Hyperactive Adverse Mechanical Stress Responses in Dystrophic Heart Are Coupled to Transient Receptor Potential Canonical 6 and Blocked by cGMP–Protein Kinase G Modulation

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Rationale: The heart is exquisitely sensitive to mechanical stimuli to adapt rapidly to physiological demands. In muscle lacking dystrophin, such as Duchenne muscular dystrophy, increased load during contraction triggers pathological responses thought to worsen the disease. The relevant mechanotransducers and therapies to target them remain unclear.

Objectives: We tested the role of transient receptor potential potential canonical (TRPC) channels TRPC3 and TRPC6 and their modulation by protein kinase G (PKG) in controlling cardiac systolic mechanosensing and determined their pathophysiological relevance in an experimental model of Duchenne muscular dystrophy.

Methods and Results: Contracting isolated papillary muscles and cardiomyocytes from controls and mice genetically lacking either TRPC3 or TRPC6 were subjected to auxotonic load to induce stress-stimulated contractility (SSC, gradual rise in force and intracellular Ca2+). Incubation with cGMP (PKG activator) markedly blunted SSC in controls and Trpc3−/−; whereas in Trpc6−/−, the resting SSC response was diminished and cGMP had no effect. In Duchenne muscular dystrophy myocytes (mdx Utrophin deficient), the SSC was excessive and arrhythmogenic. Gene deletion or selective drug blockade of TRPC6 or cGMP/PKG activation reversed this phenotype. Chronic phosphodiesterase 5A inhibition also normalized abnormal mechanosensing while blunting progressive chamber hypertrophy in Duchenne muscular dystrophy mice.

Conclusions: PKG is a potent negative modulator of cardiac systolic mechanosignaling that requires TRPC6 as the target effector. In dystrophic hearts, excess SSC and arrhythmia are coupled to TRPC6 and are ameliorated by its targeted suppression or PKG activation. These results highlight novel therapeutic targets for this disease. (Circ Res. 2014;114:823-832.)

Key Words: cardiac ■ stress mechanics ■ pharmacology ■ muscular dystrophy, Duchenne ■ muscle contraction ■ myocytes, cardiac

The working heart rapidly adjusts to changes in mechanical load to adapt to physiological demand. A primary example is the augmentation of contractility that ensues when a heart is subjected to higher afterload as occurs with increased systemic resistance. This stress-stimulated contractility (SSC) response, often termed the Anrep effect,1,2 allows the heart to provide similar cardiac output, despite higher load. The mechanisms are thought to involve mechanostimulated proteins that ultimately result in a rise of intracellular calcium [Ca2+]i. Candidates for these transducers revealed in passively stretched noncontracting cells include stretch-activated G-protein–coupled receptors such as the angiotensin type 1 receptor,3 members of the transient receptor potential (TRP) superfamily of cation channels,4–7 and the recently described piezo (1 and 2) proteins.8 The SSC response, however, involves stress imposed during contraction, and here data identifying the relevant transducers remain scant. Understanding this signaling may be particularly important to disorders such as Duchenne muscular dystrophy (DMD), in which a lack of the cytoskeletal protein dystrophin results in pathologically augmented responses to systolic load, which are thought to be a core mechanism for progressive muscle disease.

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Insight into this signaling pathway may be gleaned from studies of more sustained pressure overload that identified both TRP canonical (TRPC) 3 and TRPC6 channels as modulators of the pathological cardiac response.9–11 TRPC6 is also a putative mechanosensitive channel in noncontracting cells,6,7 although this remains somewhat controversial.12 Another feature shared by TRPC3 and TRPC6 is their post-translational modification by the serine/threonine kinase, protein kinase G (PKG) at analogous residues in their intracellular N terminus (T11 and S263 for TRPC3, T70 and S322 for TRPC6, human gene).13–17 This modification reduces channel conductance in vitro and, for TRPC6, has been shown to suppress activation of a calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway and its associated hypertrophy,14,16,17 Whether PKG modification of these channels also affects mechanosensing is unknown. Intriguingly, strategies to stimulate PKG vitro and, for TRPC6, has been shown to suppress activation of a calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway and its associated hypertrophy,14,16,17 Whether PKG modification of these channels also affects mechanosensing is unknown. Intriguingly, strategies to stimulate PKG as currently being studied in experimental18–21 and human dystrophinopathy22 spawned initially by its potential effect on vasomotor function in skeletal muscle,22,23 and some early evidence supports cardiac benefits.20,21

Accordingly, the present study tested the role of TRPC3 and TRPC6 in the modulation of systolic mechanosensing in cardiac muscle and intact myocytes and whether their influence is regulated by PKG. Second, we tested whether this pathway is altered in experimental cardiac muscular dystrophy and if so, whether it is ameliorated by acute and chronic PKG activation. We reveal PKG to be a potent negative modulator of the SSC via a TRPC6-dependent mechanism. Furthermore, we show that TRPC6 mechanosignaling is excessive in dystrophin-deficient muscle and cells leading to abnormal calcium entry, excess force, and arrhythmia. All of these are blocked by targeted TRPC6 suppression and by acute or chronic PKG activation.

**Methods**

An expanded Methods section is available in the online-only Data Supplement. All studies were conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Animals and reviewed by the Institutional Animal Care and Use Committee at Johns Hopkins University, where the work was performed.

**Animals**

TRPC3 knockout (KO; Trpc3−/−) and TRPC6 KO (Trpc6−/−) mice were generated as previously described.21,22 Each TRPC KO mouse was backcrossed for ≥5 generations into a C57BL6/J background, and their cross yielded the combined TRPC3/TRPC6 double KO (Trpc3−/−/Trpc6−/−) mouse. Heterozygous mice were crossed to yield KO and littermate controls. Both KO models displayed selectivity for the TRPC channel involved, preserving normal levels of expression for other TRPC channels (Online Figure I). For all studies, age-matched gene−/− and gene+/− littermate controls at 4 to 6 months of age were used. For a model of DMD, female mice lacking dystrophin (mdx, Jackson Laboratories) were crossed with mice with a heterozygous deletion of utrophin (utrn, Jackson Laboratories) and studies performed in either mdx/utrn−/− at 4±1 months of age or in a few instances, mdx/utrn−/− animals at 6 to 10 weeks of age because of their early mortality. Male mdx/utrn−/− were crossed with female Trpc6−/− to generate male utrn−/−/Trpc6−/− and female mdx/utrn−/−/Trpc6−/−, and their cross yielded male mdx/utrn−/−/Trpc6−/−. Studies were performed at 4±1 months of age. Only male mice were studied, with some controls provided by male C57BL6/J mice as well.

**Pharmaceuticals**

A new selective dual TRPC3/TRPC6 small molecule inhibitor GSK2833503A (GSK503A, also denoted as example 19)23,24 was provided by GlaxoSmithKline Pharmaceuticals. GSK503A has an IC50=21 nmol/L for TRPC3 and 3 nmol/L for TRPC6. Corresponding IC50 for volgate-gated calcium (Cav1.2) and sodium (Nav1.5), hERG, and TRP vanilloid TRPV1 and TRPV4) channels are 10000, 3300, >50 000, 6300, and 12,500, respectively. Cell permeable cGMP analog (8-pCPT-cGMP) and cAMP analog (8-Br-cAMP) were obtained from Sigma, and purified sildenafil from Pfizer. For in vivo studies, sildenafil citrate (Revatio, Pfizer) was compressed into soft rodent chew (Transgenic Dough Diet, Bio-Serv) and provided at a dose of 200 mg/kg per day for 2 months as described previously.25 This dose yields a free plasma concentration in the range of 30 to 50 nmol/L, well within the selective range for phosphodiesterase (PDE) 5A.

**Papillary Muscle Studies**

Papillary muscle studies were performed as previously described.26 Briefly, hearts were rapidly excised and placed in modified Krebs–Henseleit solution containing 30 mmol/L, 2.3-butadienone monoxime. The Krebs–Henseleit solution contained (in mmol/L) 141 NaCl, 50 dextrose, 25 NaHCO3, 5 HEPES, 5 KCl, 1.2 NaH2PO4, 1 MgSO4, and 2.0 CaCl2, pH adjusted to 7.35, and bubbled with 95% O2 and 5% CO2. Thin papillary muscle strips with chordae tendineae were dissected from the right ventricle. The muscle was connected to a force transducer (Scientific Instruments GmbH, Heidelberg, Germany) at one end and mechanical anchor at the chorda tendineae end, allowing the muscle to contract auxtonically. Calcium was measured by Fura-2AM (340 nm and 380 nm excitation, 510 nm emission). Fluorescence was collected by photomultiplier tube (R1527, Hamamatsu, Japan) with background recorded before dye loading. Fura-2AM (50 μg) was dissolved in 25 μL dimethyl sulfoxide and 25 μL Pluronic to which 2.755 mL Krebs–Henseleit, 4.3 mg/L tetrakis-(2-pyridylmethyl)ethylenediamine, and 5.0 mg/L cremophor were added. Muscles were loaded with this solution for 30 minutes. The muscle length (Lmax) generating maximal developed force (max–min force; ΔF) was determined and then length reduced to 92% of this value. On achieving steady state developed force (ΔF), muscles were stretched to 98% Lmax and force and Ca2+ were recorded for 10 minutes to assess the SSC. Figure 1 displays a schematic for the SSC response and its analysis. Developed force rose immediately on stretch (ΔF1), Frank–Starling Mechanism (FSM) and was indexed by ΔF1/ΔF. The subsequent more gradual rise in developed force (ΔF2−ΔF1) assessed SSC.

**Isolated Cardiac Myocyte Studies**

SSC analysis was also performed in isolated loaded cardiac myocytes. Cell isolation was performed as described (online-only Data Supplement Methods).22 Myocytes were incubated for 15 minutes with 1 μmol/L indo1-AM (Invitrogen, Molecular Probes, Carlsbad, CA) in Tyrodes (1.0 mmol/L Ca2+). The ratio of fluorescence emitted at 405 nm and 485 nm measured [Ca2+]i. Myocytes were then mounted on a custom force-length control system as previously
described. Rod-shaped quiescent single cardiomyocytes were selected, and a pair of carbon or glass fibers (6 μm at one end, 20 μm at the other) coated with a biological adhesive (MyoTak) was attached to both ends using micromanipulators. The thin fiber was compliant and its position digitally controlled by a piezoelectric translator (Physik Instrumente, P-841–80), whereas the other fiber was rigid and served as a mechanical anchor. Cells were electrically stimulated at 0.15 to 0.2 Hz with 15-ms pulses. Cardiomyocyte sarcomere length and fiber tip displacements were recorded at 120 Hz and analyzed in real time using IonOptix (MA) equipment and software. Cells were then stretched to increase sarcomere length by ≈4%, and active and passive force (F) was determined from fiber bending moment given by F=K(ΔLp−ΔLf), where K is the effective fiber stiffness; ΔLp, the change in distance between the 2 fibers; and ΔLf, the displacement of piezoelectric translator. Components of the SSC were determined as for papillary muscle data.

Acute Transaortic Constriction In Vivo Studies
Adult Trpc6−/− or littermate control mice were anesthetized with 3% isoflurane, the chest opened between ribs 2 to 4, and a 26-G needle placed on the transverse aorta. A micro pressure-volume catheter (Millar Instruments, TX) was inserted through a left ventricular apical stab and positioned so that the distal tip lay in the proximal aortic root. Instantaneous pressure–volume loops were recorded using custom-developed hardware and software (WinPVAN). After obtaining rest data, the aorta was constricted with a 6.0 suture around the aorta and 26-G needle to increase ventricular afterload. Pressure–volume data were recorded during the initial 15 minutes after aortic banding.

Statistical Analysis
Statistical analyses used 1-way or 2-way ANOVA or ANCOVA for normally distributed data with equal variance among groups. For other data, we used a Kruskal–Wallis test. Post hoc analysis used a Tukey–Kramer test or Mann–Whitney test as appropriate. Analysis was performed using SigmaStat Ver 13 and Systat version 10 software.

Results
cGMP/PKG Activation Markedly Suppresses SSC
To test whether PKG modifies systolic mechanotransduction in heart muscle, SSC was first measured in isolated cardiac papillary muscles with or without incubation with membrane permeable cGMP. On exposure to 6% auxotonic stretch, cGMP/PKG stimulation blocks cardiac muscle or cell contractility enhancement with stress increase. A–G, Force and calcium transients in isolated cardiac muscle pretreated with or without 8-pCPT-cGMP. Right, Summary data for stress-stimulated contractility (SSC) response (n=12–14/group, *P<0.05 vs +veh by ANCOVA). D–G, Force and calcium in auxotonically contracting cardiomyocytes. The SSC was suppressed by exposure to cGMP, but this was prevented by inhibition of PKG (DT3, 0.2 μmol/L, n=5–24/group, *P<0.05 vs +veh, #P<0.05 vs +cGMP). Veh indicates vehicle.

![Figure 1. Stress-stimulated contractility (SSC).](image)

![Figure 2. Cyclic GMP/protein kinase G (PKG) stimulation blocks cardiac muscle or cell contractility enhancement with stress increase.](image)
muscles exhibited an immediate rise in developed force reflecting length-dependent activation (FSM) followed by a gradual 20% to 30% rise in contractility (SSC) accompanied by high peak [Ca\(^{2+}\)] transient that occurred during the ensuing 10 minutes (Figure 2A). The FSM was unaltered, whereas the SSC response was markedly suppressed by preincubation with 8-pCPT-cGMP (1.0 mmol/L; Figure 2B and 2C).

Cyclic GMP-PKG modulation. Cyclic GMP-PKG can also target vascular cells and fibroblasts potentially influencing the muscle response; therefore, we tested whether its regulation of the SSC was myocyte autonomous. Isolated myocytes were attached to carbon or glass microfibers, and the same protocol was performed. With a rise in auxotonic stress, we again observed an immediate rise in developed force (FSM) followed by SSC (Figure 2D). Incubation with cGMP (0.1 mmol/L) did not alter the FSM but markedly depressed the SSC (Figure 2E). When myocytes were pretreated with the PKG inhibitor DT3 (0.2 µmol/L), the SSC response was markedly suppressed by preincubation with 8-pCPT-cGMP (1.0 mmol/L; Figure 2F).

The rapid FSM response was similar in all groups (Online Figure III). In Trpc3−/− muscles exhibiting 10 minutes (Figure 3A), whereas Trpc6−/− muscle had a significantly blunted force and Ca\(^{2+}\) SSC response (Figure 3B). The rapid FSM response was similar in all groups (Online Figure III). In Trpc3−/− muscle, incubation with 8-pCPT-cGMP also still profoundly inhibited the SSC as in littermate controls (Figure 3C; summary data Figure 3E). However, in Trpc6−/− mice, the SSC response remained unaltered despite cGMP incubation (Figure 3D), whereas littermate controls displayed marked suppression (Figure 3F). Thus, TRPC6, not TRPC3 contributes to the SSC response and is required for its suppression by PKG.

Because vascular cells and fibroblasts also express TRPC6, we again tested its role in isolated cardiomyocytes. Controls showed a robust SSC response that was cGMP inhibited, whereas Trpc6−/− cells displayed a blunted SSC with no change in the response after cGMP incubation (Figure 4A). As an alternative to gene deletion, we also tested the role of TRPC6 with a new selective small molecule TRPC3/6 blocker (GSK503A, 5 µmol/L). Given that the SSC was unaltered by TRPC3 gene deletion, GSK503A effects were interpreted as dependent on TRPC6. GSK503A depressed the SSC by ≈30%, similar to results from Trpc6−/− cells (Figure 4B). As a negative control, we tested GSK503A in cardiac myocytes lacking both Trpc3 and Trpc6 and found no SSC effect, supporting its selectivity (Figure 4B).

Last, we tested the relevance of TRPC6 mechanosensing in the intact heart. Hearts in situ were subjected to 15 minutes of increased afterload induced by proximal aortic constriction and pressure–volume loops recorded (Figure 4C). In both groups, the rise in afterload was first manifest by loops rapidly becoming taller and narrower and shifting rightward (shown by the response after 1 minute). In controls, the loops then gradually shifted leftward, reflecting increased contractility countering the persistently high afterload. However, this latter response was reduced in Trpc6−/− hearts. Mean data for maximal rate of pressure rise (dP/dt\(_{max}\)) and relaxation time constant (τ) are shown in Figure 4D. Controls with a rise in contractility and little delay in relaxation after high afterload contrasted to Trpc6−/− mice that displayed a fall in contractility and delayed relaxation. Taken together, SSC modulation by cGMP requires TRPC6 and is independent of TRPC3.

Figure 3. Transient receptor potential canonical (TRPC) 6 not TRPC3 contributes to stress-stimulated contractility (SSC) and is required for SSC suppression by cGMP/protein kinase G (PKG). A and B, Force–time tracings and calcium transient examples from cardiac trabeculae exposed to mechanical stress from mice lacking either Trpc3 or Trpc6. C and D, Similar tracings in muscle exposed to 8Br-pCPT-cGMP. Exposure to cGMP depressed the SSC response in Trpc3−/− but not in Trpc6−/− muscle. E and F, Summary results for SSC force or calcium temporal response (n=4–13/group). Unlike Trpc3−/−, Trpc6−/− muscle responses in both parameters were not significantly altered by the addition of cGMP (both P>0.05). Significant differences between responses based on ANOVA, denoted by the symbols: *P<0.05 Ctrl+veh, **P<0.05 vs Trpc3−/−+veh. F, *P<0.01 vs Ctrl+veh, †P<0.05 Trpc6−/−+veh vs Ctrl+veh, ††P<0.05 Trpc6−/−+cGMP vs Ctrl+cGMP. Ctrl indicates control; and veh, vehicle.
these results indicate that TRPC6 is an important mechanotransducer in cardiomyocytes and in vivo heart and is required for the modulation of cGMP-PKG suppression of afterload SSC.

**Hyperactive SSC and Arrhythmia in Myocytes Lacking Dystrophin/Utrophin Are Related to TRPC6 and Suppressed by PKG Activation**

We next asked whether TRPC6-dependent systolic mechanostimulation was abnormal in cells lacking dystrophin and utrophin (mdx/utrn+−) and whether this too could be modulated by PKG activation. The combined mutation model was used because the absence of dystrophin alone produces a mild phenotype in mice, in part, caused by compensatory upregulation of utrophin. Mice fully lacking both genes display a severe skeletal and cardiac pathology with early mortality (6–8 weeks), whereas partial utrophin deletion still leads to earlier and more prominent heart disease (Online Figure IV) but can be more easily studied. In mdx/utrn+− (and mdx/utrn−−) cells, the SSC force and corresponding [Ca2+]i transient increase were markedly amplified compared with controls (Figure 5A). Stressed cells frequently displayed arrhythmia minutes after load was increased, in some instances leading to cell demise. This exacerbated SSC response in myocytes from the DMD-model heart was fully blocked by preincubation with cGMP (Figure 5B and 5C).

**Figure 4. Genetic deletion or pharmacological inhibition of transient receptor potential canonical (TRPC) 6 blocks cardiac cell and whole heart stress-stimulated contractility (SSC) response.**

A, Trpc6−/− cardiomyocytes display a similar SSC response, despite cGMP exposure in contrast to controls (n=6–8/group, *P<0.001 vs Ctrl+veh, †P<0.05 Trpc6−/−+veh vs Ctrl+veh, ‡P<0.01 Trpc6−/−+cGMP vs Ctrl+cGMP by ANCOVA). B, TRPC3/6 inhibitor (GSK503A) blocks SSC in wild-type but not in Trpc3−/−/6−/− cells (n=7–24/group, *P<0.05 vs Ctrl+veh). C, Intact heart response to acute pressure overload depicted by pressure–volume loops. Trpc6−/− mice had a depressed inotropic response versus controls. See text for details. D, Summary results for maximal peak rate of pressure rise (dP/dtmax, contractility) and relaxation (time constant, τ) normalized to baseline; n=5 to 8/group, *P<0.05 vs Ctrl by ANCOVA. Ctrl indicates control; LV, left ventricular; and Veh, vehicle.

**Figure 5. Dystrophin/utrophin deficiency amplifies stress-stimulated contractility (SSC; force and Ca2+) and associated arrhythmia; all are suppressed cGMP.**

A, Example force and calcium transients from control, mdx/utrn−−, and mdx/ utrn−− myocytes after stress increase. B, Abnormal force/calcium response is prevented in both normal and Duchenne muscular dystrophy myocytes by cGMP. C, Summary data; n=5 to 24/group, *P<0.001 Ctrl+cGMP vs Ctrl+veh, #P<0.001 mdx/ utrn−−+cGMP vs mdx/utrn−−+veh, †P<0.01 mdx/utrn−−+cGMP vs Ctrl+veh by ANCOVA. Ctrl indicates control; and veh, vehicle.
In MD cells, gene expression for Trpc6 was elevated ≈3-fold compared with controls (Figure 6A), whereas expression of Trpc1 and Trpc3 was unchanged. Poor signal/noise and antibody specificity combined with low expression levels precluded detection of protein by immunoblot; however, evidence for a functional role of TRPC6 was obtained by incubating DMD cells with GSK503A. This reversed amplified force and Ca2+ SSC responses to control levels (Figure 6B).

Figure 6C summarizes the effect of cGMP or GSK503A incubation on stress-induced arrhythmia. In DMD cells, arrhythmia prevalence was 6× higher than control but was restored to normal by cGMP or GSK503A but not cAMP (n=12–28/group, *P<0.002 vs Ctrl+veh, #P<0.02 vs mdx/utrm+veh, †P=0.06 vs Ctrl+cGMP, ‡P<0.001 vs mdx/utrm+cGMP). D, Myocyte SSC response (+cGMP) in myocytes from mdx/utrm+/− hearts. Genetic deletion of Trpc6 in mdx/utrm+/− reversed amplified SSC responses present in mdx/utrm+− (dashed lines, from Figure 5C) and eliminated sensitivity of SSC to cGMP. Data from n=6 to 10/group, *P<0.01 vs mdx/utrm+−+cGMP by ANCOVA. Ctrl indicates control; and veh, vehicle.

Acute and Chronic Treatment of Dystrophic Myocytes and Hearts by PDE5A Inhibition Blocks Hyperactive SSC and Arrhythmia

Activation of PKG can be pharmacologically achieved in vivo by stimulating cGMP synthesis (nitrates or natriuretic peptides) or by blocking its hydrolysis by PDEs such as PDE5A. The latter is more amenable to chronic therapy (eg, sildenafil [SIL]) and has been shown to improve skeletal muscle fatigue18 and blunt progressive cardiac dysfunction in mdx mice.20 Incubation of normal cells with SIL alone (1 µmol/L for 10 minutes) did not alter the SSC (Figure 7A). However, the efficacy of PDE5A inhibition is itself dependent on how much cGMP is present, and resting isolated cells have low levels. We, therefore, next identified a low cGMP dose (1/10th that used previously, eg, 0.01 mmol/L) that had no effect itself on the SSC (Figure 7B); however, when combined with SIL, the SSC was suppressed (Figure 7C; summary data Figure 7G). In mdx/utrm+−, SIL alone was effective (Figure 7D), potentially because of higher basal PDE5A activity in the model (3.5±0.5-fold compared with controls; P<0.001; Figure 8E). Combining SIL with low-dose cGMP further suppressed the SSC (Figure 7F and 7G).

To test if chronic PDE5A inhibition also ameliorated abnormal myocyte mechanoresponses in DMD, mdx/utrm+− mice...
were treated for 2 months with SIL (200 mg/kg per day PO). This therapy blunted progressive chamber hypertrophy (Figure 7A and B) and reduced myocardial volume (Figure 7C). Cardiac function (fractional shortening) also declined slightly in the placebo group but was maintained in SIL-treated mice (Online Figure V). Fetal gene markers of pathological hypertrophy (Nppa and Trpc6) gene expression were elevated in DMD myocardium and were significantly reduced with SIL treatment (Figure 8D). Changes in Trpc6 were associated with modest directionally similar changes in calcineurin protein expression, although regulator of calcineurin-1 gene expression was similar in all groups (Online Figure VI). DMD mice displayed 4-fold higher myocardial PDE5A activity compared with controls and this declined with SIL treatment (Figure 8E). By contrast, PDE1 activity was similar to controls and unaltered by SIL (Figure 8E).

Figure 8F displays the effects of chronic SIL treatment on cellular mechanostimulation, the primary focus of this study. SIL-treated cells displayed an essentially normal SSC response compared with those receiving placebo. When further incubated with GSK503A, the SSC response was unaltered in cells from SIL-treated DMD hearts. This is consistent with the notion that chronic SIL therapy had already reversed TRPC6 hyperactivity.

Discussion

This investigation reveals TRPC6 as a modulator of systolic load–induced contractility increases in cardiomyocytes, muscle, and intact hearts and that it is also required for PKG-mediated suppression of this mechanical response. The normally adaptive mechanism is pathologically amplified in heart muscle lacking dystrophin/utrophin, resulting in dysregulated force and [Ca\(^{2+}\)] and arrhythmogenicity. Hyperactive mechanosensing in DMD is suppressed greatly by inhibiting or genetically deleting TRPC6 or by activating the cGMP/PKG pathway so long as TRPC6 is present. These results provide new insight into normal stress-induced contractility adaptations and reveal a novel therapeutic target for the dystrophic heart.

The SSC (or Anrep) response has been recognized for more than a century, yet its mechanisms have remained uncertain. Some attribute it to stretch-activated Gq protein–coupled receptors (GqPCRs, eg, angiotensin-II and endothelin-1) which via reactive oxygen species\(^{35}\) generate extracellular signal–regulated kinase 1/2 activation of the Na\(^+\)/H\(^+\) exchanger 1. The latter in turn results in higher [Ca\(^{2+}\)] via reverse mode Na\(^+/\)Ca\(^{2+}\) exchange.\(^{2}\) Other studies support activation of stretch-activated channels\(^{34}\) that themselves conduct Na\(^+\) and Ca\(^{2+}\), with [Ca\(^{2+}\)]\(^{2}\) being further enhanced by the Na\(^+/\)Ca\(^{2+}\) exchange, Na\(^+\)/H\(^+\) exchanger 1, or Ca\(^{2+}\) released from internal stores.\(^{35}\) The current results help reconcile these mechanisms by placing TRPC6 as an important upstream mechanosensor. TRPC6 is stimulated by GqPCRs, and in other cell types, can also activate extracellular signal–regulated kinase 1/2\(^{36,37}\) upstream of Na\(^+\)/H\(^+\) exchanger 1 and conduct Na\(^+\) that in turn triggers Ca\(^{2+}\) entry via the Na\(^+/\)Ca\(^{2+}\) exchange.\(^{38-40}\)

The mechanosensing capacity of TRPC6 was first revealed by Spassova et al\(^{7}\) in studies of smooth muscle cells exposed to osmotic or direct membrane passive stretch. TRPC6 mechanosensing was later reported in quiescent adult cardiac myocytes subjected to plasma membrane shear stress.\(^{8}\) Some, however, have questioned this role and attributed earlier data to stretch-activated GqPCRs and artifacts from TRPC6 overexpression in heterologous systems.\(^{12}\) The present data combine results from physiologically loaded myocytes, muscle, and intact hearts and use gene deletion and selective TRPC3/6 blockade to support the role of TRPC6 as a mechanosensor in both the normal heart and particularly hearts lacking dystrophin. The experiments did not involve artificial overexpression models, and although more proximal GqPCR stretch activation could still play a role, gene deletion and targeted pharmacological blockade studies strongly implicate TRPC6 as a critical node in the stress response.

Modulation of TRPC channel function by PKG was first reported for TRPC3 in human embryonic kidney 293 cells\(^{13}\) and later in TRPC6 in similar cells\(^{15}\) and cardiac myocytes.\(^{14,16}\) For TRPC6, this modification not only reduced cation conductance but also blunted associated signaling via calcineurin/NFAT activation triggered by Gq-coupled agonists.\(^{14,16}\) Phosphosilenced or phosphomimetic TRPC6 mutants display gain or loss of channel function, respectively, with respect to conductance and associated NFAT signaling.\(^{14,16}\) The current data add mechanosignaling to the list of TRPC6-mediated responses modified by cGMP/PKG, and this influence seems...
separable from effects on hypertrophic signaling. First, we identified its role in normal cells exposed to rapid stress that is unlikely to involve hypertrophic cascades. Second, we found that TRPC6 modulation of the SSC was greatly amplified in DMD, despite only modest evidence of calcineurin/NFAT activation in the same hearts. Last, TRPC3 was uninvolved with mechanoactivation or PKG modulation of SSC, yet it has been previously linked to calcineurin/NFAT activation and hypertrophy in chronic pressure-overloaded hearts.10,11,41

Abnormal mechanoresponses in DMD skeletal muscle have long been considered fundamental to the disease. Stretch imposed on contracting DMD muscle induces enhanced [Ca\textsuperscript{2+}] and a decline in tetanic force, and these abnormalities are reduced by lowering Ca\textsuperscript{2+} in the bathing solution or by blocking stretch-activated channels. TRPC1 has been proposed as a relevant stretch-activated channel,44,45 although TRPC6 is also expressed and may be involved.46 The current study focusing on the heart reveals a key role of TRPC6 to altered mechanosensing. Afterload imposed on the heart during systole has a parallel with stretch imposed during skeletal muscle contraction. The consequential amplified rise in [Ca\textsuperscript{2+}] is thought to stimulate myocardial damage. This rise has been linked to plasma membrane defects and altered sarcoplasmic reticulum calcium handling,49,50 and the current study adds TRPC6 hyperactivity to this list.

The current results counter the notion that DMD myocytes are subject to stress-induced membrane tears that nonselectively leak cations because SSC force/Ca\textsuperscript{2+} responses were both normalized by selectively blocking TRPC6 or activating PKG. However, membrane instability stemming because of a lack of dystrophin could well trigger TRPC6 hyperactivity, and surfactants that can stabilize the plasma membrane may also suppress such hyperactivity. Interestingly, muscle cell permeability of Evans Blue in mdx declines from treatment with a stretch-activated channel inhibitor (streptomycin) or with chronic SIL therapy.21

Benefits in DMD skeletal muscle from PKG activation have been reported, with most data showing improved vascular perfusion.18,21,22 Although some benefit has also been observed in the heart,20 mechanisms have remained unclear. While long-term studies remain to be performed, the present data support that a PKG-TRPC6 targeting mechanism is likely involved. The findings that SSC was near fully blocked by cGMP/PKG so long as Trpc6 was expressed yet more modest suppression was observed when TRPC6 was inhibited or genetically deleted suggest that phosphorylated TRPC6 may interact and impair other proteins as well. This would be consistent with the observation that TRPC channels often form heterotetramers. The specific nature of such interactions remains to be elucidated.

There are several limitations of the current study. First, we were unable to measure TRPC6 phosphorylation by PKG directly in adult myocardium or isolated myocytes so as to relate this modification with the physiological behavior. As noted, this was because of low expression levels of the channel (even with enhanced gene expression) and poor antibody signals for both total
and particularly phospho-TRPC6 from these tissues. Indeed, published data stem almost exclusively from human embryonic kidney 293 cells overexpressing the protein.17 Nonetheless, such data confirm a strong influence of PKG-phosphorylation on the channel, suppressing GqPCR or DAG-stimulated conductance and hypertrophy signaling.14,16,17 The failure of PKG to modulate the SSC response in control or DMD myocytes without Trpc6 strongly supports this interaction. The chronic SIL study also supports targeting because cells from PKG-treated DMD hearts displayed no further effect from TRPC3/6 blockade. Another limitation resides with the new small molecule TRPC3/6 inhibitor that could not be tested in vivo, even for acute studies, because of high plasma binding and extremely rapid metabolism.26 This compound has no known influence on TRPC6 phosphorylation and suppressed GqPCR-signaling in cells expressing a TRPC6 phosphomimetic (Trpc6T70E,S322E), indicating independence from this modification.

In conclusion, we identify TRPC6 as a cardiac systolic mechanosensor, primary target of PKG modulation of SSC, and a major contributor to mechanostimulation pathology in the DMD heart. Combined, Duchenne and Becker muscle diseases involving the absence or deficiency of dystrophin account for 80% of all muscular dystrophies. Although the major focus of prior research has been on often catastrophic skeletal muscle disease, the heart is potently involved as well, and heart failure is now a major cause of mortality.51 The present results identify a novel and important role of TRPC6 and its modulation by PKG that may provide a new therapeutic avenue for these diseases.

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- Stretch of contracting cardiac muscle increases calcium entry and augments force; termed stress-stimulated contractility.
- Calcium entry from muscle stretch increases in dystrophin-deficient forms of muscular dystrophy (DMD), leading to cell damage.
- Mechanosensitive transient receptor potential canonical (TRPC) channels are expressed in heart, and their hormone receptor–activated conductance is blocked by protein kinase G (PKG).

What New Information Does This Article Contribute?

- PKG potently blocks stress-stimulated contractility in a TRPC6-dependent manner.
- Increased mechano-stimulated force and arrhythmia in DMD cells are prevented by genetic or pharmacological suppression of TRPC6 or by PKG activation.
- Chronic PKG activation in DMD hearts by phosphodiesterase 5 inhibition reverses hyperactive mechanostimulation in DMD myocytes.

Understanding stress-stimulated contractility in the heart is important because it plays a role in normal adaptations to afterload and is also potentially relevant to forms of muscular dystrophy in which dystrophin is effectively absent. In the latter case, membrane instability is thought to augment stress-induced myocyte calcium entry, contributing to disease progression. Here, we show that increases in intracellular calcium and force that follow a rise in mechanical stress imposed on contracting heart muscle are blocked by PKG activation. This modulation requires the presence of TRPC6. In a mouse model of DMD, we find these responses are hyperactivated and often lead to arrhythmia. Either TRPC6 inhibition, using gene deletion or selective pharmacological inhibition, or acute PKG activation blocks this pathological behavior. We also found that chronic treatment with sildenafil, a cGMP-selective phosphodiesterase type 5 inhibitor, improved measures of dystrophic cardiac function and molecular remodeling and improved calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload. J Am Coll Cardiol. 2009;53:207–215.

What Is Unknown?
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Supplemental Material

Hyperactive Adverse Mechanical-Stress Responses in Dystrophic Heart Are Blocked by cGMP-PKG Modulation of TRPC6

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Supplemental Methods

Cardiomyocyte Isolation
Hearts were quickly removed from the chest after euthanasia. Retrograde perfusion is performed with Ca\(^{2+}\)-free HEPES buffer containing (in mmol/L) 140 NaCl, 5.4 KCl, 0.33 NaH\(_2\)PO\(_4\), 0.5 MgCl\(_2\), 11 glucose, 5 HEPES (pH7.4) at 1.0 ml/min for 3 min, followed by an enzyme solution containing collagenase (1.0 mg/ml collagenase type II, Worthington), protease (0.05 mg/ml, type XIV, Sigma), and 0.1 mmol/L Ca\(^{2+}\) for 7 min. The ventricular tissue was then cut into small pieces and filtered with 250-µm nylon mesh. The calcium concentration of Tyrode solution was gradually increased to 1.0 mmol/L for physiologic analysis.

Echocardiography
In vivo cardiac geometry and function were serially assessed by transthoracic echocardiography (Acuson Sequoia C256, 13 MHz transducer; Siemens) in conscious mice. M-mode LV end-systolic and end-diastolic cross-sectional diameter (LVESD, LVEDD), and the mean of septal and posterior wall thicknesses were determined from an average of 3–5 cardiac cycles. LV fractional shortening (%FS) and LV mass were determined using a cylindrical model as previously described \(^1\).

Polymerase Chain Reaction (PCR)
Quantitative PCR was used to assess fetal and hypertrophic gene expression and TRPC1, 3, or 6 mRNA expression in cells and myocardial tissue. RNA was extracted with TRIzol (Invitrogen) and cDNA synthesized by Taqman Reverse Transcription Reagents and protocol (Applied Biosystems). Quantitative real-time PCR was performed with the 7900HT (Applied Biosystems) or the CFX384 PCR detection system (Bio-Rad) with sample duplicates. Taqman primers and probes for rat and mouse were obtained from Applied Biosystems. Gene expression was calculated using the \(\Delta\DeltaCt\) method, and normalized to GAPDH or 18s rRNA expression.

PDE5A, PDE1 and PKG activity
PDE5 and PDE1 activity was assessed by fluorescence polarization assay (Molecular Devices), following manufacturer’s instruction and as previously described \(^1\). Briefly, heart tissue was homogenized in lysis buffer (Cell Signaling Technologies) and sonicated. Protein concentration was determined used the bichinchoninic acid (BCA) protein assay (Pierce). Complete reaction buffer containing 0.1 % BSA and 1 mmol/L DTT and cGMP substrate solution (100 nmol/L final concentration) were freshly prepared according to manufacturer’s instruction and added to a 384 well plate (sample input ~3 µg total protein). Lung tissue lysates were used as positive control and to generate a standard curve. Sildenafil was added at 1µmol/L final concentration and samples incubated for 1h at 22 °C in the dark. IMAP binding buffer was prepared according to the manufacturer, added to the wells and incubated for another 60-120 minutes. Fluorescence polarization was measured at 485nm excitation and 525 emission on a SpectraMax M5 microplate reader (Molecular Devices). For determination of PDE1 activity Ca\(^{2+}\) (50 µmol/L) and Calmodulin (5U) were added and the PDE1 inhibitor PF4822163 (Pfizer, 1µmol/L) was used. PKG activity was assessed
by EIA colorimetric assay (CycLex). Briefly, kinase reaction buffer containing 2.5 mmol/L ATP and 500 µmol/L cGMP was prepared according to the manufacturer and added to protein lysates prepared from myocardial tissue in a 96 well reaction plate and incubated at 30°C for 20 minutes. After washing HRP conjugated anti-phospho specific antibody was added to the wells for 60 minutes at 22°C. After washing substrate reagent was added to the wells for 5-15 minutes and finally the reaction was halted using stop solution and absorbance at 450/550 nm was measured using the SpectraMax M5 microplate reader (Molecular Devices). cGK positive control was obtained from CycLex and used per manufacturer instructions.

**Immunoblot**

Heart tissue was lysed with RIPA buffer and subjected to SDS-PAGE using Nupage gels (Invitrogen) under reducing and denaturing conditions and transferred to nitrocellulose membranes. The antibodies against Pan-Calcineurin A and Gapdh were obtained from Cell Signaling Technologies (#2614) and Abcam (#ab9484), respectively. Fluorescence labeled secondary antibodies were obtained from Licor. Membranes were scanned on an infrared imaging system (Odyssey, Licor) and quantification of band intensity performed using Odyssey Application Software 3.0.

**WGA Staining for Myocyte Cross Sectional Area**

Myocardium was fixed with 4 % paraformaldehyde, paraffin embedded, sectioned into 4 µm slices, deparaffinized, rehydrated, and subjected to citrate-based head-mediated antigen retrieval. Slides were incubated with 5 µg/ml Alexa Fluor 488-conjugated wheat germ agglutinin (Invitrogen) overnight at 4 °C and mounted using Prolong Gold mounting medium (Invitrogen). Image acquisition was performed on a Zeiss LSM510-META laser scanning confocal microscope. Myocyte cross sectional area was analyzed blinded to the treatment group using an automated algorithm with NIH Image J 1.47i software. At least 1000 cells from 3-8 areas per heart were analyzed.

Online Figure I. Trpc1, 3 and 6 gene expression in TRPC knock-out mice and littermate controls. N=4-8/group. Gene deletion in TRPC3 or 6 knock-out mice did not alter the expression of the other two TRPC channels.
Online Figure II. Force/calcium in auxotonically contracting cardiomyocytes. Cyclic-GMP suppression of SSC is blocked by PKG-inhibition (Rp-8-CPT-cGMP, 10 µM, n=6-24/group, * p<0.01 vs +veh, # p<0.05 vs +cGMP)
**Online Figure III.** FSM in muscle from control and mice lacking Trpc3 or Trpc6. N=4-13/group. There is no difference among groups.
Online Figure IV. Echocardiographic analyses of cardiac morphology and function in control, *mdx/utrn*+/− and *mdx* mice up to 12 months of age. * p<0.05 vs Ctrl, # p<0.05 vs *mdx*. The *mdx* model which lacks dystrophin shows a modest increase in LV mass and wall thickness and no chamber dysfunction. However, the combined *mdx* – utrophin heterozygous deletion (*mdx/utrn*+/−) develops more ventricular hypertrophy and chamber dilation (end-diastolic dimension: LVEDD, end-systolic dimension: LVESD, fractional shortening: FS).
Online Figure V. Echocardiographic analyses of cardiac function in *mdx/utrn*+/− treated with sildenafil and placebo for 60 days. Chronic PDE5A inhibition by sildenafil prevents deterioration of cardiac function (fractional shortening) observed in *mdx/utrn*+/− placebo group. n=14-17/group, *p<0.05 vs baseline.
A) Western blot of calcineurin A (n=4-5/group, * p<0.01 vs Ctrl, # p<0.05 vs placebo).

B) mRNA expression of Rcan1 (n=3-5/group, p=n.s. for 1-way ANOVA).

Online Figure VI. Calcineurin A and Rcan1 expression in hearts of mdx/utrn<sup>+/−</sup> mice treated with sildenafil and placebo for 60 days. A) Western blot of calcineurin A (n=4-5/group, * p<0.01 vs Ctrl, # p<0.05 vs placebo). B) mRNA expression of Rcan1 (n=3-5/group, p=n.s. for 1-way ANOVA).