhythmic phenotype observed in these mutant mice. Indeed, we previously reported significant electric remodeling in Cx43-deficient hearts.38 These observations reinforce recent suggestions that the intercalated disc may act as a functional unit, with cross talk between connexins, scaffolding proteins such as ankyrins, and various sodium and potassium channel subunits.39

There are several limitations to the present study. Although these experiments provide definitive evidence linking Cx43 phosphorylation state to gap junction stability and resistance to GJR, additional work will be required to more precisely identify what steps in the connexin life cycle are primarily responsible for this salutary behavior. One hypothesis for the increased stability seen with CK1\textsubscript{H9254} phosphorylated Cx43 is that phosphorylation at these sites confers protection from degradative pathways. Gap junctions are dynamic structures whose degradation involves internalization and eventual breakdown by both lysosomal43–45 and proteasomal45–48 pathways. Ubiquitin conjugation to gap junctional Cx43 is thought to initiate endocytosis. The ubiquitin protein ligase Nedd4 is known to bind the carboxyl-terminus of Cx43, and the phosphorylation status of Cx43 may modulate this interaction.49 Previous studies have shown that treatment of rat epithelial cells with epidermal
growth factor and 12-O-tetradecanoylphorbol-13-acetate induces ubiquitination of Cx43, and this process is associated with hyperphosphorylation of Cx43 by mitogen-activated protein kinase and protein kinase C.50 These findings illustrate the close relationship between Cx43 phosphorylation status and ubiquitination, as well as the importance of specific target residues. It is conceivable that phosphorylation at serines 325/328/330 interferes with endocytosis and subsequent degradation of gap junctions, whereas dephosphorylation at these sites enhances degradation. Our studies using heterologous expression systems have suggested the half-lives of the various mutant Cx43 proteins differ from WT Cx43; however, attempts to perform comparable studies in intact hearts with translational inhibitors have not been revealing. Alternatively, the major effect of Cx43 phosphorylation could involve trafficking. The S3E modification might augment (and conversely, the S3A mutation might inhibit) the direct targeting of connexons to the adherens junction.51 Additional studies will be required to distinguish among these potential mechanisms.

Inasmuch as the phosphorylation status of Cx43 reflects the balance of various kinases and phosphatases such as CK1δ and PP2A, we measured the abundance of these 2 enzymes. Given the recent report of increased expression of PP2A and enhanced association of PP2A with Cx43 in a model of nonischemic heart failure, we anticipated a similar finding in the hypertrophied hearts; however, we found no change in the abundance of either of CK1δ or PP2A in hearts with pressure-overload hypertrophy and no evidence of increased interaction between PP2A and Cx43 (data not shown). Although this does not exclude changes in enzymatic activity, additional studies will be required to clarify the mechanisms responsible for the altered phosphorylation state of Cx43 we observed in hypertrophied hearts.

In summary, these data provide the first in vivo evidence to date of a mechanistic link between Cx43 phosphorylation status, gap junction expression, and arrhythmic susceptibility. Our results provide additional evidence indicating that modulation of Cx43 phosphorylation status may be a rational antiarrhythmic strategy.52

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Disclosures
None.

References
Novelty and Significance

**What Is Known?**

- Gap junctions comprise intercellular channels that electrotonically couple cardiomyocytes with one another and facilitate impulse propagation and normal rhythmicity in the heart.
- Diverse disease-causing stimuli promote abnormal expression of gap junctions, i.e., pathological gap junction remodeling (GJR), and various lines of experimental evidence indicate that GJR contributes to increased arrhythmic propensity.
- Connexin43 (Cx43), the major cardiac gap junction protein, is posttranslationally phosphorylated by numerous kinases, and these posttranslational events are thought to regulate channel assembly, membrane trafficking, gating, and turnover.
- In vitro studies indicate that phosphorylation of Cx43 by casein kinase 16 (ck16) at serines 325, 328, and 330 may promote gap junction assembly, which suggests this event may be an important regulator of intercellular coupling and cardiac rhythmicity.

**What New Information Does This Article Contribute?**

- Using genetically engineered knock-in mice with site-specific mutations introduced into the Cx43 gene, we demonstrate that phosphomimetic mutants of Cx43 at CK1δ-dependent target sites enhance gap junction formation and confer resistance both to pathological GJR and to the induction of ventricular arrhythmias.
- Conversely, inhibition of phosphorylation at these same target sites diminishes gap junction formation and confers enhanced arrhythmic susceptibility.

**Histological pathologic studies have demonstrated abnormal expression of gap junctions in diverse cardiac disease states, including most acquired and a number of inherited cardiomyopathies. At the molecular level, this process of GJR is associated with hypophosphorylation of Cx43, the major gap junctional protein. Although the expression and function of various kinases and phosphatases that act on Cx43 have been examined in vitro, the precise roles of these regulatory pathways in the context of the intact organism remain unknown. Using genetically engineered mice with site-specific mutations introduced into the Cx43 protein that either mimic (glutamic acid) or prevent (alanine) phosphorylation, we tested the role of CK1δ-dependent phosphorylation of Cx43 on gap junction expression and function. The phosphomimetic mutant mice were resistant to GJR and induction of ventricular arrhythmias, whereas the phosphoresistant mutant mice showed the opposite phenotype. These data demonstrate for the first time a mechanistic link between posttranslational phosphorylation of Cx43 and gap junction formation, remodeling, and arrhythmic susceptibility in vivo.**
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SUPPLEMENTAL MATERIAL

EXPANDED MATERIALS AND METHODS

Generation of Cx43 Phospho-mutant Constructs

Site-directed mutagenesis was performed to introduce the S3E and S3A (residues 325,328,330) into the same gene-targeting vector previously used to conditionally inactivate Cx43 in the heart and other lineages.1–3

Animal models

All animal procedures were carried out in accordance with Public Health Service guidelines for the care and use of laboratory animals and approved by the New York University School of Medicine Institutional Animal Care and Use Committee. Cx43 phospho-mutant targeting vectors were introduced into the 129/Sv-derived ES cell line R1.1,4 Screening for correct recombinants was performed by Southern blot and PCR analysis, also as previously described.5 For each construct, two correctly targeted ES clones were injected into C57BL/6 blastocysts. Highly chimeric male mice were crossed with wild-type (WT) CD1 females to generate F1 Cx43S3A^WT and Cx43S3E^WT heterozygous mutant mice. These were subsequently bred to generate homozygous Cx43S3A/S3A and Cx43S3E/S3E mice. Both heterozygous and homozygous intercrosses were maintained and for all experiments, WT littermates were used as controls.

Echocardiography

Left ventricular dimensions and function were assessed by echocardiography as previously described using an ATL 5000CV Ultrasound System (Philips Medical, Bothell, WA).1

Western Blot Analysis

Total protein lysates and Triton X-100 insoluble pellet fractions were prepared from the apical two-thirds of the ventricle as previously described.6 Primary antibodies included a rabbit polyclonal anti-Cx43 (Sigma c6219), which recognizes all forms of Cx43; mouse monoclonal anti-Cx43 (Zymed 13-8300), which has been reported to bind selectively to non-phosphorylated Cx43.7 Equivalency of protein loading was verified by probing for GAPDH. Signals were visualized and quantified using the Odyssey Imaging System (Li-Cor, Lincoln, NE).

Immunofluorescence, Confocal Microscopy, and Cx43 Quantification

Immunofluorescent staining was performed on formalin-fixed, paraffin embedded sections using rabbit polyclonal anti-Cx43 antibody (Sigma c6219) and mouse anti-N-cadherin antibody (Invitrogen). Secondary antibodies were FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunostained sections were examined using a Leica DMI6000B confocal laser-scanning microscope (Leica, Heidelberg, Germany). Gap junction remodeling was quantified by determining the extent of colocalization of Cx43 and N-cadherin at the intercalated discs using images collected at 63x magnification (CoLocalizer Pro software, CoLocalization Research Software). Calculations were performed on 7–9 sections from 3 hearts for each genotype.

Heart Isolation and Optical Mapping

High-resolution optical mapping experiments were performed as previously described, using a CMOS video camera (Ultima-L; SciMedia, Inc).1,8 Most studies were performed with the addition of blebbistatin (5 µM; BIOMOL International, LP) to reduce motion artifacts. Epicardial
Conduction velocity (CV) and optical action potential duration (APD) measurements were obtained from the left ventricular surface at basic cycle length (BCL) of 100 ms with 3 ms stimuli at twice diastolic threshold. APD measurements were obtained using an S1-S2 protocol consisting of a 10 beat S1 drive cycle at 100 ms BCL followed by a single S2 extrastimulus. APD values were calculated from the single S2 beat. APDs were measured for each pixel at 50% (APD50) of repolarization. APD dispersion was calculated as the standard deviation of APD values for all pixels.

**Electrophysiology Studies (EPS)**

EPS was performed as previously described. Briefly, male mice 3-4 months of age were anesthetized with isofluorane and intubated. A cut-down of the right internal jugular vein was performed, and a 2-French octapolar catheter was advanced into the right atrium and ventricle using electrocardiogram guidance and pacing capture to confirm intracardiac location.

Standard pacing protocols (single, double and triple extrastimulation and burst pacing (BP)) were performed to test for ventricular tachycardia (VT) inducibility. Briefly, drive trains of 8 paced beats (S1 x 8) were delivered at rates of 140ms and 100ms and followed by a single, double and triple extrastimuli (S2, S3, or S4) brought down to a minimum coupling interval of 30ms. Ventricular burst pacing was performed as eight 50ms and four 30ms cycle length trains applied once every 3s, up to a maximum 1min time limit of total stimulation. VT was defined as ≥ 4 consecutive beats.

**Transverse aortic constriction**

Transverse aortic constriction was performed on male mice at 3-4 months of age as previously described.

**No-Flow Ischemia Protocol**

Langendorff global ischemia model was performed as previously described. Briefly, hearts were excised, transferred to a Langendorff apparatus, and perfused via an aortic cannula. After a 10-minute stabilization interval of normoxic perfusion, hearts were made ischemic by cessation of perfusion for 30 minutes. Hearts were then flash frozen in liquid nitrogen and stored at -80° Celsius prior to protein isolation for Western blot analysis.

**Statistical Analysis**

Data are presented as mean ± SEM and analyzed by Student's t-test. For multiple groups, one way ANOVA was performed with post-hoc Newman-Keuls test. Arrhythmia inducibility was analyzed by Fisher's exact test. P values less than 0.05 were considered statistically significant.

**References**


### Supplemental Table I

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<tr>
<td>WT, Base (n=5)</td>
<td>0.07</td>
<td>0.11</td>
<td>0.38</td>
<td>0.22</td>
<td>0.074</td>
<td>0.104</td>
<td>41.84 +/- 0.75</td>
<td>5.537 +/- 0.142</td>
</tr>
<tr>
<td>WT, TAC (n=20)</td>
<td>0.07</td>
<td>0.11</td>
<td>0.40</td>
<td>0.31</td>
<td>0.077</td>
<td>0.115</td>
<td>23.22 +/- 0.87</td>
<td>8.488 +/- 0.179</td>
</tr>
<tr>
<td>S3A, Base (n=5)</td>
<td>0.06</td>
<td>0.10</td>
<td>0.39</td>
<td>0.23</td>
<td>0.066</td>
<td>0.100</td>
<td>39.32 +/- 0.83</td>
<td>5.935 +/- 0.148</td>
</tr>
<tr>
<td>S3A, TAC (n=17)</td>
<td>0.08</td>
<td>0.12</td>
<td>0.43</td>
<td>0.33</td>
<td>0.074</td>
<td>0.108</td>
<td>22.24 +/- 1.10</td>
<td>8.661 +/- 0.294</td>
</tr>
<tr>
<td>S3E, Base (n=5)</td>
<td>0.06</td>
<td>0.10</td>
<td>0.38</td>
<td>0.22</td>
<td>0.077</td>
<td>0.117</td>
<td>42.07 +/- 2.09</td>
<td>5.845 +/- 0.056</td>
</tr>
<tr>
<td>S3E, TAC (n=16)</td>
<td>0.07</td>
<td>0.11</td>
<td>0.39</td>
<td>0.30</td>
<td>0.074</td>
<td>0.111</td>
<td>24.91 +/- 0.88</td>
<td>8.086 +/- 0.118</td>
</tr>
</tbody>
</table>

**Echocardiographic and Physiologic Comparison of WT and Mutant Mice at Baseline and Post-TAC.** LVAW, LV anterior wall; LVID, LV internal dimension; LVPW, LV posterior wall; d, diastole; s, systole.