Disulfide-activated protein kinase G Iα regulates cardiac diastolic relaxation and fine-tunes the Frank–Starling response

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The Frank–Starling mechanism allows the amount of blood entering the heart from the veins to be precisely matched with the amount pumped out to the arterial circulation. As the heart fills with blood during diastole, the myocardium is stretched and oxidants are produced. Here we show that protein kinase G Iα (PKGIα) is oxidant-activated during stretch and this form of the kinase selectively phosphorylates cardiac phospholamban Ser16—a site important for diastolic relaxation. We find that hearts of Cys42Ser PKGIα knock-in (KI) mice, which are resistant to PKGIα oxidation, have diastolic dysfunction and a diminished ability to couple ventricular filling with cardiac output on a beat-to-beat basis. Intracellular calcium dynamics of ventricular myocytes isolated from KI hearts are altered in a manner consistent with impaired relaxation and contractile function. We conclude that oxidation of PKGIα during myocardial stretch is crucial for diastolic relaxation and fine-tunes the Frank–Starling response.
Protein kinase G I\(\alpha\) (PKG\(\alpha\)) can be activated via the classical NO/cyclic guanosine monophosphate (cGMP) pathway or via a cGMP-independent pathway involving oxidants\(^{1,2}\). Reactive oxygen species (ROS) promote formation of a reversible intermolecular disulfide bond between the two subunits of the PKG\(\alpha\) homodimer at Cys42 (refs 3, 4). This redox mechanism operates in blood vessels to control vasotone and blood pressure in vivo\(^{5,6}\). However, PKG\(\alpha\) is also expressed in heart muscle where the significance of Cys42 oxidation is less clear.

The amount of blood that enters the heart is continuously changing, for example with postural alterations and breathing. As the heart fills with blood during diastolic relaxation, myocardial cells become stretched. The magnitude of stretch is proportional to the volume of blood that enters the ventricle, and the force of the subsequent contraction is proportional to the degree of stretch. This mechanism, which ensures that the amount of blood pumped out (stroke volume) is synchronized with the amount that enters (venous return), is known as the Frank–Starling or Maestrini law of the heart\(^{7–10}\). This mechanism also enables beat-to-beat matching of left ventricular to right ventricular output.

Widely accepted molecular mechanisms that contribute to the Frank–Starling response include stretch-induced alterations in myofilament overlap, myofilament Ca\(^{2+}\) sensitivity, and actin-myosin cross-bridge cycling\(^{11,12}\). Recently, it was discovered that there is an increased production of ROS during diastolic stretch, termed X-ROS signalling, which is involved in regulation of cardiac Ca\(^{2+}\) cycling\(^{13}\). Removal of cytosolic Ca\(^{2+}\) to trigger diastolic relaxation occurs predominately via the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase 2a (SERCA2a), which transfers Ca\(^{2+}\) into the lumen of the SR to be stored before the next contraction\(^{14}\). SERCA2a activity is regulated via interactions with its reversible inhibitor phospholamban (PLN); when PLN is phosphorylated at Ser16, its inhibitory action on SERCA2a is relieved and Ca\(^{2+}\) sequestration into the SR is increased. Myocardial relaxation is potentiated which enhances filling of the heart\(^{15,16}\).

Here we identify PLN Ser16 as a direct target of disulfide PKG\(\alpha\) in the heart by an unbiased chemical genetic phosphoproteomic experiment utilizing analogue-sensitive PKG\(\alpha\) mutants\(^{17}\). We investigate the functional significance of disulfide PKG\(\alpha\)-dependent phosphorylation of PLN using a Cys42Ser PKG\(\alpha\) knock-in (KI) transgenic mouse and find that PKG\(\alpha\) oxidation occurs during stretch to contribute to the Frank–Starling response and is a key determinant of cardiac output.

### Results

**Disulfide-activated PKG\(\alpha\) phosphorylates PLN at Ser16.** A chemical genetic phosphoproteomic method utilizing analogue-sensitive PKG\(\alpha\) mutants was performed to identify direct cardiac substrates of PKG\(\alpha\) (ref. 18). Phosphopeptide abundance—directly relating to the amount of substrate phosphorylation—was determined by a label-free quantitative analysis\(^{19,20}\) (Supplementary Dataset 1). Substrate phosphorylation was assessed when PKG\(\alpha\) was activated by the Cys42 disulfide bond or via the classical pathway with cGMP, and compared with phosphorylation when PKG\(\alpha\) was in its basal ‘unactivated’ state (control; Fig. 1a). Eighty five direct substrates of PKG\(\alpha\) were identified and the 29 substrates that had a statistically significant change in phosphorylation upon activation of the kinase are listed in Table 1. Phosphorylation of 28 of these proteins was significantly increased when PKG\(\alpha\) was activated by cGMP. Intriguingly, the abundance of a phosphopeptide from the cardiac protein phospholamban (PLN pSer16; RApStIEMPQQAR) was significantly increased relative to control when PKG\(\alpha\) was activated by Cys42 oxidation rather than by cyclic nucleotide binding, indicating that PLN Ser16 is a selective target of disulfide PKG\(\alpha\).

We compared basal phosphorylation of PLN Ser16 in isolated, buffer-perfused hearts from C42S PKG\(\alpha\) KI mutant mice (which cannot form the activating intermolecular disulfide bond) to Ser16 phosphorylation in wild-type (WT) hearts (Fig. 1b). PLN Ser16 phosphorylation was significantly lower in the KI hearts, consistent with the proteomic evidence that PLN is a substrate of disulfide-activated PKG\(\alpha\). We observed no change in phosphorylation of PLN Thr17 in the KI tissue suggesting that disulfide PKG\(\alpha\) is highly selective for Ser16. As well as Ser16 phosphorylation, the oligomeric state of PLN was altered in the myocardiun of the KI, as indicated by a three-fold increase in the pentamer/monomer ratio of total PLN in samples that were not boiled before western blotting.

Given that PLN plays a central role in cardiac excitation–contraction (EC) coupling and Ca\(^{2+}\) homeostasis, we analysed several other key proteins involved in these processes to determine whether their expression or phosphorylation status was altered in the KI myocardium (Fig. 1c). However, we observed no changes for any of the indices measured, including cardiac troponin I (cTnI) Ser22/23, cardiac myosin binding protein C (cMyBP-C) Ser282, ryanoide receptor 2 (RyR2) Ser2808, phospholemman (FXDY1) Ser63, Ser68 and Ser69, myosin light chain 2 (MLC2) Ser19, heavy chain cardiac myosin, slow myosin heavy chain and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK2-\(\beta\)γ/δ) Thr282.

**Impaired Frank–Starling mechanism in C42S PKG\(\alpha\) hearts.** To investigate the functional significance of oxidized PKG\(\alpha\)-dependent phosphorylation of PLN, we began by assessing the Frank–Starling relationship of perfused ex vivo hearts from WT or C42S PKG\(\alpha\) KI mice. The systolic pressure (SP), rate of contraction (+dp/dt), and rate of relaxation (−dp/dt), were monitored as the end-diastolic pressure (EDP), that is, cardiac preload, was sequentially increased. The KI hearts displayed a markedly different Frank–Starling profile from that of the WT hearts (Fig. 2a). A statistically significant elevation of EDP was required for the KI hearts to achieve the same SP as the WTs. For example, at an EDP of 4 mm Hg the WT heart generated a SP of ~60 mm Hg, whereas the KI only generated a SP of ~30 mm Hg. Furthermore, the KI hearts had significantly slower rates of contraction and relaxation than the WT at a given EDP.

**Effect of stretch on PLN Ser16 phosphorylation.** We assessed the effect that EDP had on PKG\(\alpha\) oxidation state and PLN Ser16 phosphorylation in WT and KI ex vivo hearts by Western blot. Increasing EDP from 0 mm Hg to 5 mm Hg, thus increasing diastolic stretch, significantly increased oxidation of PKG\(\alpha\) to the disulfide dimer in WT hearts (Fig. 2b). As expected, this oxidation event was absent in hearts that harboured the PKG\(\alpha\) C42S mutation. An increase in EDP was also associated with a significant elevation in PLN Ser16 phosphorylation in WT myocardium, whereas Ser16 phosphorylation in the C42S mutant tissue was unchanged (Fig. 2c).

Subcellular fractionation of myocardial tissue from the WT and KI mice was also performed to see if increased stretch was associated with translocation of PKG\(\alpha\). Indeed, we observed a statistically significant increase in the amount of WT PKG\(\alpha\) in the particulate fraction—where the SR is enriched and PLN and SERCA2a are located (Fig. 2d). However, the amount of C42S PKG\(\alpha\) in the SR-enriched fraction from KI heart tissue did not change. This observation is consistent with the
phosphoproteomic data which revealed that disulfide PKGIz directly interacts with PLN.

### Oxidized PKGIz binds to the cytoplasmic domain of PLN.

To explore the PLN-PKGIz interaction further we carried out isothermal titration calorimetry (ITC), titrating the cytoplasmic domain of PLN (residues 1–23; PLN$_{1-23}$) against the oxidized (WT) and reduced (C42S mutant) forms of PKGIz. A sigmoidal binding isotherm was fitted to the integrated titration data for oxidized PKGIz which is consistent with one PKGIz disulfide dimer binding to one PLN peptide with a $K_d$ of $\sim 7$ µM (Fig. 2e). In contrast, integrated heats for the mutant kinase, recorded under the same experimental conditions, could not be fitted to a sigmoid-shaped binding curve, therefore a dissociation constant for the C42S PKGIz-PLN$_{1-23}$ complex could not be derived from our experiments. Although the ITC data here does not exclude the possibility of an interaction between mutant PKGIz and PLN, it does suggest that the interaction between reduced, unactivated PKGIz and PLN is markedly weaker than the interaction between oxidant-activated PKGIz and PLN. Using the MicroCal isotherm simulation tool, we estimated that the $K_d$ for the mutant kinase is at least five-fold higher than the $K_d$ for WT PKGIz disulfide dimer.

### Ca$^{2+}$ handling in myocytes from C42S PKGIz KI hearts.

Experiments were performed in ventricular myocytes isolated from adult WT or C42S PKGIz KI hearts, comparing intracellular calcium ([Ca$^{2+}$]) dynamics between genotypes. Specimen transients (Fig. 3a) are clearly consistent with significantly altered Ca$^{2+}$ handling in the cells from KI animals. Quantitative analysis of the transients showed the KI was significantly deficient in their systolic [Ca$^{2+}$], transient and SR Ca$^{2+}$ content evoked by application of caffeine, whereas the diastolic [Ca$^{2+}$] concentration was the same between genotypes (Fig. 3b–d). Normalization of the [Ca$^{2+}$] transients allowed direct comparison of their decay phase (indicative of SERCA2a activity) between genotypes (Fig. 3e). The dashed lines show single exponential fits which were used to determine the rate constants for the decay of...
[Ca^{2+}]_o, which was significantly slower by ~50% in cells from KI mice (Fig. 3f).

Diastolic relaxation in C42S PKGIZ mutant mice in vivo. We performed a comprehensive echocardiography study to assess the myocardial contractile function of WT and KI mice in vivo. The complete list of measurements is given in Supplementary Table 1. Importantly, the KI, which is resistant to PKGIZ oxidative activation, had a significant decrease in the transmural early (E) to late (A) peak flow velocity wave ratio compared with WT, indicating impaired diastolic relaxation (Fig. 4a)\(^2\). The mitral annulus early diastole tissue motion (E) to mitral annulus late diastole tissue motion (A) wave ratio was also decreased in the KI, providing further evidence for abnormal myocardial relaxation when the Cys42 PKGIZ disulfide bond cannot form.

In vivo cardiac performance was further assessed by analysis of pressure-volume (PV) loops obtained from a catheter inserted in the left ventricle (LV) of the WT and KI mice; the complete list of measurements can be found in Supplementary Table 2. The KI hearts had significantly increased EDPs and were slower in both their contraction and relaxation rates (Fig. 4b). Furthermore, the reduced end-systolic pressure-volume relationship in the KI reveals a deficiency in contractile performance, and the elevated end-diastolic pressure-volume relationship indicates that the ventricle of the KI is stiffer. Compelling evidence for diastolic impairment in the KI is further provided by an elevated isovolumic relaxation constant, Tau, which is a preload-independent measure of diastolic function; a higher value indicates slower relaxation\(^2\). The Frank-Starling mechanism in C42S PKGIZ mice in vivo. Based on the data described so far, we concluded that oxidation of PKGIZ is an important mechanism for obtaining the appropriate degree of filling during the relaxation phase of the cardiac cycle. According to the Frank-Starling law—which couples end-diastolic volume (EDV) to cardiac output on a beat-to-beat basis—impaired filling in the KI should attenuate the force of the following contraction. To test this hypothesis, we varied the preload (EDV) of WT and KI mice by mechanical occlusion of the following contraction. To test this hypothesis, we varied the preload (EDV) of WT and KI mice by mechanical occlusion of the venae cava and recorded high-resolution PV data. Consequently, EDP, EDV, SP, + dp/dt and − dp/dt could be determined for individual cardiac cycles as indicated in the representative trace in Fig. 4c. Thus, intra-beat relationships could be calculated, for example EDP versus SP, that pertained directly to the Frank-Starling response in vivo.

A representative scatter plot of SP versus EDP for an individual WT and KI mouse is shown in Fig. 4d. Two hundred heartbeats were analysed for each mouse and slopes were averaged in order to obtain the mean intra-beat relationships for EDP versus SP, EDP versus the rate of contraction, and EDP versus the rate of relaxation. On average there was a ~14 mm Hg increase in SP for a 1 mm Hg change in EDP in WT, while there was only a ~10 mm Hg increase in SP per unit change in EDP in the KI. The intra-beat relationships between EDP and + dp/dt and − dp/dt were also significantly decreased in the KI. Additionally, the spread of data was strikingly greater in the scatter plots of EDP versus SP for the KI mice. Hence, we calculated the mean coefficient of determination (R^2) for each intra-beat relationship to assess quantitatively the variability of the data, thus providing a measure of how tightly linked EDP and cardiac output were in the KI mice compared with WT. R^2 for each relationship was

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**Table 1 | Direct substrates of PKGIZ identified by a quantitative phosphoproteomic screen.**

| UniProt ID | Protein Description | Phospho site | Log2 fold change |
|-----------|---------------------|--------------|-----------------|
| P61016    | Cardiac phospholamban | S16          | −0.81           |
| P01086    | 1-phosphatidylinositol 4,5-bisphosphate phosphodiester gamma-1 | S1233 | 4.50* |
| P3083      | 6-phosphofructokinase, liver type | S775 | 4.15* |
| Q99068    | Alpha-2-macroglobulin receptor-associated protein | S245 | 2.64* |
| P27653    | C-1-tetrahydrofolate synthase, cytoplasmic | T545 | 3.68* |
| Q55156    | CAP-Gly domain-containing linker protein 2 | S353 | 2.05* |
| Q991D4    | CLP-associating protein 2 | S436 | 1.79* |
| Q991X8    | Cytoplasmic dynein 1 light intermediate chain 1 | T408 | 3.76* |
| Q991X8    | Cytoplasmic dynein 1 light intermediate chain 1 | S412 | 4.15* |
| FILP64    | E3 ubiquitin-protein ligase TRIP12 | S1073 | 1.63* |
| Q99080    | G-protein-signaling modulator 1 | S567 | 2.93* |
| P97541    | Heat shock protein beta-6 | S16 | 2.61* |
| P15865    | Histone H1.4 | S36 | 1.30* |
| D328N0    | Histone H1.5 | T35 | 3.90* |
| P62804    | Histone H4 | S48 | 2.70* |
| P555E0    | Leucine-rich PPR motif-containing protein, mitochondrial | S656 | 4.57* |
| P43244    | Matrin-3 | T150 | 3.58* |
| P39426    | Microtubule-associated protein 1A | S460 | 2.41* |
| Q537W5    | Microtubule-associated protein 4 | T899 | 3.69* |
| P19332    | Microtubule-associated protein tau | S525 | 3.90* |
| Q522R4    | Mitochondrial ribonuclease P protein 1 | T377 | 1.98* |
| E9PT78    | Myosin light chain kinase 3 | S155 | 3.16* |
| P18437    | Non-histone chromosomal protein HMG-17 | S29 | 3.65* |
| P53125    | Polymerase I and transcript release factor | T304 | 3.05* |
| P53125    | Polymerase I and transcript release factor | S302 | 3.16* |
| Q8BVU2    | Protein NDRG2 | S332 | 3.42* |
| Q6345    | Protein SET | S7 | 3.80* |
| Q658B7    | Trifunctional enzyme subunit beta, mitochondrial | S198 | 3.44* |
| P23693    | Troponin I, cardiac muscle | S167 | 3.49* |

All proteins that displayed a statistically significant log2 fold change in phosphorylation upon PKGIZ activation by cGMP or Cys42 disulfide bond are listed (*P<0.05, Dunnett’s test; n=4).
under the same experimental conditions. The cytoplasmic domain of PLN, this is markedly weaker than for oxidized PKGI disulfide-activated PKGI bars show s.e.m. and R much more variable as illustrated by significantly decreased contraction rates were diminished. Additionally, the data were positive relationships between EDV and the relaxation and as much SP as WT hearts per unit change in EDV and the significant increase in the amount of PKGI is, stretch, according to the Frank–Starling law. However, the responses were significantly reduced in the KI hearts compared with WT (* P<0.05; n = 8). The hearts of C42S PKGI between EDP and contractile function was distinctly impaired in hearts but not in the KI (* P<0.05; n = 5). (e) Phosphorylation of PLN Ser16 was also significantly increased in the WT but not KI hearts with increased diastolic stretch (* P<0.05; n = 5). (d) Subcellular fractionation of WT and KI hearts perfused at different EDPs followed by immunoblotting revealed a significant increase in the amount of PKGI in the particulate fraction from stretched WT myocardium compared with the particulate fraction from unstretched WT myocardium (* P<0.05; n = 5). No stretch-dependent changes in PKGI abundance were observed in fractions from the KI tissue. Error bars show s.e.m. and P values were determined by t-test. (e) ITC analysis of the interaction between the cytoplasmic domain of PLN (residues 1–23) with disulfide-activated PKGI and the C42S mutant. A sigmoidal binding isotherm can be fitted to the titration data for oxidized PKGI which is consistent with one PKGI disulfide dimer binding to one PLN peptide with a Kd of ~7μM. Although the ITC data for C42S PKGI also suggests a direct interaction with the cytoplasmic domain of PLN, this is markedly weaker than for oxidized PKGI as the integrated data cannot be fitted to a sigmoid-shaped binding curve under the same experimental conditions.

Figure 2 | Isolated hearts from C42S PKGI KI mice have impaired Frank-Starling responses. (a) Curves showing the variation in SP, rate of contraction (+ dp/dt), and rate of relaxation (− dp/dt) as a function of EDP for Langendorff-perfused WT and KI hearts. Cardiac performance increased with EDP, that is, stretch, according to the Frank-Starling law. However, the responses were significantly reduced in the KI hearts compared with WT (P<0.05; n = 8). (b) Immunoblotting showed that oxidation of PKGI to the disulfide dimer increased with increasing stretch (from 0 mm Hg to 5 mm Hg EDP) in the WT hearts but not in the KI (* P<0.05; n = 5). (c) Phosphorylation of PLN Ser16 was also significantly increased in the WT but not KI hearts with increased diastolic stretch (* P<0.05; n = 5). (d) Subcellular fractionation of WT and KI hearts perfused at different EDPs followed by immunoblotting revealed a significant increase in the amount of PKGI in the particulate fraction from stretched WT myocardium compared with the particulate fraction from unstretched WT myocardium (* P<0.05; n = 5). No stretch-dependent changes in PKGI abundance were observed in fractions from the KI tissue. Error bars show s.e.m. and P values were determined by t-test. (e) ITC analysis of the interaction between the cytoplasmic domain of PLN (residues 1–23) with disulfide-activated PKGI and the C42S mutant. A sigmoidal binding isotherm can be fitted to the titration data for oxidized PKGI which is consistent with one PKGI disulfide dimer binding to one PLN peptide with a Kd of ~7μM. Although the ITC data for C42S PKGI also suggests a direct interaction with the cytoplasmic domain of PLN, this is markedly weaker than for oxidized PKGI as the integrated data cannot be fitted to a sigmoid-shaped binding curve under the same experimental conditions.

significantly reduced in the KI mice, indicating that coupling between EDP and contractile function was distinctly impaired in the hearts of C42S PKGI KI mice. The intra-beat relationships for EDV versus SP, − dP/dt and + dP/dt were also determined and the findings mirrored those reported for EDP (Fig. 4e). Namely, the KI hearts did not develop as much SP as WT hearts per unit change in EDP and the positive relationships between EDV and the relaxation and contraction rates were diminished. Additionally, the data were much more variable as illustrated by significantly decreased R² values. EDV provides a surrogate index of myocardial stretch during diastole; thus the intra-beat relationships of SP and + dP/dt versus EDV can be used as an in vivo readout of the Frank–Starling response. This data therefore provides further evidence that the C42S PKGI KI mouse has a robust impairment in its ability to fully invoke the Frank–Starling mechanism.

Force generated by the heart in C42S PKGI KI mice. We hypothesized that disconnection between diastolic filling and cardiac output in the KI would result in a more variable developed pressure (that is SP–EDP). Thus, we derived the variance in the developed pressure amplitude over 1,000 consecutive heartbeats for mice of each genotype from PV data obtained with a catheter inserted in the LV. Indeed, the developed pressure—the force generated with each heartbeat—was ~ three-fold more variable in the KI (Fig. 4f). We performed a similar analysis on radiotelemetry data collected from conscious, freely moving WT and KI mice that had a pressure catheter inserted into the aorta. Similar to the data obtained with the PV catheter, the aortic pulse pressure of the KI was ~ 2.2-fold more variable than that of the WT. These results further corroborate the importance of the PKGI redox control mechanism in regulation of cardiac output.

Discussion PKGIz activity can be stimulated by reversible oxidation of Cys42 or by cGMP binding1,2. cGMP inhibits formation of the Cys42 intermolecular disulfide bond3,4 and, similarly, oxidation has been shown to attenuate cGMP-dependent PKGIz substrate phosphorylation23, consistent with discrete PKGIz signalling.
were determined by Ca²⁺ (ventricular cardiomyocytes. The Cys42 intermolecular disulfide of PKGI pathways that diverge depending on the stimulus. To investigate PKGI substrates using a chemical genetics phosphoproteomics approach. The majority of proteins we identified, including the known PKGI target heat shock protein beta 6 Ser16 (HspB6) (ref. 24), were reproducibly phosphorylated by cGMP-activated kinase. However, the crucial cardiac protein PLN was selectively and reproducibly phosphorylated at Ser16 by disulfide-activated PKGI—not by cyclic nucleotide-activated kinase. This observation is in agreement with studies showing PKG can phosphorylate PLN (ref. 25), but this is not induced by stimuli that increase intracellular cGMP in cardiac muscle.

PKG isoforms are known to target binding partners via their N-terminal leucine zipper domains where the differing patterns of surface charge are important for regulating the interactions. The Cys42 intermolecular disulfide of PKGI is located within the leucine zipper domain and is likely to alter the physicochemical properties of this region. Depending on the substrate, formation of the Cys42 disulfide bond will promote or inhibit kinase-substrate interactions, resulting in an increase or decrease in phosphorylation regardless of cGMP binding. Indeed, we found by ITC that disulfide-activation of PKGIz, in the absence of cyclic nucleotide, increased its affinity for the cytoplasmic domain of PLN at least five-fold. Analysis of myocardium from WT or C42S PKGIz KI mice—which cannot form the activating disulfide bond—revealed a significant deficit in PLN Ser16 phosphorylation in the mutant tissue, consistent with our biochemical data. The reduced phosphorylation of PLN Ser16 produces substantial alterations in Ca²⁺ handling. PLN binds to SERCA2a, the pump that mediates Ca²⁺ reuptake into the SR, and reduces its activity. Therefore, SERCA2a activity influences the rate of decay of the Ca²⁺ transient and the amount of Ca²⁺ stored in the SR (SR Ca²⁺ content). The latter controls the amplitude of the Ca²⁺ transient, therefore SERCA2a activity also controls the amplitude of the Ca²⁺ transient. PLN Ser16 phosphorylation reduces inhibition of SERCA2a resulting in increased activity, hastening the rate of decay of the Ca²⁺ transient, increasing both the SR Ca²⁺ content and the Ca²⁺ transient amplitude. On the basis of these considerations, it is not surprising to find that the lower levels of PLN Ser16 phosphorylation observed in the C42S PKGIz KI are associated with a substantial reduction in the rate of decay of the Ca²⁺ transient, the SR Ca²⁺ content, as well as the Ca²⁺ transient amplitude. PLN can be phosphorylated at Ser16 by cAMP-dependent protein kinase (PKA) following adrenergic stimulation. In addition, phosphorylation of Thr17 by CaMKII also increases SERCA2a activity. The main physiological function of CaMKII-mediated phosphorylation is to adapt SERCA2a function to increases in heart rate. Identification of PLN Ser16 as a selective target of oxidized PKGIz raised a question about the functional significance and role of this phosphorylation event. We reasoned that oxidants produced during diastolic stretch (X-ROS signals) may trigger disulfide-activation of PKGIz and subsequent phosphorylation of PLN Ser16. This stretch-dependent myocardial oxidant signalling should be deficient in the hearts of the KI mice, because of the inability of C42S PKGIz to transduce X-ROS signals into Ser16 PLN phosphorylation via the disulfide activation pathway. Indeed, we observed that cardiac stretch promoted oxidation of PKGIz in WT hearts, and was associated with translocation of the kinase to the SR fraction, and an increase in PLN Ser16 phosphorylation. These events were absent in the KI hearts, as hypothesized. On the basis of these observations, we speculated that the relationship between stretch and systolic contraction, that is, the Frank-Starling mechanism, might be impaired in the KI hearts. Indeed, contractile responses were markedly impaired in isolated KI hearts compared with WT as EDSP (stretch) was progressively increased up to 10 mm Hg. The reduction in contractile responses is due both to impaired systolic function secondary to smaller Ca²⁺ transients because of the lower SR Ca²⁺ content, as well as diastolic dysfunction secondary to the slower Ca²⁺ transient decay, which will impair cardiac filling. These observations clearly delineate a novel role for the oxidized PKGIz-PLN-SERCA2a axis in the modulation of the Frank-Starling mechanism, which traditionally has been attributed to modulation of myofilament properties. At this point it is important to highlight an apparent discrepancy in our data. The observation that the systolic Ca²⁺ transient was substantially lower in the KI cardiomyocytes compared with WT would be anticipated to manifest itself as attenuated cardiac systolic force development in isolated heart preparations. However, this is not the case, as left ventricular systolic output measured by a PV catheter is identical between genotypes. This potential discrepancy can be fully reconciled by the fact that the KI is able to achieve the same
systolic output as the WT in vivo at the cost of a sustained elevation in EDP. Similarly, isolated KI hearts can generate the same systolic pressure as the WT, but again this is only achieved by increasing their EDP above that required in WT. Thus at an EDP of 4 mmHg KI hearts only generate ~50% of the systolic output of WT, matching very well the proportional deficit in the systolic Ca\(^{2+}\) amplitude measured in isolated unloaded cardiomyocytes. Essentially, the alterations to Ca\(^{2+}\) cycling observed in the KI reflect the inability to engage the Frank-Starling mechanism as effectively as WT myocardium.

At very high EDP the preload will overcome the diastolic dysfunction and it is conceivable that additional mechanisms, not defined here, involving cardiac myofilaments or SR Ca\(^{2+}\) load adaptation, participate to normalize systolic function.

We observed an increase in the pentamer/monomer ratio of PLN in the KI myocardium compared with the WT, which may contribute to decreased diastolic relaxation and impaired myocardial relaxation in the KI. We observed an increase in the pentamer/monomer ratio of PLN in the KI myocardium compared with the WT, which may contribute to decreased diastolic relaxation and impaired myocardial relaxation in the KI. However, it is difficult to make firm conclusions about the significance of this observation, as there is evidence for refs 16, 30, as well as against ref. 31, oligomerization mediating activation of SERCA2a. We should...
also consider that there are other regulatory mechanisms that participate in the control of SERCA2a activity, such as dephosphorylation of PLN by protein phosphatase 1, and by differential interactions with SR membrane proteins including sarcolin and DWORF (refs 32–34).

An in vivo comparison of cardiac function in the WT and KI mice by echocardiography and ventricular PV loop analysis revealed diastolic dysfunction in the KI, consistent with the ex vivo heart preparations and intracellular Ca\(^{2+}\) measurements. Taken together, our data supports a role for the PKG1z Cys42 disulfide bond in stretch-induced enhancement of myocardial relaxation to obtain the appropriate amount of ventricular filling during diastole (Fig. 4g). Analysis of high-resolution PV data from the WT and KI mice showed that as preload increased, the SP and rate of contraction for the next beat increased, as anticipated due to the Frank–Starling law of the heart. However, not only was the intra-beat relationship between preload and contractility diminished in the KI mice, systolic pressures and contraction rates were also more random for a given EDV. As a result, the pulse pressure was more variable in the KI. We conclude that oxidation of PKG1z is involved in coupling ventricular filling with cardiac output on a beat-to-beat basis, that is, it contributes to the Frank–Starling mechanism.

Deficiencies in the cardiac response to preload in the C42S PKG1z KI mice are due, at least in part, to insufficient phosphorylation of PLN Ser16 during ventricular filling. Removal of cytosolic Ca\(^{2+}\) is therefore slower, which means that the myocardium cannot relax properly and a reduced SR Ca\(^{2+}\) load also leads to diminished contractility\(^{35}\). Passive tension is also likely to play a role in the diminished Frank–Starling responses of the KI hearts due to increased interactions of the giant elastic protein titin with Ca\(^{2+}\). Ca\(^{2+}\) binding to titin is known to increase passive tension of the myocardium, making the ventricle stiffer and thus harder to fill\(^{32}\). Furthermore, because impaired relaxation results in inadequate extension of the sarcomeres, the myocardial Ca\(^{2+}\) sensitivity—which is dependent on sarcomere length\(^{11,12,29}\)—will be reduced in the KI cardiomyocytes and also contribute to the decreased contractility observed in the KI hearts\(^{11,12,36}\).

Other molecular mechanisms that facilitate Frank–Starling responses may also be altered in the absence of the PKG1z oxidation pathway. For example, phosphorylation of the sarcomeric proteins cTnI and cMyBP-C is involved in length-dependent activation of myofilaments\(^{37}\). However, we did not observe changes in phosphorylation of key residues in these proteins in the KI hearts. Basal phosphorylation of several other phosphosites involved in cardiac EC coupling and Ca\(^{2+}\) handling including phospholemman Ser68 and CaMK2 Thr282 was also unchanged in the KI hearts, suggesting that these residues are not central to the X-ROS/PKG1z oxidation/enhanced myocardial relaxation pathway. S-nitrosylation of PLN is another modification that has been shown to modulate the Frank–Starling mechanism and it is possible that this modification has a role in X-ROS signalling\(^{38}\). However, we cannot envisage how the PKG1z Cys42Ser mutation could affect PLN S-nitrosylation and it is unlikely that this redox modification contributes to the diminished Frank–Starling mechanism observed in the KI mice.

In conclusion, fundamental to the Frank–Starling law of the heart is an initiating diastolic stretch which induces events that result in a systolic contraction of appropriate force. Here, as summarized in Fig. 4g, we show that this crucial relaxation step is significantly mediated by oxidative activation of PKG1z which phosphorylates phospholamban to enhance diastolic relaxation. Furthermore, in the absence of this redox control mechanism, as is the case in the KI, the pressure amplitude the heart generates from beat-to-beat is erratic.

Methods

**Animals and tissues.** All procedures were performed in accordance with the United Kingdom Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. The KI mice constitutively expressing PKG1z C42S were generated on a pure C57BL/6 background by TaconicArtemis (Germany) as described previously\(^5\). All mice used in this study were male and age and body weight–matched.

**Method for identification of direct substrates of PKG1z.** We employed a chemical genetic method\(^{10,39}\) to identify direct substrates of cGMP-activated PKG1z and disulfide-activated PKG1z in heart tissue. This approach involves mutation of the ATP binding-site of the kinase so that it can accept a ‘bulky’ N6-alkylated ATP analogue for example, N6- phenethyl ATP. An oxygen atom on the γ-phosphate is also replaced with a sulfur atom—giving an N6-alkylated ATP’s analogue—so that the mutant kinase catalyses the transfer of a γ-phosphate group (\(\text{PO}_{4}\)) to its substrates instead of a phosphate. The thiophosphate group is nucleophilic, providing a basis for substrates of the analogue-sensitive kinase to be purified by a ‘covalent capture’ protocol. LC–MS/MS allows identification of the substrate and localization of the phosphorylation site. Thiophosphorylated proteins can also be detected with a specific antibody\(^39\). Analogue-sensitive mutants of PKG1z were confirmed using the Omnia kinase assay kit (ThermoFisher). Detailed protocols are given below.

**Recombinant WT and analogue-sensitive PKG1z mutants.** A pCDNA3 expression vector encoding human FLAG-tagged PKG1z (ref. 41) underwent site-directed mutagenesis to generate constructs for untagged WT, C42S, M438G and C42S/M438G PKG1z. Mutations were introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer’s instructions. Expression and purification of the PKG1z mutants were performed according to a published method\(^{32}\) as follows: suspension of expression FrexStyle 293-F cells (ThermoFisher Scientific) were transfected with the appropriate PKG1z construct using the stable cationic polymer polyethyleneimine (PEI) as a transfection reagent\(^36\). After ~72 h cells were harvested by centrifugation (400g; room temperature; 15 min), re-suspended in ice cold lysis buffer (25 mM 4-(2-aminoethyl)-3-[3-(4-morpholinyl)-propyl]-carbodiimide (EDTA); 100 mM NaCl; 10 mM benzamidine hydrochloride; and 10 mM diethiothreitol (DTT)) and frozen in liquid N\(_2\). Cells were lysed by three freeze (liquid N\(_2\))-thaw (37°C) cycles and the lysate was clarified by centrifugation at 140,000g and 4°C for 30 min. The soluble protein fraction was loaded onto a pre-equilibrated 5–20% (2–aminoethylamino)lactamose (SEPHACRYL S-300) column (8–AEA–AM) agarose; BioLog, Germany) followed by washing with 20 column volumes of lysis buffer. The column was further washed with lysis buffer + 3 M NaCl (5 column volumes) and PKG1z was then eluted with lysis buffer + 150 mM NaCl and 500 μM cAMP. Removal of cAMP and buffer exchange was achieved by dialysis against 25 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 0.1% Tween 20 and EDTA-free protease inhibitor cocktail solution (Merck Millipore). DTT was absent in buffer so that the activating disulfide bond would not be reduced and so that the PKG1z M438G mutant would be oxidized to disulfide dimer in the presence of air (confirmed by SDS-PAGE). Protein concentration was determined by Pierce BCA assay (ThermoFisher) and enzyme activity was confirmed using the Omnia kinase assay kit (ThermoFisher).

**Thiophosphorylation of PKG1z Substrates in Heart Homogenate.** Male Wistar rats (9–10 weeks; body weight 300–330 g) were euthanized by intraperitoneal injection of sodium pentobarbionate (200 mg kg\(^{-1}\)) with heparin (500 US units). Hearts were flushed in the chest with ice cold Krebs buffer and the left ventricle was excised and immediately transferred to ice cold homogenization buffer (2 ml g\(^{-1}\) heart: 0.4 mM sodium phosphate buffer pH 7.4, 150 mM NaCl, 0.1% Tween 20 and EDTA-free protease inhibitor cocktail (Roche)). Tissue was homogenized with a Ystral homogenizer and the homogenate was clarified by centrifugation at 50,000g and 4°C for 30 min. The protein concentration of the soluble fraction was determined by BCA assay and then adjusted to 20 mg ml\(^{-1}\). Four thiophosphorylation reactions were set up: (1) with cGMP-activated PKG1z, (2) with disulfide-activated PKG1z M438G, (3) with unactivated, basal, PKG1z C42S/M438G and (4) a ‘no kinase’ control. The total reaction volume was 200 μl and the mixtures consisted of 25 mM Tris pH 7.5; 10 mM MgCl\(_2\); 100 μM cGMP (added to the cGMP-activated PKG1z reaction only); 0.4 mM ATP; 6 mM GTP; 1 mM N6-phenethyladenosine 5’-O-(3-thiophosphate) (6-Fu-ATP-Y-S; BioLog, Germany); 100 μl of heart homogenate (soluble fraction) and 20 μg of the appropriate analogue-sensitive PKG1z (not added to the
control reaction). The kinase reactions proceeded for 1 h at 30 °C, after that time they were quenched by addition of 220 mM EDTA, 10 μl of each mixture was taken and further processed with 10 mM p-nitrophenyl phosphate (PNP; Abcam) to check the reaction by Western blot using the anti-phosphothesate ester antibody (51-8) (ab92570; Abcam; working concentration of 1:5,000 and secondary antibody used at 1:10,000). The protocol was repeated four times; each time with a fresh rat heart (that is, four biological replicates; 16 kinase reactions in total).

Samples were frozen in liquid N2 and stored at −80 °C until the ‘covalent capture’ procedure.

Covalent capture of thiophosphorylated peptides. Thiophosphorylated peptides were isolated and converted to phosphopeptides for analysis by LC–MS/MS according to a published method30 as follows: proteins were denatured by the addition of 120 mg solid urea (80% w/v) and 10 mM tris(2-carboxyethyl) phosphine (TCEP), with incubation at 55 °C for 1 h. Samples were diluted 2 × with MS buffer A (H2O and 0.1% (v/v) formic acid) at a flow rate of 25 nMl per min. The MS buffer was added with 10 mM p-nitrobenzyl mesylate (PNBM; Abcam) to the lock mass option was enabled using the polydimethylcyclosiloxane ion (m/z 97.97, 48.985 and 32.65667 was enabled. Target ions selected for CID. For phosphopeptide analysis, multi-stage activation for

Isothermal titration calorimetry. ITC experiments were carried out on a high sensitivity MicroCal iTC200 microcalorimeter (Malvern Instruments, UK). A synthetic N-terminally acetylated peptide corresponding to the N-terminal cytosolic domain of human phospholamban, PLN (1–23) (700 μM) were titrated into the reaction cell containing WT disulfide PKG1 or the C42S mutant (70 μM) at 150 or 180 s intervals. Integrated heat data was fitted to a theoretical titration curve using a nonlinear least-squares minimization algorithm in the Microcal-Origin 7.0 software package as previously described.

Isolated cardiomyocyte Ca2+ measurements. Ventricular myocytes were isolated from 3 to 4-month-old C57B6 PKG1−/− mice and their WT littermates using an enzymatic digestion technique as described previously44,45 as dissolution of dicarboxylic acids and hearts excised and placed in ice-cold isolation solution containing (in mM) NaCl, 134; HEPES, 10; Glucose, 11.1; NaH2PO4, 1.2; MgSO4, 1.2 and Taurine, 50; KI mice and their WT littermates using

To measure cytosolic Ca2+ content was estimated from the amplitude of the Ca2+ transient levels, rate of decay of the Ca2+ transient and SR Ca2+ content48. SR Ca2+ content was estimated from the amplitude of the Ca2+ transient evoked by application of 10 mM caffeine.
Echocardiography. Mice were anesthetized and examined by echocardiography using a high-resolution Vevo 770 echocardiography system (VisualSonics) with a RMV-707B transducer operating at 30 MHz. High-resolution images were obtained for offline measurements with Vevo Software (VisualSonics). For the assessment of diastolic function, an apical four-chamber view was acquired by positioning the transducer as parallel to the mitral inflow as possible. Tissue motion velocity was assessed by spectral pulsed-wave Tissue Doppler imaging, obtained from the mitral septal annulus in the parasternal short axis view. LV diastolic function was assessed by the measurement of the LV transmitral early peak flow velocity (E) to LV transmitral late peak flow velocity (A) wave ratio and mitral annulus early diastolic tissue motion (E') to mitral annulus late diastole tissue motion (A') wave ratio.

In vivo pressure-volume analysis and blood pressure analysis. Invasive pressure-volume analysis real-time pressure volume loops were obtained using the ADVantage system (Science Inc., Canada) which uses a miniature 1.2 Fr admittance catheter. In our experiments measurements of LV function were performed in the more physiological closed chest mode when a catheter was placed in LV by retrograde approach. Briefly, mice were anesthetized, right internal carotid artery was exposed and catheterized. The 1.2 Fr catheter was advanced towards the heart and inserted into the LV cavity via the aortic valve. In order to analyse the effect of preload changes, inferior vena cava occlusion was performed. Blood pressure and heart rate were assessed by telemetry in conscious mice. Mice were anesthetized with isoflurane and a TA11PA-C10 probe catheter (Data Science International) was implanted into the aortic arch via the left carotid artery. Blood pressure variability was assessed from a continuous telemetric blood pressure record made in undisturbed (telemetered) animals in a quiet room. Thousand beats were chosen for analysis in each mouse.

Statistics. All results relating to the WT and KI mice are presented as mean ± s.e. of the mean (s.e.m). Differences between groups were assessed by ANOVA followed by post-hoc t-test. Differences were considered significant at the 95% confidence level. In PV loop analysis measurements > 2 s.d.s from the mean were excluded, which resulted in one mouse per group being omitted.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Author contributions

J.S. and O.P. contributed equally to this study. J.S. designed and performed the majority of the in vitro kinase experiments, as well as the work up of samples for analysis by mass spectrometry, with assistance from E.D.M. M.R.C. performed, analysed and interpreted ITC studies with assistance from J.S. who also prepared the recombinant kinase. J.W., J.S., P.R.C. or J.F.T. designed, performed or interpreted data from the phosphoproteomics mass spectrometry analysis. O.P. designed and performed the majority of the biochemical analyses of cardiac tissue with assistance from F.C. O.P. also performed, analysed and interpreted the isolated heart and echocardiography studies. O.R. and O.P. performed the blood pressure analysis. A.B. performed the pressure-volume analysis and analysed and interpreted the data with O.P. M.J.S. and M.S.M. analysed and interpreted data from the in vivo and ex vivo cardiac function studies. K.K., N.H., D.J.G. A.G. and L.V. designed, performed or interpreted data from isolated myocyte calcium measurements. P.E. conceived and coordinated the study, designed experiments, analysed and interpreted the data. P.E. also wrote the paper with J.S. and O.P., with input or editing from all authors.

Additional information

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