Adrenergic Signaling Strengthens Cardiac Myocyte Cohesion

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Rationale: The sympathetic nervous system is a major mediator of heart function. Intercalated discs composed of desmosomes, adherens junctions, and gap junctions provide the structural backbone for coordinated contraction of cardiac myocytes.

Objective: Gap junctions dynamically remodel to adapt to sympathetic signaling. However, it is unknown whether such rapid adaption also occurs for the adhesive function provided by desmosomes and adherens junctions.

Methods and Results: Atomic force microscopy revealed that β-adrenergic signaling enhances both the number of desmoglein 2-specific interactions along cell junctions and the mean desmoglein 2-mediated binding forces, whereas N-cadherin-mediated interactions were not affected. This was accompanied by increased cell cohesion in cardiac myocyte cultures and murine heart slices. Enhanced desmoglein 2-positive contacts and increased junction length as revealed by immunofluorescence and electron microscopy reflected cAMP-induced reorganization of intercellular contacts. The mechanism underlying cAMP-mediated strengthening of desmoglein 2 binding was dependent on expression of the intercalated disc plaque protein plakoglobin (Pg) and direct phosphorylation at S665 by protein kinase A: Pg deficiency as well as overexpression of the phospho-deficient Pg-mutant S665A abrogated both cAMP-mediated junctional remodeling and increase of cohesion. Moreover, Pg knockout hearts failed to functionally adapt to adrenergic stimulation.

Conclusions: Taken together, we provide first evidence for positive adhesiotropy as a new cardiac function of sympathetic signaling. Positive adhesiotropy is dependent on Pg phosphorylation at S665 by protein kinase A. This mechanism may be of high medical relevance because loss of junctional Pg is a hallmark of arrhythmogenic cardiomyopathy. (Circ Res. 2017;120:1305-1317. DOI: 10.1161/CIRCRESAHA.116.309631.)

Key Words: cardiac function ■ cell adhesion molecule ■ desmosome cardiomyopathy ■ intercellular junction ■ sympathetic nervous system

The myocardium predominantly consists of cardiac myocytes, which require strong coupling to maintain heart function. Adjacent cardiac myocytes are linked via intercalated discs (ICDs) consisting of desmosomes, adherens junctions, and gap junctions.1 Desmosomes, together with adherens junctions, provide cardiac myocyte cohesive strength necessary for strong mechanical coupling. They consist of the transmembrane adhesion molecules desmoglein 2 (Dsg2) and desmocollin 2 (Dsc2), which are linked to the desmin intermediate filament system via the plaque proteins Pg (plakoglobin), Pkp2 (plakophilin 2), and Dp (desmoplakin).2 The adhesion molecule of the adherens junction—the classical cadherin N-cadherin (N-Cad)—is linked to the actin filament cytoskeleton via α-catenin and β-catenin.3 However, in adult myocardium, components of desmosomes and adherens junctions are predominantly intermingled in a mixed type of junction referred to as area composita.4 Gap junctions are composed of connexins, of which connixin 43 (Cx43) is most abundant. Connexins of adjacent cardiac myocytes form a pore to provide the exchange of ions and other small molecules.1 It is known that the components of the ICD act as a functional unit, and the desmosomal components are crucial for ICD integrity.1,3 For instance, desmosomal components, such as Dp, Dsg2, Pkp2, and Pg, regulate gap junction formation, anchorage, and expression.5-10 Additionally, the voltage-gated sodium channel with its subunit Na1.5 is located at the ICD, and its association with desmosomal components like Dsg2 and Pkp2 seems to be important for current density and sodium channel properties.11-13 This is reflected by the dramatic effect of mutations in ICD molecules, causing arrhythmogenic cardiomyopathy, a disease associated with arrhythmias, leading to sudden cardiac death among young people.11-13 Recent data indicate that

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In the current study, we provide first evidence that sympathetic stimulation increased cardiac myocyte cohesion via phosphorylation of plakoglobin at serine 665 by protein kinase A. Increased intermyocyte cohesion seems to be important for the positive inotropic and chronotropic effect of adrenergic stimulation. Recent data demonstrated that the β-adrenergic receptor is also located at the ICD and showed desmosomal adhesion to be crucial for sufficient adaption of cardiac myocytes to β-adrenergic signaling. Dsg2 binding is important because cardiac myocyte–specific depletion of Dsg2 or its adhesive domain caused heart failure, and interfering with Dsg2-adhesion reduced basal and cAMP-stimulated heart rate.5,15 Despite their biological significance, binding forces of adhesion molecules in ICDs, such as Dsg2, as well as their regulation, are unknown.

The sympathetic nervous system via its second messenger cAMP modulates heart function by induction of positive inotropic, lusitropic, chronotropic, dromotropic, and bathmotropic effects.16,17 Increased conduction velocity and gating function, for instance, is a result of enhanced expression of gap junction proteins driven by sympathetic signaling.18–20 Recent data demonstrated that the β-adrenergic receptor is also located at the ICD and showed desmosomal adhesion to be crucial for sufficient adaption of cardiac myocytes to β-adrenergic signaling.5 In the current study, we provide first evidence that sympathetic signaling vice versa enhances cardiac myocyte cohesion in a positive adhesiotropic manner by recruiting Dsg2 to and strengthening of ICDs, a process dependent on Pg expression and phosphorylation at serine 665 by protein kinase A (PKA).

Statistics
For comparison of 2 independent sample groups, 2-tailed unpaired Student’s t test was performed. In case of multiple group comparisons, 1-way analysis of variance followed by Bonferroni’s post hoc test was applied. Statistical significance was assumed at P<0.05. Data are expressed as mean±standard error of mean (SEM). Statistical comparisons were performed using Prism 5 (GraphPad Software, La Jolla, CA).

Results
Dsg2-Mediated Binding Events Are Enhanced at Cell–Cell Contacts in Response to β-Adrenergic Signaling
Atomic force microscopy (AFM) was applied to simultaneously study the topography of living cardiac myocytes, the distribution of transmembrane adhesion molecules, and their binding properties.21,22 Recombinant Dsg2- or N-Cad-Fc chimeras5,23 were linked to AFM probes to investigate the
distribution of the desmosomal and classical cadherins on living cells of the murine cardiac myocyte cell line HL-1. A functionalized tip of a flexible AFM cantilever is repeatedly approached to and indented into the cell surface and retracted again (Figure 1A, left). Binding events between cellular adhesion molecules and the tip-linked proteins are detectable by monitoring the deflection of the cantilever with measurement of the force required to rupture these interactions (referred to as unbinding force; Figure 1A, right). For adhesion measurements, a region of 1.25 μm × 5 μm containing 16×64 measurement points was selected bridging the border of 2 adjacent cells (Figure 1B). Under baseline conditions, a homogenous

Figure 1. β-Adrenergic signaling results in redistribution of desmoglein 2 (Dsg2)-mediated binding events to cell borders. A, Schematic of atomic force microscopy (AFM) setup with functionalized cantilever (left). Right, Representative force–distance curves with (black plot, asterisk in [II]) and without (gray plot) binding event. Please see Online Data Supplement for more detail. B, AFM topography image (right) selected from the brightfield image (green box in left, dashed lines denote cell borders), scale bar: 10 μm. For adhesion measurements, regions of 5 μm × 1.25 μm spanning a cell border were defined (green boxes and asterisks, right). C, Representative images of distribution of Dsg2-mediated binding events before and after adrenergic stimulation at cell–cell contacts (each white pixel in binding event and every blue pixel in the overlay images indicates one binding event, green line denotes the cell border), scale bar: 1 μm. D, To calculate the distribution ratio of Dsg2-mediated binding events, a 10–pixel-wide region at cell–cell contact was defined as the cell border (CB, green striped area). The distribution ratio is defined as the binding frequency (percentage of binding events) at the CB vs remaining cell surface (CS; n=6), *P<0.05 vs control, scale bar: 1 μm. E, Mean numbers of Dsg2-mediated binding events per measurement area (n=6); ns P>0.05 vs control. F, Scatter blot of unbinding forces of all binding events from experiments in C–E. Green and blue bars indicate means±SEM (n>1500 from 6 independent experiments) *P<0.05. F/R indicates forskolin/rolipram; and Iso, isoproterenol.
distribution of Dsg2-mediated binding events was detectable both at cell borders, as well as on the cell surface away from these areas (Figure 1C). This is in line with the existence of cadherins not connected to intercellular junctions, which are thought to form a reservoir for the turnover of these junctions.26 To enhance sympathetic signaling, the β-adrenergic agonist isoprenaline (Iso) or a combination of the adenyl cyclase activator forskolin and the phosphodiesterase-4 inhibitor rolipram (F/R) were applied. Förster resonance energy transfer and ELISA (enzyme-linked immunosorbent assay measurements) confirmed increased cAMP levels after treatment with both mediators in HL-1 cells after 10 and 60 minutes, respectively (Online Figure I). β-Adrenergic stimulation by Iso or F/R caused redistribution of Dsg2 binding events toward cell borders, whereas the total number of binding events was not changed (Figure 1C through 1E). Dsg2-specific binding was demonstrated by significant reduction in the number of binding events after incubation with a monoclonal antibody targeting the extracellular domain of Dsg2 (Online Figure IIA and IIB). Blocking of specific Dsg2–Dsg2 interactions by monoclonal antibody targeting the extracellular domain of Dsg2 was confirmed in cell-free environment with a Dsg2-coated tip probed on a Dsg2-coated mica (Online Figure IIC). To quantify changes in the localization of binding events, we defined the ratio of the percentage of Dsg2-mediated binding events on the cell border (10-pixel-width area at cell–cell contact) versus the percentage on the surface (remaining cell area; Figure 1D). This distribution ratio was significantly increased after addition of F/R or Iso, respectively. In sharp contrast, no redistribution of N-Cad binding was induced by increased cAMP (Online Figure IID and IIE). Interestingly, after adrenergic stimulation, the force necessary to disrupt the binding between tip-coupled Dsg2 and the surface of HL-1 cells significantly increased ∼10% (Figure 1F).

β-Adrenergic Stimulation Increases Cardiac Myocyte Cohesion In Vitro and Ex Vivo

To determine whether adrenergic stimulation is effective to modulate cardiac myocyte cohesion, we used a dispase-based dissociation assay in vitro5 and established a dissociation assay in murine heart slice cultures. The fragment numbers after application of defined mechanical stress to HL-1 monolayers were significantly reduced under both Iso and F/R treatment for 60 minutes (Figure 2A), demonstrating increased cohesion of cardiac myocytes by β-adrenergic stimulation in vitro. To evaluate whether adrenergic signaling would not only alter overall cell cohesion but also the quality of binding, we studied whether hyperadhesion also exists in cardiac myocytes. This phenomenon, describing an enhanced adhesion state independent of Ca2+, has been established for keratinocytes.27 Cardiac myocyte cultures pretreated with Iso or F/R were incubated with the Ca2+-chelator ethylene-bis(oxyethylenenitrilo)tetraacetic acid for 90 minutes before shear stress was applied. Under these conditions, adrenergic signaling largely blunted the effect of ethylene-bis(oxyethylenenitrilo)tetraacetic acid, indicating that cAMP rendered cardiac myocytes hyperadhesive (Figure 2B). To measure intercellular adhesion ex vivo, we modified the dispase-based dissociation assay for heart slices: transversal murine heart slices were treated as indicated and incubated with a dispase-collagenase mix afterward to dissolve integrin binding and subsequently subjected to defined mechanical stressing. The number of intact single cardiac myocytes released from the slices was counted and used as an indirect measurement of cardiac myocyte cohesion. These single cardiac myocytes were viable, contracted spontaneously, and displayed an intact sarcomere system and ICD (Online Figure III). To validate this assay, ethylenebis(oxyethylenenitrilo)tetraacetic acid 10 mmol/L was applied for 60 minutes before incubation with dissociation buffer to disrupt total cadherin binding, which drastically enhanced the number of cardiac myocytes released from the slices (>6-fold of control; Figure 2C, right). The absolute numbers of cells released from consecutive slices under control conditions did not vary significantly (data not shown). In line with the dissociation assays performed in cultured cells, numbers of intact
dissociated cardiac myocytes were decreased by Iso or F/R treatment for 60 minutes (Figure 2C), indicating enhanced cohesion of cardiac myocytes by sympathetic signaling ex vivo.

**β-Adrenergic Stimulation Increases Desmosomal Molecules at Cell–Cell Border In Vitro and Ex Vivo**

Increased adhesion in response to β-adrenergic signaling was paralleled with pronounced junctional reorganization. Under control conditions in HL-1 cells, Dsg2 immunostaining appeared in a punctuated fashion along cell borders, while N-Cad staining displayed a continuous linear signal at cell–cell contacts (Figure 3A). After incubation with Iso or F/R, the areas with Dsg2 staining at the cell border were significantly enhanced, and mean length of the single Dsg2 signal was elongated at cell borders, whereas N-Cad staining was unchanged (Figure 3A through 3D; Online Figure IVA). To exclude the possibility of increased Dsg2 staining at the cell border through uncovering of epitopes detected by the Dsg2 antibody, HL-1 cardiac myocytes were transfected with a green fluorescent protein–tagged Dsg2 construct (Dsg2-GFP) and treated with Iso or F/R, respectively. Exogenous Dsg2-GFP presented comparable effects as endogenous Dsg2 detected by immunostaining with signal increase at cell borders and reduction on the cell surface, visible in color-coded z-stack height projections (Online Figure IVB). Similar to the appearance in HL-1 cells, Dsg2 displayed a punctuated staining at the ICD in cardiac slice cultures, whereas N-Cad was distributed linearly. Enhancing cAMP levels significantly increased the area of Dsg2 signal compared with the area of N-Cad staining at the ICD (Figure 3E). Similar results were obtained when whole murine hearts were perfused ex vivo with 2 μM Iso in a Langendorff setting (Online Figure IVC). On the ultrastructural level, the plaques of intercellular junctions of HL-1 cells were elongated under these conditions as shown by transmission electron microscopy (Figure 3F).

Combined with the results from AFM experiments, these findings demonstrate a rapid translocation of Dsg2 to cell–cell contacts as a result of enhanced sympathetic signaling, which correlated with strengthened cardiac myocyte cohesion.

To investigate the underlying mechanisms by which cAMP causes Dsg2-mediated positive adhesiotropy, we biochemically characterized composition of Dsg2-containing complexes. The total protein amounts of Dsg2 or of the other desmosomal components Pg, Dp, and Pkp2 were not altered by F/R treatment (Online Figure VA). Furthermore, no changes in the composition of desmosomal complexes were detectable by immunoprecipitation of Pg, Dp, and Pkp2 with Dsg2 after cAMP enhancement (Online Figure VA). Also, Triton-X-extraction assay with separation of the cytoskeletal-bound proteins from the noncytoskeletal-bound proteins showed no difference in the distribution of desmosomal proteins between the 2 pools (Online Figure VB).

We further explored if changes in the mobility of Dsg2 molecules in desmosomal cell contacts at the cell border contribute to adrenergic effects and performed fluorescence recovery after photobleaching experiments with Dsg2-GFP-transfected HL-1 cardiac myocytes. Interestingly, after photobleaching of a cell junction area exhibiting a homogeneous Dsg2-GFP signal, there was nearly no Dsg2-GFP signal recovery detectable under control conditions within 180 s. Measuring a second comparable Dsg2 signal of the same cell after adding F/R to the culture media for 10 minutes showed a significantly higher recovery of the fluorescence intensity after bleaching with an increased mobile fraction of Dsg2 molecules (Figure 3G and 3H). This indicates an increased mobility of Dsg2 molecules in cell contacts after adrenergic stimulation, which may contribute to the increase of Dsg2 signals at cell borders and the increase of cell cohesion. Besides Dsg2, Pg, and Dp staining along cell junctions was enhanced after treatment with Iso or F/R (Figure 3I and 3J). In contrast, Pkp2 and Dsc2 did not undergo comparable translocation (Figure 3K). In summary, these data are in agreement with the concept that enhanced mobility of Dsg2 which, together with Pg and Dp, shifts toward cell junctions, which may increase cardiac myocyte cohesion in response to β-adrenergic signaling.

**Pg Deficiency Abrogates the β-Adrenergic Effects on Cell Cohesion, Dsg2 Distribution, and the Physiological Response After β-Adrenergic Challenge**

We observed translocation of Pg together with Dsg2 on adrenergic stimulation. Pg is well established to be essential for cardiac myocyte function and was shown to be downregulated in many arrhythmogenic cardiomyopathy patients.29 To study the role of Pg on cAMP-enhanced cardiac myocyte cohesion and function, we generated cardiac-specific Pg-deficient mice (Pg KO) similar to a previous approach.7,8 These mice were recapitulating the arrhythmogenic cardiomyopathy phenotype with development of a progradent cardiac hypertrophy, fibrosis, and cardiac dilatation as described earlier (Online Figure VIA through VIC). Intriguingly, in Pg-deficient hearts of 2-week-old mice, before fibrosis and hypertrophy became evident, Dsg2 was largely reduced, whereas levels and localization of Dsc2, N-Cad, or other desmosomal and adherens junction components was not altered except a significant increase of β-catenin presumably to compensate for loss of Pg (Online Figures VII, VIE, and VIF).7,8,29 These data indicate that Pg may be important for turnover and function of Dsg2 and that loss of Dsg2 occurred prior to cardiac hypertrophy and fibrosis. These findings are similar to previous studies showing that distribution of N-Cad in contrast to Dsg2 was not altered by Pg deficiency.7,8 However, in the first report, localization of Cx43, Pkp2, and Dp at the ICD was impaired in addition to Dsg2. Because in contrast to our mouse model, Pg depletion was induced between the age of 6 to 8 weeks, it is possible that loss of these ICD components are a result of the different knockout approaches.

Next, we exposed transversal heart slices of 2-week-old mice to dissociation assays. Under baseline conditions, the mean number of single cells from Pg-deficient heart slices was higher than that from wild-types (WT), but differences were not significant (22.67±3.68 versus 16.42±3.46; P=0.238). However, in contrast to WT controls, both Iso and F/R were not effective to increase cell cohesion in slices from Pg KO animals (Figure 4A). This suggests that Pg is required to enhance Dsg2-mediated cardiac myocyte cohesion in response to β-adrenergic signaling. To investigate whether Pg is required for cardiac myocytes to adapt to β-adrenergic challenge on the
Figure 3. β-Adrenergic signaling causes junctional plaque reorganization. A–C, Immunostaining and quantitative analysis of HL-1 cells for desmoglein 2 (Dsg2; red) and N-cadherin (N-Cad; green), scale bar: 20 μm. White boxes mark zoomed areas, scale bar: 2 μm. For additional information about the analysis and calculation, see Online Figure IVA (n=90 cells of 3 independent experiments), *P<0.05 vs control. D, Analysis of length of N-Cad immunostaining signals at the membrane per single HL-1 cell before and after isoprenaline (Iso) or forskolin/rolipram (F/R) treatment (n=90 cells of 3 independent experiments); ns P>0.05 vs control. E, Quantitative analysis of Dsg2 and N-Cad immunostaining in murine heart slices (n>95 ICDs of 3 independent experiments), *P<0.05 vs control. F, Transmission electron microscopy images of cell junctions of HL-1 cells, scale bar: 500 nm (representative images of 3 independent experiments). G and H, Analysis of fluorescence recovery after photobleaching on cell–cell contacts of Dsg2-GFP transfected HL-1 cardiac myocytes with representative kymograph showing the relative fluorescence intensity over time with prebleaching period, bleaching (indicated with black area) and start of recovery measurement at time point 0 (n=22 of 5 independent experiments), *P<0.05 vs corresponding control time point. I, Double immunostaining of HL-1 cardiac myocytes for Dsg2 and plakoglobin (Pg). Representative deconvoluted images in xy direction processed out of z-stack of 0.13 μm thin xy slices (n=3), scale bar: 15 μm. J, Representative images of double immunostaining of HL-1 cells for Dsg2 and desmoplakin (Dp) and (K) staining for plakophilin 2 (Pk2) and desmocollin 2 (Dsc2), scale bars: 15 μm (n=3).
physiological level, we performed ex vivo heart perfusion in a Langendorff setup. Basal pulse pressure was similar in retrograde-perfused 10-week-old WT and Pg KO hearts. However, Pg KO hearts failed to increase the pulse pressure amplitude in response to Iso treatment, which rapidly occurred in WT hearts (Figure 4B). This indicates reduced inotropy in murine hearts lacking Pg. Also the positive chronotropic response was blunted in Pg-deficient hearts, which did not respond with increased heart rate after Iso treatment (Figure 4C). Perfused hearts were further analyzed by immunostaining for N-Cad and Dp. After 10-minute Iso perfusion, the ICDs of Pg KO appeared widened or even ruptured, which was absent in Pg KO hearts not challenged in a Langendorff setup (Figure 4D). This indicates that Pg KO ICDs fail to sufficiently adapt to the...
increased adhesive demand elicited by β-adrenergic signaling, which may explain the reduced inotropy.

To study the effects of Pg depletion on cAMP-enhanced cardiac myocyte cohesion in more detail, Pg expression was suppressed by small interfering RNA (siRNA) specific for Pg and nontargeting siRNA in HL-1 cardiac myocytes. In dissociation assays, Pg silencing significantly increased the number of fragments from 7.2±0.7 to 15.4±3.1 under control conditions and, thus, reduced basal cardiac myocyte cohesion. Moreover, similar to the Pg-deficient mouse model, Pg silencing blunted the positive impact of adrenergic signaling on cell cohesion and reduced the amount of Dsg2 in HL-1 cells (Figure 5A and 5B). To test Pg-dependency of the Dsg2 shift toward the cell border in response of F/R treatment by AFM, siRNA specific for Pg and nontargeting siRNA were labeled with the fluorophore cy3 and transfected into HL-1 cardiac myocytes to detect Pg knockdown in living cells (Figure 5C).

AFM measurement with a Dsg2-coated tip were performed

Figure 5. Desmoglein 2 (Dsg2) redistribution and increased cell cohesion by β-adrenergic signaling is plakoglobin (Pg)-dependent. A, Dissociation assays of nontargeting siRNA (siNT)- or Pg-specific siRNA (siPg)-treated HL-1 cells with and without cAMP enhancement (siNT n=8; siNT+F/R n=6; siPg n=7; siPg+F/R n=6). Right, Photographs of representative shear-stressed monolayers. B, Western blot corresponding to experiments in A to confirm protein silencing. Values demonstrate band density normalized to α-tubulin (α-Tub) and expressed as fold of siNT±SEM (n=5). C, Immunostaining of HL-1 cells transfected with cy3-tagged siPg confirmed Pg knockdown in transfected cells in comparison to cy3-tagged siNT. Asterisk marks representative cell containing no siPg-cy3 with normal Pg staining at the membrane. Membrane was labeled with sulfo-NHS-biotin and stained with Streptavidin-Alexa 488 (n=3), scale bar: 10 μm. D, Atomic force microscopy (AFM) setup for Pg silencing experiments. HL-1 cells transfected with cy3-tagged siPg or siNT were visualized by overlay of red cy3 fluorescence with the brightfield image (left, scale bar: 10 μm). An overview AFM topography image with overlay of cy3 signal (right, in area of green box in left, scale bar: 5 μm) was created for orientation and selection of a region containing cell borders of 2 siRNA transfected cells for AFM measurements (green box in right). E and F, AFM adhesion measurements and distribution ratio of Dsg2-mediated binding events at borders of 2 adjacent HL-1 cells transfected with cy3-tagged siNT or siPg (n=6), scale bar: 1 μm. *P<0.05, ns P>0.05. F/R indicates forskolin/rolipram.
on areas, including the cell–cell border of 2 cardiac myocytes containing siRNA (Figure 5D). Pg silencing completely blocked the redistribution of Dsg2-mediated binding events to the cell border, indicating the Dsg2 shift toward the cell border to be dependent on Pg (Figure 5E and 5F).

β-Adrenergic Effects on Dsg2 Distribution and Cardiac Myocyte Cohesion Are Dependent on Phosphorylation of Pg at S665

To elucidate possible mechanisms by which cAMP regulates Dsg2 binding in Pg-dependent manner, we investigated whether the effects of adrenergic signaling were mediated by PKA or Epac/Rap1. The cAMP-mediated increase of cell cohesion was blocked after PKA inhibition by preincubation with the PKA inhibitor H89 for 60 minutes, whereas the PKA activator 6-Bnz-cAMP strengthened cell cohesion similar to F/R in vitro (Online Figure VIII A). Cohesion of cardiac myocytes treated with a combination of siRNAs for the PKA catalytic subunits α and β to specifically inhibit PKAβ failed to increase after F/R treatment compared with nontargeting siRNA (Online Figure VIII B). Ex vivo dissociation assay revealed similar results as in vitro experiments (Online Figure VIII C). The selective activation of the Epac/Rap1 pathway by O-Me-cAMP had an inconsistent but not significant effect, indicating that effects of adrenergic signaling on cell cohesion were primarily PKA dependent (Online Figure VIII A). Nevertheless, our data do not rule out an additional effect of Epac signaling, which may also indirectly enhance signaling of PKA.31,32 In line with the important role of PKA, preincubation with H89 abrogated F/R-mediated effects on reorganization of Dsg2 staining at HL-1 cell junctions (Online Figure VIIID), as well as on the redistribution of Dsg2-mediated binding events as detected by AFM (Online Figure VIII E and VIII F). In contrast, 6-Bnz-cAMP promoted junctional redistribution of Dsg2 comparable to F/R treatment (see Figure 3A). These data suggested that PKA-mediated phosphorylation of Pg may be important. Because phosphorylation of desmosomal proteins is known to modulate desmosomal function and assembly,33,34 we used the Phos-tag approach to visualize changes in the phosphorylation state of junctional proteins in response to increased cAMP. Here, in an SDS-PAGE, phosphorylated proteins migrate slower compared with unphosphorylated molecules, and the lowest band represents unphosphorylated proteins or the basal phosphorylation state. Changes in phosphorylation are reflected by a change in the ratio between the upper band(s) versus the lowest band. An Iso- and F/R-induced increase of Pg phosphorylation was visible as increased ratio of upper (**) versus lower (*) migrating band, which was reduced by siRNAs for the PKA catalytic subunits α and β to specifically inhibit PKA (Figure 6A). In contrast, Dp phosphorylation did not significantly change (Figure 6B). No clear phosphorylation bands were obtained for Dsg2 and Pkp2 (data not shown). Indeed, phosphopeptide-specific mass spectrometry identified 2 phosphorylation sites within the Pg sequence unambiguously localized to S182 and S665, respectively. After normalization of phosphopeptide abundances to the amount of Pg in the respective sample, we observed a 2-fold increase in phosphorylation of S665 on F/R stimulation, whereas S182 phosphorylation levels remained unchanged (Figure 6C).

To ascertain the role of Pg-S665 phosphorylation, we generated the phospho-deficient mutant Pg-GFP-S665A with Pg-GFP-WT as control (Figure 6D). Phosphorylation of Pg at this site in response to cAMP elevation was confirmed by the absence of increased phosphorylation of Pg-GFP-S665A transfected in HL-1 cardiac myocytes (Figure 6E). According to the pkPS Predictor (http://mendel.imp.ac.at/pkaPS),35 S665, among other less likely sites, is predicted as most probable PKA-specific phosphorylation site in Pg. To test this, we performed in vitro kinase assays with the PKA catalytic subunit in comparison to protein kinase Cα. In accordance with >1 putative PKA phosphorylation motif, recombinant WT Pg showed 2 additional bands (p1 and p2) in presence of PKA but not PCKα, probably representing 2 sites phosphorylated by PKA in vitro (Figure 6F). In contrast, phosphorylation state p2 was absent in phospho-deficient Pg S665A mutant, which demonstrates that S665 is directly phosphorylated by PKA.

When we tested the functional effects of the phospho-deficient mutant in HL-1 cells, Pg-GFP-S665A transfection in contrast to Pg-GFP-WT largely prevented F/R-mediated increase in cell cohesion (Figure 6G). In these experiments, the amount of exogenous Pg was roughly 30% of the endogenous Pg. Also the increase of Dsg2 staining at the cell junctions by adrenergic stimulation was reduced at joint cell borders of cells expressing the phospho-deficient mutant compared with WT controls (Figure 6H). Finally, in AFM measurements on a region spanning the cell–cell border of 2 transfected cardiac myocytes, expression of Pg-GFP-S665A but not of Pg-GFP-WT prevented the shift of Dsg2-mediated binding events from the cell surface toward the cell border (Figure 6I and 6J) and abrogated the increase in the mean unbinding forces (Figure 6K).

Altogether, these data demonstrate that positive adhesiotropy to strengthen cardiac myocyte cohesion associated with Dsg2 translocation to cell borders is mediated by phosphorylation of Pg at S665 by PKA.

Discussion

In the present study, we describe a signaling mechanism regulating cardiac myocyte cohesion and provide first evidence for enhancement of cardiac myocyte cohesion as a novel function of adrenergic signaling in the heart, a phenomenon we refer to as positive adhesiotropy. Mechanistically, cAMP-induced translocation of Dsg2 to cell borders was found to be associated with ICD plaque reorganization and cardiac myocyte hyperadhesion. We provide evidence that cAMP-mediated recruitment of Dsg2 to cell borders as well as enhanced Dsg2 binding strength and overall cardiac myocyte cohesion are dependent on PKA-mediated phosphorylation of Pg at S665.

From studies on keratinocytes, it is known that desmosomal contacts form signaling hubs to regulate signaling pathways, including p38MAPK, protein kinase C, and Src, which control cell cohesion and migration.26,36,37 For cardiac myocytes, data on the pathogenesis of arrhythmic cardiomyopathy also reveal that desmosomal components in the ICD regulate signaling mechanisms important for differentiation and conduction of excitation in cardiac myocytes such as glycogen synthase kinase 3β or Wnt.38,39 However, it is unclear
Figure 6. Positive adhesiotropy is dependent on phosphorylation of plakoglobin (Pg) at S665. A and B, Separation of phosphorylated proteins by Phos-tag immunoblotted for Pg or desmoplakin (Dp) in HL-1 cell lysates transfected with nontarget siRNA (siNT)- or siRNA pools specific for protein kinase A (PKA) catalytic subunits α and β (siPKA). Phosphorylation is visible with 30 μM Phos-Tag as upper band (marked with **) vs lower band (marked with *). Values in the top row show densitometric analysis of 30 μM Phos-Tag blots expressed as ratio of upper (**) vs lower (*) band (fold of siNT). Blots from gels without Phos-Tag reagent (0 μM) served (Continued)
how intercellular cohesion of cardiac myocytes is regulated. We provide evidence that adrenergic signaling enhances cardiac myocyte cohesion and Dsg2 binding strength, as well as recruitment of Dsg2 to intercellular contacts, which become elongated under these conditions. The result that β-adrenergic signaling fosters desmosomal intercellular cohesion is in line with previous studies from keratinocytes. We found that the effects of β-adrenergic signaling and Dsg2 redistribution were dependent on Pg expression and its phosphorylation at S665 by PKA. Thus, depletion of Pg as well as expression of a Pg-mutant phospho-deficient at S665 abolished Dsg2 recruitment, enhancement of Dsg2 binding strength, as well as of overall cardiac myocyte cohesion. Similar to regulation of desmosomes in keratinocytes, PKA activity was required for recruitment of Dsg2-specific binding events, formation of Dsg2-positive cell contacts, and for increased cell cohesion. Moreover, in vitro kinase assays revealed that Pg phosphorylation at S665 is directly mediated by PKA but not protein kinase Ca. Immunoablotting of HL-1 cell lysates transfected with Pg-GFP-WT or Pg-GFP-S665A and immunoblotting for GFP to specifically label exogenous Pg. Phosphorylation is visible as upper band (compared with baseline status; n=5). In vitro kinase assay of recombinant glutathione S-transferase-tagged Pg proteins (Pg-GST-WT, Pg-GST-S665A) incubated with PKA catalytic subunit or protein kinase Ca (PKCα), respectively. Lower phosphorylation state is marked with p1, while p2 marks higher phosphorylation state compared with the unphosphorylated protein nonphos (n=6 different bacterial clones). Dsg2 localization did not change in response to adrenergic stimulation, it is possible that the proteins shift together as a complex, which would explain why neither the association of desmosomal plaque proteins with Dsg2 nor the cytoskeletal anchorage of desmosomal components was altered by adrenergic signaling. In addition, increased cAMP rendered cardiac myocyte cohesion independent of Ca++; a state referred to as hyperadhesions and previously known in keratinocytes only. This result indicates that the enhanced binding strength of Dsg2 molecules is paralleled by a qualitative change of overall cell cohesion toward a mature Ca++-independent state. The hyperadhesive state is assumed to be achieved by conformational rearrangement and the configuration of the desmosomal cadherin extracellular domains associated with changes in the binding patterns and organization of desmosomal molecules. It is further hypothesized that hyperadhesion is regulated by phosphorylation of desmosomal components, which is assumed to generate a signal that leads to a conformational change in the extracellular domains of desmosomal, rather than of classical cadherins. Because we found that Pg was phosphorylated on adrenergic stimulation, it is possible that phosphorylation of Pg induces the recruitment of Dsg2 together with Pg and Dp to stabilize ICDs which, by altered clustering of Dsg2, may induce hyperadhesion and increased overall cardiac myocyte cohesion. However, more refined studies on the mechanisms underlying hyperadhesion are required. Besides impaired enhancement of intercellular adhesion, we observed that Pg-deficient hearts in Langendorff experiments failed to respond to adrenergic challenge with adequate increase of pulse pressure and heart rate and exhibited rupture of ICDs after 10 minutes of perfusion with Iso. We cannot exclude that the reduced cardiac response to adrenergic stimulation in Pg-deficient hearts is because of secondary changes, such as hypertrophy and fibrosis, occurring in transgenic mice. Alternatively, these data may indicate that

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**Figure 6 Continued.** as control to exclude protein fragmentation (siNT n=8 or 7; siPKA n=10 or 8; siPKA+F/R n=8 or 6; siPKA+F/R n=7 or 5; siNT+Iso n=7 or 6; siPKA+Iso n=6 or 5 for Pg or Dp, respectively). D, Schematic diagram of the green fluorescent protein (GFP)-tagged Pg mutant with phospho-deficiency at serine 665 (Pg-GFP-S665A) and corresponding wild-type mutant (Pg-GFP-WT). E, Phos-tag SDS-PAGE in HL-1 cell lysates transfected with Pg-GFP-WT or Pg-GFP-S665A and immunoblotting for GFP to specifically label exogenous Pg. Phosphorylation is visible as upper band (compared with baseline status; n=5). F, In vitro kinase assay of recombinant glutathione S-transferase-tagged Pg proteins (Pg-GST-WT, Pg-GST-S665A) incubated with PKA catalytic subunit or protein kinase Ca (PKCα), respectively. Lower phosphorylation state is marked with p1, while p2 marks higher phosphorylation state compared with the unphosphorylated protein nonphos (n=6 different bacterial clones). G, Dissociation assay of HL-1 cardiac myocytes, transfected with indicated constructs. Right, Corresponding Western blot analysis to confirm transfection and expression of the plasmids (control n=8, F/R n=11). Values indicate ratio of exogenous vs endogenous Pg analyzed by densitometric measurements of corresponding bands. H, Immunoablotting of HL-1 cell lysates transfected with Pg-GFP plasmids (green) and counterstaining of desmoglein 2 (Dsg2; red). Arrows indicate cell borders of 2 adjacent cells, expressing the corresponding plasmid. Arrowheads indicate cell borders of cells not expressing the plasmid (n=3), scale bar: 15 μm. I and J, Atomic force microscopy (AFM) measurements with corresponding distribution ratio of Dsg2-mediated binding events in areas containing the joint cell border of 2 HL-1 cells expressing the indicated plasmid. Each white/blue pixel indicates one binding event, green line denotes the cell border (n=6). Scale bar: 1 μm. K, Scatter plots of unbinding forces of Dsg2-mediated single binding events, bars indicate mean±SEM (n=1300 from 6 independent experiments). *P<0.05; ns P>0.05. F/R indicates forskolin/rolipram; and Iso, isoprenaline.
the positive adhesiotropic function is closely coupled with positive inotropic and chronotropic effects of β-adrenergic signaling and is crucial for ICD integrity. Taking into account that Dsc2 and Dp similar to N-Cad were present at ICDs in Pg-deficient hearts, which did not exhibit a significant positive adhesiotropic, inotropic, and chronotropic response, our data suggest that Pg via regulation of Dsg2 binding is important for β-adrenergic modulation of heart function. Sympathetic signal transduction has been shown to enhance conduction velocity and gating function of gap junction by upregulation, as well as fostering the accumulation of gap junction components at cell–cell borders.18–20 In this context, it has been proposed that cAMP via Epac/Rap1 and protein kinase C signaling causes phosphorylation of Cx43.45,46 Because the data presented here reveal that cAMP elevation not only strengthens electric but also mechanical coupling of cardiac myocytes and given the fact that the ICD acts as a functional unit,1,47 our results suggest that the positive effect of cAMP on gap junction communication may at least in part be mediated indirectly by enhancing desmosomal adhesion, especially because desmosomal proteins of the ICD have been shown to regulate gap junction structure and function on several levels. In fact, previous data showed that desmosomal adhesion is crucial for maintenance of basal heart rate and adequate responses of murine hearts to sympathetic activity.6,13,14 This could be attributed to regulation of expression, membrane targeting, and cytoskeletal anchorage of Cx43 and Na,1.5 by virtual all the different desmosomal components.5–13,48

Taken together, our study reveals that adrenergic signaling strengthens Dsg2 binding and overall cardiac myocyte cohesion. Because the β–adrenergic receptor is directly located at ICDs and Dsg2 binding is important for cardiac myocytes to maintain basal heart rate,6 desmosomal adhesion seems to be critical for sufficient electric coupling of cardiac myocytes under resting conditions, and positive adhesiotropy of cardiac myocytes in response to adrenergic stimulation may be important for adaption of heart function to sympathetic activity.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL:

Experimental Procedures:

Mediators and reagents. Isoprenaline hydrochloride (Iso, Sigma-Aldrich, Munich, Germany) was dissolved in H₂O and applied at concentrations of 2µM. The combination of the adenylyl cyclase activator forskolin and phosphodiesterase-4 inhibitor rolipram (F/R, Sigma-Aldrich) was applied at concentrations of 5µM and 10µM, respectively. Iso and F/R were incubated for 60min, unless otherwise stated. The protein kinase A (PKA)-inhibitor H89 (Sigma-Aldrich) was dissolved in H₂O and pre-incubated at 20µM 60min before addition of F/R. The cAMP analogues 6-Bnz-cAMP, a selective PKA-activator, and 8-pCPT-2’-O-methyl-cAMP (O-Me-cAMP), a selective Epac-activator, (both Biolog, Bremen, Germany) were dissolved in H₂O and applied for 120min at concentration of 0.1mM. The Ca²⁺ chelator Ethylene-bis(oxyethylenenitriilo)tetracetic acid (EGTA) (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) and applied for 60min at 10mM for dissociation assay ex vivo or as indicated.

Cell culture. The murine cardiomyocytes cell line HL-1 was kindly provided by William Claycomb (LSU Health Sciences Center, New Orleans, USA) and maintained in Claycomb medium (Cat # 51800C) supplemented with 10% fetal bovine serum (Cat # F2442, batch 058K8426), 100µM norepinephrine, 100µg/ml penicillin/streptomycin and 2mM L-glutamine (all purchased from Sigma-Aldrich) at 37°C, 5% CO₂ and 100% humidity. Cells were seeded on glass coverslips for immunostaining and AFM experiments or in 24-well plates for dissociation assays, TEM and Western blot analysis after pre-coating with 25µg/ml bovine fibronectin in 0.02% gelatin solution (both Sigma-Aldrich). After seeding for experiments, cells were incubated in Claycomb medium without norepinephrine to avoid basal adrenergic stimulation and additionally supplemented with 1.8mM Ca²⁺ to provide sufficient cadherin binding. Unless otherwise stated, cells were grown to confluency within seven days.

Mouse models. Animal handling and sacrifice was in accordance with guidelines of the European Commission and local university regulations. For experiments without genetically modified mice, adult male 12-week-old Balb/c mice were used. To generate heart-specific Pg knockout mice (Pg KO), Juptm1.1Glr/J mice (The Jackson Laboratory, Bar Harbor, USA), which homozygously express loxP sites flanking exon 1 of the junctional plakoglobin (Jup) gene, were crossed with B6.FVB-Tg(Myh6-cre)2182Mds/J mice (The Jackson Laboratory), which heterozygously express Cre under the control of the cardiac-specific alpha myosin-heavy chain promotor, to generate heart-specific heterozygous Jup knockout animals. These F1 animals were bred with the parental Juptm1.1Glr/J line to generate homozygous Pg KO animals. Pg deficiency was shown to result in cardiomyocyte death and fibrosis of the heart muscle evident in older mice. To exclude the possibility of interference due to reduced number of cardiomyocytes because of fibrosis, we used 2-week-old mice for dissociation assay before fibrosis became evident (see Online Fig. VI).

Murine heart slice culture. Mice were sacrificed by cervical dislocation and the heart was immediately dissected, placed in ice-cold oxygenated cardiac slicing buffer (136mM NaCl, 5.4mM KCl, 1mM MgHPO₄, 0.9mM CaCl₂, 30mM 2,3-Butanedionemonoxime, 5mM HEPES)
supplemented with 10mM d(+)-glucose until contractions subsided. The heart was then embedded in 37°C low melt agarose (Carl Roth, Karlsruhe, Germany) dissolved in cardiac slicing buffer. By immediately cooling down on ice, the agarose hardened and the sample was cut in 300µm sections for immunostaining or Western blot analysis and in 200µm sections for dissociation assays using a LeicaVT1200S vibratome (Leica Biosystems, Nussloch, Germany; amplitude: 1mm; speed: 0.07mm/s). During cutting, the embedded heart and freshly cut sections were maintained in ice-cold cardiac slicing buffer supplemented with 10mM d(+)-glucose. For immunostaining and Western blot analysis, sections were transferred to sterile 0.4µm membranes of organotypic tissue culture plate inserts (Millipore, Tullagreen, Ireland) and incubated in a 6-well plate in 1.2ml cardiac slice medium (DMEM F12 nutrient mixture (1:1) supplemented with 20.4% knockout serum replacement, 1% minimum essential medium non-essential amino acids, 0.1% 2-mercaptoethanol, (all purchased from Life Technologies, Carlsbad, California, USA), 2mM L-glutamine, 10U/L : 10µg/ml penicillin and streptomycin) at 37°C, 5% CO₂ and treated as indicated. After treatment, cardiac slices were washed in PBS on cell culture filters. For immunostaining, slices were mounted in tissue freezing medium (Leica) and snap-frozen in liquid nitrogen. For Western blot analysis, slices were lysed in SDS-lysis buffer (see section “Western blot analysis”).

Dissociation assay ex vivo. 200µm thin murine cardiac slices were prepared as described above. Slices were gently washed in PBS and carefully transferred to 500µl pre-warmed cardiac slices medium in a 24-well-plate (one slice per well). Mediators or EGTA were added as indicated. To avoid interference due to different size or location in the heart, consecutive slices were used for controls and treatment, respectively, and resulting single cell numbers after application of shear stress were compared to the respective control slice. The slice to slice variability of the number of released single cells was roughly 1% (data not shown). After incubation and one washing step with PBS, slices were transferred in 200 µl dissociation buffer (liberase DH 0.065U/ml, Roche, Mannheim, Germany; dispase II 2.5U/ml, Sigma-Aldrich) in Hanks' balanced saline solution ((HBSS), AppliChem, Darmstadt, Germany) per well and incubated at 37°C for 25min. 150µl HBSS was added per well and slices were subjected to mechanical shear stress by repeatedly pipetting up and down with an electrical pipette for a defined number of times. Cells were left to settle for 5min and the amount of intact viable single cardiomyocytes in five crosswise-located fields of view were immediately counted using an inverted microscope (Axio, Carl Zeiss, Oberkochen, Germany). The viable cells were identified by an intact sarcomere system, spontaneous contractility and typical cell appearance. Viability was double-checked by Thiazolyl Blue Tetrozolium Bromide staining (Sigma-Aldrich). The number of dissociated single cardiomyocytes was used as an indirect measurement for intercellular adhesion. For immunostaining of single cardiomyocytes, dissociated cells were seeded on laminin-coated (from murine sarcoma basement membrane, Sigma-Aldrich) glass coverslips for 120min at 37°C, 5% CO₂. Cells were gently washed in PBS and fixed in 2% paraformaldehyde (PFA). Immunostaining for F-Actin, N-Cad and Dsg2 was performed similar to the procedure for HL-1 cells (see section “Immunostaining”).

Dissociation assay in vitro. HL-1 cardiomyocytes were grown to confluency in 24-well-plates and treated as indicated. After washing in PBS twice, cells were incubated with 200µl dissociation buffer per well at 37°C until detachment of intact cell monolayers from the well bottom occurred.
The dissociation buffer was replaced with 350µl HBSS and the monolayers were mechanically stressed by shaking on an orbital shaker at 1250rpm for 5min. After fixation by adding 500µl 2% PFA per well, the total number of resulting fragments was determined using a binocular stereo microscope (Leica) with 1.25x zoom. The number of fragments is an indirect measure for intercellular adhesion. To test for hyperadhesion, i.e. Ca^{2+} insensitivity of cell cohesion, the dissociation assay was modified as follows: Confluent HL-1 monolayer were released from well bottom by dissociation buffer and pre-incubated with indicated mediators for 30min in Claycomb medium containing 1.8mM Ca^{2+}. Subsequently medium was changed to a low- Ca^{2+} Claycomb medium containing 5mM EGTA and the corresponding mediators for 90min. After replacing medium with HBSS, monolayers were subjected to mechanical shear stress with further treatment as described above.

**Atomic Force Microscopy (AFM) experiments.** For measuring simultaneously topography, distribution of transmembrane adhesion molecules and their binding properties a Nanowizard® III atomic force microscope (JPK instruments, Berlin, Germany) mounted on an optical fluorescence microscope (Axio Observer D1, Carl Zeiss) was used. Recombinant Dsg2-Fc was purified from cell culture supernatant of stably Dsg2-Fc expressing Chinese hamster ovary cells as described previously. N-Cad-Fc was purchased from R&D Systems (Abingdon, UK). Flexible Si_{3}N_{4} AFM cantilevers (MLCT probes, Bruker, Calle Tecate, CA, USA) were coated with bifunctional polyethylene glycol spacers (acetal-PEG-NHS, Gruber Lab, Institute of Biophysics, Linz, Austria) to link recombinant Dsg2-Fc or N-Cad-Fc (at concentrations of 0.1 mg/ml) as described before. HL-1 cells were incubated in supplemented Claycomb medium with 1.8mM Ca^{2+} added and without norepinephrine at 37°C. For experiments, the pyramidal-shaped D-tip (nominal spring constant: 0.03N/m) with a tip radius of 20nm was used. To create AFM images and perform adhesion measurements SPM Control v.4 software (JPK instruments) was used. For visualization of the cells, a brightfield image was acquired with a 63x objective and an overview AFM image was created using a force-curve based quantitative imaging (QI) mode with following settings: setpoint 0.2nN, pulling speed 50µm/s, z-length 1500nm. For adhesion measurements, a cell-cell border containing region of 5µm x 1.25µm was selected and measurement were performed on a 64 x 16 pixel grid in force mapping (FM) mode with following settings: setpoint 0.2nN, extend speed 5µm/s, extend delay 0.1s. Each pixel acquired while scanning the selected region of interest contains the information of one force-distance curve (see also Fig. 1A). For each of these pixels, the tip repeatedly approaches the cell surface, indents the cell membrane until the defined set point is reached (Fig. 1A [I]) and retracted again. During this movement, the deflection of the flexible cantilever is detected by a laser beam with regard to its relative position over the cell surface, creating a force-distance plot (Fig. 1A). In case of a binding event, the tip is held back by the interacting molecules and the cantilever is deflected downwards (Fig. 1A [II]). When the force exerted by the pulling tip exceeds the binding force, the bond ruptures, the cantilever rapidly bends back into the neutral position (unbinding event, asterisk in Fig. 1A) and is further retracted to a predefined height before a novel approach is started one pixel lateral to the previous one. The amount of the cantilever deflection can be converted into forces acting on the cantilever using Hooke’s law. The spring constant k was determined separately by the thermal noise method for each tip. The amplitude of the “jump” in the force plot during an unbinding event represents the strength of the bond. For all experiments, the same cell-cell border was measured before and after addition of mediators (Iso, F/R or H89).
or antibody (Dsg2 mAb, monoclonal rabbit anti-Dsg2, Abcam, Cambridge, UK # ab124683). Measurements for cell free conditions were performed in HBSS containing 1.8mM Ca²⁺ at 37°C on cleaved mica sheets (SPI Supplies, West Chester, USA) functionalized with Dsg2 as described above for AFM probes.

**Immunostaining.** Confluent HL-1 cells were treated with corresponding mediators and washed with PBS. For membrane staining in siRNA experiments (Fig. 5) 160µM sulfo-NHS-biotin (Thermo Scientific, Rockford, USA) was added to the medium 20min before fixation and washed briefly with PBS. All cells were fixed in PFA in PBS for 10min, permeabilized with 0.1% Triton X-100 in PBS for 5min, washed in PBS and blocked with 3% bovine serum albumin/10% normal goat serum (BSA/NGS, Sigma-Aldrich) in PBS for 60min. Heart tissue was cut with a cryostat (HM500OMV, Microm, Walldorf, Germany) in 8µm thin sections. Probes were heated to 37°C for 10min, washed in PBS, fixed in 2% PFA for 10min at room temperature, permeabilized with 1% Triton-X-100 for 60min, washed in PBS and blocked with BSA/NHS for 60min. Primary antibodies were diluted in PBS and incubated at 4°C in a humidified chamber over night. The following primary antibodies were applied: polyclonal rabbit anti-Dsg2 (Progen Biotechnik, Heidelberg, Germany, #610121), monoclonal mouse anti-N-Cad (BD Biosciences, Heidelberg, Germany, #610921), monoclonal mouse anti-Dsc2/3 (Life Technologies, #32-6200), monoclonal mouse anti-Pg (Progen, #61005), monoclonal rabbit anti-Desmin (Abcam, #ab32362), rabbit anti-Cx43 (Sigma-Aldrich, #SAB4501175), polyclonal rabbit anti-Dp1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, USA #sc-33555), monoclonal mouse anti-Dp1/2 (Progen, #61003), monoclonal mouse anti-Pkp2 (Progen, #651167), monoclonal mouse anti-β-Cat (BD Biosciences, #610154), polyclonal rabbit anti-α-T-Cat (Proteintech Group, Inc. Chicago, USA, #13974-1-AP). Cy2-, cy3- and cy5-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Dianova, Hamburg, Germany) and Alexa Fluor®488 phalloidin (Life Technologies) to visualize F-Actin were incubated for 60min at room temperature. Streptavidin-Alexa Fluor®488 (Life Technologies) was applied to visualize biotin membrane staining. 1.5% n-propyl gallate/60% glycerol (Sigma-Aldrich) in PBS was used as mounting medium. Samples were imaged with the Leica SP5 confocal microscope (Leica) equipped with a 63x oil objective using LAS-AF software (Leica) and analyzed with ImageJ software (NIH, Bethesda, USA). For deconvolution, z-stacks of 0.13µm thick xy-sections were created and processed as described below.

**Western blot analysis.** Western blot analysis was performed according to standard procedures. Cells were washed in PBS and lysed in ice-cold SDS-lysis buffer (25mM HEPES, 2mM EDTA, 25mM NaF, 1% sodiumdodecylsulfate (SDS), pH7.4) supplemented with protease-inhibitor cocktail (Complete-O, Roche). Samples were sonificated and protein concentration was determined by BCA protein assay kit (Pierce/Thermo Scientific, Waltham, Germany) according to manufacturer’s protocol. Equal amounts of protein were denaturazed in Laemmli buffer at 95°C for 5min and loaded on a 10% SDS-polyacrylamide gel. PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) was used as protein size standard. After electrophoresis, proteins were transferred on nitrocellulose membrane (Amersham, GE Healthcare Life Sciences, Freiburg, Germany) by the wet-blot method and blocked in 5% non-fat milk in TBS-T buffer (20mM Tris-base, 137mM NaCl, 0.0475% Tween, pH7.6). Primary antibodies were diluted in 5% non-fat milk or bovine serum albumin in TBS-T buffer according to manufacturer’s instructions
and incubated at 4°C overnight. Polyclonal rabbit anti-PKA catalytic subunit α + β (Thermo, #PA5-13737), monoclonal mouse anti-Dsg1/2 (Progen, #61003), monoclonal mouse anti-Pg (Progen, #61005), polyclonal rabbit anti-Dp1/2 (Santa Cruz, #sc-33555), monoclonal mouse anti-Pkp2 (Progen, #651167), monoclonal mouse anti-α-Tub (Abcam, #ab7291) monoclonal rabbit anti-Desmin (Abcam, #ab32362), polyclonal rabbit anti-Cx43 (Sigma-Aldrich, #SAB4501175), polyclonal rabbit anti-Dp1/2 (Santa Cruz, #sc-33555), monoclonal mouse anti-Pkp2 (Progen, #651167) monoclonal mouse anti-β-Cat (BD Biosciences, #610154), monoclonal mouse anti-GAPDH (Aviva Systems Biology, San Diego, USA, #OAEA00006) and polyclonal rabbit anti-α-CAT (Proteintech, #13974-1-AP) served as primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Dianova) were incubated at room temperature for 120 min. The ECL-method (0.5% luminol, 0.22% para-cumaric acid, 0.03% H₂O₂, 100mM Tris-HCl) was used for chemiluminescence-based protein visualization. Densitometric band analysis was performed with ImageJ software (NIH).

**Immunoprecipitation (IP).** After treatment, confluent HL-1 cells were washed in ice-cold PBS and incubated in modified RIPA-buffer (10mM Na₂HPO₄, 150mM NaCl, 1% Triton X-100, 0.25% SDS, 1% Na deoxycholate, pH 7.2) supplemented with leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride and phosphatase inhibitors (Roche) on ice for 30 min. Cells were scraped off and homogenized by pulling up and down with 20G and 27G needles ten times each. Equal amounts of lysate and either monoclonal rabbit anti-Dsg2 (Abcam, #ab124683) or normal rabbit IgG (Santa Cruz, #sc-2027) were incubated overnight. Afterwards, lysates were incubated with pre-cleared magnetic Dynabeads® Protein G (Life Technologies) for 120 min at 4°C. After several washing steps, beads were resuspended in Laemmli buffer and proteins were denaturized at 95°C for 10 min. The whole amount was loaded on 10%-polyacrylamide gels and subjected to gel electrophoresis and protein detection as described in the section “Western blot analysis”.

**Triton-X-extraction assay.** Confluent HL-1 cells were treated as indicated and protein fractionating was carried out using standard procedures. Cells were incubated with Triton buffer (0.5% Triton X-100, 50mM MES, 25mM EGTA and 5mM MgCl₂) on ice for 10 min under gentle shaking. To separate a cytoskeleton-anchored (Triton-insoluble, pellet) and a cytoskeleton-unanchored (Triton-soluble, supernatant) protein fraction, the collected cell lysate was centrifuged (10 min at 14,500g, 4°C), the supernatant collected and the pellet lysed in SDS-lysis buffer. The same protein amounts were loaded on 10%-polyacrylamide gels for cytoskeletal as well as non-cytoskeletal fraction and subjected to gel electrophoresis and protein detection as described in the section “Western blot analysis”.

**Phos-tag SDS-PAGE.** To detect protein phosphorylation, a Manganese(II)-Phos-tag™ SDS-PAGE (Wako Chemicals GmbH, Steinbach, Germany) was performed. The Phos-tag compound is added to the gel and leads to reduced migration speed of phosphorylated proteins in comparison to their lower- or non-phosphorylated counterparts. This results in a shift of phosphorylated proteins according to the number of phospho sites. After treatment, HL-1 cells were washed in ice-cold PBS and lysed in modified RIPA-buffer supplemented with leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride and phosphatase inhibitor (Roche). Manganese(II)-Phos-tag™ SDS-PAGE was performed according to manufacturer’s instructions.
using a freshly prepared 6%-polyacrylamide gel containing 0mM (as control to exclude protein fragmentation) or 30mM Phos-Tag™ ALL-107 (Wako Chemicals). Lysates were sonificated, mixed with Laemmli buffer and loaded on the gels. To remove Mn²⁺ from the gels after electrophoresis, gels were washed twice in transfer buffer supplemented with 10mM EDTA for 10min and in transfer buffer without EDTA for 10min. For further procedure see "Western blot analysis". For protein transfer, a PDVF-membrane (Bio-Rad Laboratories, Munich, Germany) was used.

Proteomics sample preparation for mass spectrometry (MS). Pg was purified from control or F/R treated HL-1 lysates by IP using monoclonal mouse anti-Pg (Progen, #61005) for protein pull down as described in the section “Immunoprecipitation”. Proteins bound to beads were treated with 5mM dithiothreitol to reduce disulfide bonds and subsequently alkylated using 20mM iodoacetamide. Proteins were cleaved with trypsin for 12h at 25°C. Following tryptic digest, peptide mixtures were desalted on reversed-phase C18 stage tips. 90% of the peptide mixture was mixed with 2M glyclic acid for phospho peptide enrichment on TiO₂, loaded onto the prepared TiO₂ resin (Glygen). Eluted phospho peptide mixtures were separated on an Ultimate 3000 nano-RP-HPLC (Thermo Scientific) online coupled to a LTQ-Orbitrap classic mass spectrometer (Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition mode with one survey scan for precursor mass detection and up to 6 MS/MS experiments per cycle. CID-MS/MS experiments were conducted with multi-stage activation for neutral loss masses of 32.7, 49 and 98. For MS acquisition, monoisotopic precursor selection and dynamic exclusion for 30s were enabled. Tryptic peptide mixtures were separated on an Ultimate 3000 nano-RP-HPLC (Thermo Scientific) online coupled to a 6600 TripleTOF qTOF mass spectrometer (Sciex) or a QExact HF mass spectrometer (Thermo Scientific). Both mass spectrometers were operated in data dependent acquisition mode enabling up to 25 or 10 MS/MS experiments per duty cycle for the qTOF and the QExact, respectively. All experiments were performed in triplicates.

Analysis of mass spectrometry data. Raw data were searched with MaxQuant vs. 1.5 against a mouse proteome data base (Uniprot, May 2015) and all phospho peptide hits for the target proteins were manually curated and revealed a Pg sequence coverage of 72%. For quantitation of the phosphorylation, peak areas for the most abundant charge stated for unmodified peptides and phospho peptides of the target protein were extracted from the raw data of the tryptic peptide mixture before phospho peptide enrichment. In case the peptide was not identified in the same experiment, signal was integrated according to accurate mass and retention time tag from other experiments. Signal strength of unmodified peptides was used for normalization of the protein amount between control and treatment experiments of each biological replicate. Differences in phosphorylation in both conditions were calculated from the normalized phospho peptide intensities.

Generation and mutagenesis of Pg plasmids. cDNA encoding full-length human wild type plakoglobin (hPg)⁹ was cloned into eGFP-tagged destination vector (pDEST-eGFP-N1, Addgene plasmid # 31796, gift from Robin Shaw, Cedars-Sinai Heart Institute, Los Angeles, USA 10). To generate the pDEST-hPg-eGFP construct, Gateway recombination technology (Thermo Scientific) using pDONR221 as an intermediate vector was used. Specifically, hPg cDNA with
flanking attB recombination sequences was generated by polymerase chain reaction (PCR). The following primers were used:
Forward:
5’GGGGACAAGTTTGACTACAAAAAGCGAGCTTCGAAGGAGATAGACATgtaggggtgatgaacctgat
gg-3’
Reverse:
5’-GGGGACCACTTTGTACAAGAAAGCGGGTCCggccagcatgtggtctgcagtgg-

where the gateway recombination sequences are in capital letters, and the sequences complementary to the hPg cDNA are in lowercase letters. The PCR product together with pDONR221 vector, both bearing flanking sequences compatible for recombination, were used in BP reaction to generate the pDONR221-hPG entry vector. Subsequent LR reaction between the entry (pDONR221-hPg) and the destination (pDEST-eGFP) vectors resulted in creation of the final pDEST-hPg-eGFP construct. For studies with phospho-deficient Pg mutant at serine 665 (see Fig. 6), site-directed mutagenesis with substitution of serine 665 to alanine was performed with pDEST-hPg-eGFP to generate pDEST-hPG-eGFP-S665A (short: Pg-GFP-S665A) and pGEX4T1-hPg (short: Pg-GST-WT) to generate Pg-GST-S665A plasmid. pDEST-hPg-eGFP and Pg-GST-WT (kind gift of Ritva Tikkanen, Institute of Biochemistry, Justus-Liebig Universität Gießen, Gießen, Germany)11 were used as a PCR template with primers containing the mutation corresponding to alanine (Forward: 5’-CGGAAGCGCGTGGCCGTGGAGCTCACC-
Reverse: 5’-GGTGAGCTCCACGGCCACGCCTCCG-3’). Validation of all constructs was performed by sequencing analysis.

Plasmids and transfection. The vector encoding for C-terminally eGFP-tagged human wild type Dsg2 (Dsg2-GFP)12 was kindly provided by Katja Gehmlich (BHF Centre of Research Excellence Oxford, University of Oxford, Oxford, UK). The FRET sensor Epac1-cAMPs containing CFP and YFP as fluorophores was kindly provided by Viacheslav O. Nikolaev (University Medical Center Hamburg-Eppendorf, Germany) and Martin J. Lohse (Julius-Maximilians-Universität Würzburg, Germany).13 All other vectors were generated as described in the section “Generation and mutagenesis of Pg plasmids”. For immunostaining and FRAP experiments, HL-1 cells were transfected with indicated endotoxin-free vector DNA at final concentration of 2µg/ml using TurboFect™ (Life Technologies) according to manufacturer’s instructions and incubated for 24h. For AFM experiments, cells were transfected with indicated DNA at final concentration of 2.5µg/ml using FuGENE® HD (Promega, Madison, USA) according to manufacturer’s instructions and incubated for 24h. To increase transfection efficiency for dissociation assays, indicated DNA plasmids were transfected by electroporation (4D-Nucleofector, Lonza, Cologne, Germany) according to manufacturer’s instructions. Confluent HL-1 cells were trypsinized, counted and electroporated in Amaxa™ SF solution (Lonza) with 100µg/ml DNA at a concentration of 3.8 x 107 cells/ml with pulse EN180. Cells were seeded and incubated in Claycomb medium with neither norepinephrine nor additional Ca2+ for 24h. Experiments were performed 72h after electroporation. For all experiments, successful transfection and expression of the plasmid was visually confirmed by green immunofluorescence signal of the eGFP-tag or Western blot analysis, respectively.

siRNA-mediated knockdown. At 60-70% confluence, HL-1 cells were transfected with ON-TARGET plus SMARTpool mouse Jup-siRNA (siPg) or a combination of ON-TARGET plus
SMARTpools mouse Prkaca- and Prkacb-siRNA (siPKA) in parallel with the same amount of non-targeting siRNA (siNT) (all Thermo Fisher Scientific/Dharmacon, Layfette, USA) using RNAiMAX® (Life Technologies) as transfection reagent following manufacturer’s instructions. The siRNA was incubated for 24h. Cells were grown to confluency within four days. Knockdown efficiency was confirmed by Western blot analysis for every experiment. For AFM measurements, fluorescent siPg and siNT were generated using the Silencer® siRNA Labeling Kit with Cy3 dye (Life Technologies) in accordance with the manufacturer’s protocol similar to a previous study.7

**GST fusion protein expression and purification.** The GST fusion proteins Pg-GST-WT and Pg-GST-S665A were expressed in competent E. coli - strain Rosetta DE3 (Millipore). The bacteria were grown at 37°C until OD_{600} 0.4–0.6 and subsequently induced with 0.3mM IPTG for 22h at room temperature. Cells were pelleted and lysed in lysis buffer (50mM Hepes pH7.4, 150mM NaCl, 1mM EDTA, 5% glycerol, 0.1% Nonidet P-40) supplemented with 100µg/ml lysozyme, aprotinin, leupeptin, pepstatin, 1mM PMSF (all from Roth) and 1mM dithiothreitol. GST proteins from the lysates were allowed to bind to glutathione magnetic beads for 1h on a rotator at 4°C, washed with PBS, eluted in 50mM tris with 10mM reduced glutathione pH8.0 (all from Sigma-Aldrich) and stored at 4°C.

**In vitro kinase assay.** In vitro phosphorylation of Pg-GST-WT and –S665A was performed by incubating 20 µg GST fusion protein with 2.500U PKA catalytic subunit (New England Biolabs GmbH, Frankfurt am Main, Germany) or 100ng PKCa (Abcam) with 40µM phorbol-12-myristate-13-acetate in 1x NEBuffer for protein kinases (New England Biolabs) supplemented with 4mM ATP (Sigma-Aldrich), aprotinin, leupeptin, pepstatin and 1× phosphatase inhibitor cocktail (Roche) in a total volume of 30 µL at 30°C for 1 hour. The reaction was terminated by heating at 95°C for 5 minutes in SDS loading buffer. Phosphorylation was analyzed by 6% Phos-Tag SDS-PAGE performed as described above.

**Fluorescence Recovery after Photobleaching (FRAP).** For FRAP studies, HL-1 cells were seeded in 8-well imaging chambers (Ibidi, Martinsried, Germany) and transfected with Dsg2-GFP as described above. FRAP measurements were performed 48h after transfection in a constant atmosphere at 37°C with 5%CO_{2} using a cage incubator (OKOLAB, Burlingame, CA). The FRAP wizard software (Leica) was used to conduct and analyze the measurements. After definition of regions of interest at the cell junction containing a desmosome, the Dsg2-GFP signal was recorded. After a short pre-bleach time the Dsg2-GFP was bleached using the 488-nm laser line at 100% transmission and the fluorescence recovery was captured over time.

**Förster resonance energy transfer (FRET).** For the detection of cAMP changes after Iso and F/R treatment, HL-1 cells were transfected for 48h with the FRET sensor Epac1-cAMPS.13 Measurements were conducted as previously described.14 In brief, the setup consisted of an inverted microscope (Carl Zeiss Microscopy) equipped with a Polychrome V light source (TILL Photonics, Martinsried, Germany), a MultiSpec-MicroImager DualView (Photometrics, Tucson, USA) a CFP/YFP dual-band filter set (AHF analysetechnik, Tuebingen, Germany) and a CoolSNAP-HQ2 digital camera (Photometrics). Measurements were performed using the MetaFluor software (Molecular Devices, Sunnyvale, USA). Cells were illuminated at 436nm for 20ms every 6s and emission intensities at 535nm and 480nm were recorded. Emissions at
535nm were corrected for CFP bleed through and direct excitation of YFP. An increase in intercellular cAMP levels causes an increase in the CFP/YFP ratio with this sensor.

Transmission electron microscopy (TEM). Confluent HL-1 cells were washed in PBS and fixed with 1% glutaraldehyde in PBS. Samples were prepared as described previously. Images were acquired with a ZEISS Libra 120 TEM (Zeiss, Oberkochen, Germany) using a CCD camera (Tröndle, Moorenweis, Germany).

Histology. Hearts were fixed in 2% PFA in PBS and embedded in paraffin; 10µm thick tissue sections were stained with Azan Heidenhein using Azokarmin G, aniline blue and orange G and embedded with DePex. The staining results in red nuclei, red-orange muscle fibers and blue collagen fibers. Samples were imaged with a Leica DMI8 microscope (Leica) equipped with a 20x objective using LAS 5 software (Leica). For analysis of fibrosis in Pg KO hearts compared to WT littermates, three transversal slices per mouse from the entire ventricle were randomly selected and analyzed. For analysis of hypertrophy, the cross-sectional area of 100 cardiomyocytes per mouse was analyzed in the same slices. Both was done by a blinded person using ImageJ software (NIH).

Langendorff heart preparation. Adult murine hearts were used for Langendorff heart preparations on a commercially available apparatus (AD Instruments, Spechbach, Germany) and analyzed using LabChart7 software as described previously. Animal handling and sacrifice was in accordance with guidelines of the European Commission and local university regulations. Adult Balb/c mice or 11-week-old Pg KO with their corresponding wild-type (WT) littermates were obtained from the institutes’ animal facilities. Isoproterenol was applied at 2µM for the indicated time period using a perfusion pump. ECG was measured via needle electrodes and pulse pressure was determined via the aortic cannula. For immunostaining, hearts were mounted in tissue freezing medium (Leica) and snap-frozen in liquid nitrogen.

ELISA-based measurements of cAMP levels. cAMP levels of confluent HL-1 cells were measured after indicated treatment using an ELISA-based cAMP enzyme immunoassay kit (Sigma-Aldrich), according to the manufacturer’s instructions similar to a previous study.

Image analysis and processing. For image processing, Photoshop CS5 (Adobe, San José, USA) was used. Origin 9.1 (Originlab, Northampton, MA, USA) and Excel (Microsoft, Redmond, WA) were applied for analyzing and creating graphs and plots. Histology, immunostained images and Western blot bands were analyzed using ImageJ software (NIH). For analyzing AFM force-distance curves and processing AFM images JPK data processing software (JPK instruments) was applied. For deconvolution of immunostained images, Huygens essential software (Scientific Volume Imaging b.v., Hilversum, Netherlands) was used.
Supplemental References:


Online Figure Legends:

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Online Figure II. Dsg2-coated tip detects Dsg2-specific binding events and cAMP enhancement has no effect on distribution of N-Cad binding. A, Representative atomic force microscopy (AFM) topography images of a Dsg2-Fc-coated cantilever probed on HL-1 cardiomyocytes before and after addition of a monoclonal antibody targeting the Dsg2 extracellular domain (Dsg2 mAb, green line denotes the cell border), scale bar: 1µm. B, Analysis of the frequency of Dsg2-mediated binding events following Dsg2 mAb addition (n=5), *P<0.05 vs. control. C, Binding frequency of Dsg2-Fc-coated AFM tips probed on mica functionalized with Dsg2-Fc before and after addition of Dsg2 mAb (n=3), *P<0.05 vs. control. D, Representative images of AFM experiments performed with N-Cad-Fc-functionlialized cantilevers on living HL-1 cardiomyocytes before and after cAMP increase (green line denotes the cell border). Scale bar: 1µm. E, Corresponding distribution ratio of N-Cad-mediated binding events on HL-1 cells (n=6), nsP>0.05 vs. control.

Online Figure III. Analysis of single cardiomyocytes released from murine heart slices during dissociation assay. A - D, Cardiomyocytes dissolved ex vivo by dissociation assays are viable before fixation, demonstrate intact cell membranes and sarcomere systems as confirmed by F-Actin (green), Dsg2 (magenta) and N-Cad (red) staining, scale bar: 25µm. Dsg2 and N-Cad are still present at the former intercalated disc (arrows B - E). E, Zoomed area marked as white box in D. Scale bar: 5µm. F, Bright field microscopy of a fixed dissociated cardiomyocyte after fixation, scale bar: 25µm.

Online Figure IV. β-adrenergic signaling causes translocation of Dsg2-GFP in HL-1 cells and ICD reorganization in ex vivo-perfused hearts. A, Schematic of the analysis and calculation of Dsg2 staining. Black circles mark representative single Dsg2 signals. B, Representative images of Dsg2-GFP expressed in HL-1 cells display recruitment to cell borders similar to endogenous Dsg2 (see Fig. 3A) (upper row), scale bar: 20µm. Lower row shows corresponding height projection of z-stack images of 0.13µm thin sections in xy-direction (n=3), scale bar: 20µm. C, Dsg2 (red) and N-Cad (green) immunostaining of murine hearts perfused ex vivo with 2µM Iso or perfusion buffer for 60min (white boxes mark zoomed areas, scale bar: 5µm), scale bar: 20µm (n=3).

Online Figure V. β-adrenergic signaling has no effect on desmosomal protein amount and desmosome composition. A, Immunoprecipitation (IP) of Dsg2 in HL-1 cardiomyocytes shows no differences in total (Input) and co-immunoprecipitated protein amounts of Dp, Pkp2 and Pg after F/R treatment. To exclude unspecific binding, IP with normal rabbit immunoglobulin G control (IgG rb) was performed in parallel (n=4). B, Separation of cytoskeleton-bound and non-
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**Online Figure VI. Characterisation of Pg KO mice.**

A, Morphology and histology of cardiac specific Pg knock out (Pg KO) mice compared to wild-type (WT) littermates at age of 2 and 11 weeks. Left panel: Cardiac morphology of freshly prepared hearts. Arrows indicate dysmorphic areas, arrow heads highlight fibrotic areas, scale bar: 2.5mm. Right panel: Azan stained heart sections to detect fibrosis. Arrow heads highlight ventricular fibrotic areas (blue staining), scale bar: 300µm (3 mice per condition). B, Area of fibrosis per transversal section in Pg KO mice compared to WT littermates at age of 2 and 11 weeks, analysed in Azan stained heart section with representative image in A. (n=3 mice per condition with 3 sections per mouse), *P<0.05, nsP>0.05. C, Analysis of cardiomyocyte hypertrophy by quantification of the cardiomyocyte cross-sectional area from cardiac histological sections. (n=3 mice per condition with 100 cardiomyocytes per mouse), nsP > 0.05, *P<0.05. D, Expression of ICD proteins in Pg KO mice compared to WT littermates at age of 2 and 11 weeks, detected by Western blot analysis. α-Tubulin (α-Tub) served as loading control (n=3 mice per condition). E, Quantification of Western blot analysis in D. Values of densitometric measurements were normalized to α-Tub as loading control and expressed as fold of a corresponding WT littermate. Every data point represents the result of one Pg KO/WT combination with horizontal black bar as mean ±SEM (n=3 mice per condition), *P<0.05 vs WT.

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**Online Figure VIII. β-adrenergic effects on cardiomyocyte cohesion and Dsg2 distribution are PKA-dependent.**

A, Dissociation assays of HL-1 cells following modulation of PKA or Epac activity (control n=18; F/R n=21; H89 + F/R n=10; 6-Bnz-cAMP n=16; O-Me-cAMP n=14), *P<0.05, nsP>0.05 vs. control. B, Dissociation assays of HL-1 cells transfected with non-target siRNA (siNT) or siRNA specific for PKA catalytic subunit α and β (siPKA) with and without cAMP enhancement (siNT n=12; siNT + F/R n=13; siPKA n=10; siPKA + F/R n=13), *P<0.05, nsP>0.05. Knockdown efficiency was confirmed by Western blot analysis (right panel). C, Dissociation assays of murine heart slices with inhibition or activation of PKA (control n=10; F/R n=8; H89 + F/R n=11; 6-Bnz-cAMP n=6), *P<0.05. D, Immunostaining of HL-1 cells after PKA-inhibition and subsequent F/R or PKA-activation by 6-Bnz-cAMP (representative images of three independent experiments), scale bar: 20µm. For corresponding F/R images see Fig. 3A. E, F, Distribution of Dsg2-mediated binding events after PKA-inhibition by H89 and subsequent cAMP elevation (n=6), scale bar: 1µm. For corresponding F/R images see Fig. 1C, *P<0.05, nsP>0.05 vs. control.
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