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Force development and intracellular Ca^{2+} in intact cardiac muscles from gravin mutant mice☆

Zhitao Li[b,1], Sonal Singh[c,1], Santosh V. Suryavanshi[a], Wengang Ding[d], Xiaoxu Shen[e], Cori S. Wijaya[a], Wei Dong Gao[f], Bradley K. McConnell[a,*]

[a] Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Texas Medical Center, Houston, TX, USA
[b] Department of Pathophysiology, Harbin Medical University, Heilongjiang, China
[c] Department of Pharmacotherapy and Translational Research, College of Pharmacy, University of Florida, Gainesville, FL, USA
[d] Department of Anesthesiology of 2nd Affiliated Hospital, Harbin Medical University, Heilongjiang, China
[e] Cardiology Department, Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing, China
[f] Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, 1800 Orleans Street, Zaye Tower 6208, Baltimore, MD 21287, USA

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ABSTRACT

Gravin (AKAP12) is an A-kinase-anchoring-protein that scaffolds protein kinase A (PKA), β_2-adrenergic receptor (β_2-AR), protein phosphatase 2B and protein kinase C. Gravin facilitates β_2-AR-dependent signal transduction through PKA to modulate cardiac excitation-contraction coupling and its removal positively affects cardiac contraction. Trabeculae from the right ventricles of gravin mutant (gravin-t/t) mice were employed for force determination. Simultaneously, corresponding intracellular Ca^{2+} transient ($[Ca^{2+}]_i$) were measured. Twitch force (T_f)-interval relationship, $[Ca^{2+}]_i$-interval relationship, and the rate of decay of post-extrasysolic potentiation (R_f) were also obtained. Western blot analysis were performed to correlate sarcomeric protein expression with alterations in calcium cycling between the WT and gravin-t/t hearts. Gravin-t/t muscles had similar developed force compared to WT muscles despite having lower $[Ca^{2+}]_i$ at any given external Ca^{2+} concentration ($[Ca^{2+}]_o$). The time to peak force and peak $[Ca^{2+}]_i$ were slower and the time to 75% relaxation was significantly prolonged in gravin-t/t muscles. Both T_f-interval and $[Ca^{2+}]_i$-interval relations were depressed in gravin-t/t muscles. R_f, however, did not change. Furthermore, Western blot analysis revealed decreased ryanodine receptor (RyR2) phosphorylation in gravin-t/t hearts. Gravin-t/t cardiac muscle exhibits increased force development in responsiveness to Ca^{2+}. The Ca^{2+} cycling across the SR appears to be unaltered in gravin-t/t muscle. Our study suggests that gravin is an important component of cardiac contraction regulation via increasing myofilament sensitivity to calcium. Further elucidation of the mechanism can provide insights to role of gravin if any in the pathophysiology of impaired contractility.

1. Introduction

A-kinase anchoring proteins (AKAPs) are scaffolding proteins that complex protein kinase A (PKA) along with other signaling molecules and target them to specific subcellular locations thereby increasing the specificity and diversity of cellular signaling (Colledge and Scott, 1999). So far fourteen AKAPs have been identified in the heart and studies have shown that disruption of AKAP/PKA interactions by the synthetic peptide Ht31 resulted in the altered distribution of PKA as well as augmentation of cell shortening in response to β-adrenergic receptor (β-AR) signaling. Interestingly, these changes were associated with a normal rate of Ca^{2+} cycling, normal intracellular Ca^{2+} transient amplitude and reduced PKA dependent phosphorylation of cardiac troponin I (cTnI) and cardiac myosin binding protein C (cMyBPC) (Fink et al., 2001; McConnell et al., 2009).

Gravin, also known as AKAP12, AKAP250 or SSeCKS, is highly

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* Correspondence to: Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Texas Medical Center, 3455 Cullen Boulevard, 460 Science and Research Building 2, Houston, TX 77204-5037, USA.
E-mail addresses: wgao3@jhmi.edu (W.D. Gao), bkmcconn@central.uh.edu (B.K. McConnell).
[1] First Co-authors.

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expressed in the heart and targets a number of signaling molecules including PKA, protein kinase C (PKC), and protein phosphatase 2B (PP2B) to the β_2-AR (Fan et al., 2001). Additionally, it has been reported that β-AR agonist treatment strengthens the gravin/β_2-AR interaction. Agonist stimulation of β-ARs enhances cardiac function, mainly through PKA-dependent phosphorylation of key components of the excitation-contraction (EC) coupling mechanism (Katz and Lorell, 2000). In addition to facilitating PKA-dependent substrate phosphorylation, gravin also plays an important role in the desensitization/resensitization cycle of the β_2-AR (Shih et al., 1999). Moreover knockdown of gravin expression has been shown to abrogate the recruitment of G-protein coupled receptor kinase 2 and β-arrestin, key proteins involved in β-AR desensitization (Lin et al., 2000; Shih et al., 1999). Additionally, we have recently identified that both in the presence and absence of acute β-AR stimulation cardiac function is enhanced in mutant gravin-t/t mice (Guillory et al., 2013). This increased cardiac function in gravin-t/t mice was coupled with reduced β_2-AR phosphorylation. Gravin-t/t mice also exhibited increased phosphorylation of cMyBPC at Ser-273 (Guillory et al., 2013), the key site for modulating cardiac function (Guggilam et al., 2013; Jin et al., 2013a) with no change in the phosphorylation levels of phospholamban (PLB), cTnI and two alternative phosphorylation sites of cMyBPC at Ser-282 and Ser-302 compared to the WT (Guillory et al., 2013). These data suggests that absence of gravin enhances the cardiac contractility via the modulation of the β-AR pathway.

In this study, we measured force development and intracellular Ca^{2+}($[Ca^{2+}]_i$) simultaneously in isolated intact cardiac trabeculae from gravin mutant (gravin-t/t) mice. Our objective was to study cardiac contraction and intracellular Ca^{2+} cycling in gravin-t/t mutant mice myocardium to better understand the functional importance of gravin and gain further insights into the molecular mechanisms for the enhanced cardiac function in gravin-t/t mice. Our results show that gravin-t/t muscles exhibited increased myofilament Ca^{2+} responsiveness while maintaining their ability to reconstitute in terms of force development and Ca^{2+} release from the sarcoplasmic reticulum (SR); suggesting that gravin also functions as regulator of myofilament Ca^{2+} sensitivity in addition to serving as a scaffolding protein and maybe involved in the modulation of the cardiac EC Coupling.

2. Materials and methods

2.1. Gravin mutant mice

Gravin mutant mice were produced using gene trap technology to remove the *Akap12* (gravin) gene (NM_031185) (Guillory et al., 2013). In brief, embryonic stem (ES) cells containing the gravin trapped vector were obtained from BayGenomics, microinjected into normal blastocysts and surgically implanted into foster mice at the University of Maryland School of Medicine Transgenic / Knockout Core Facility. The mutant gravin allele in these mice contained the gene trap vector that included a splice acceptor site immediately following exon 2, which causes early termination of the transcript. This mutant allele removes the remaining two 3′ exons (exon 3 and exon 4) and is predicted to encode from the amino-terminal to amino acid residue 89 of the 1684 residues from wild-type (WT) gravin, representing less than 6% of the full-length protein. Exons 1 and 2 of the mutant allele are expressed at WT levels. Protein chemical studies have demonstrated that this mutant allele produces less than 10% of the normal amount of gravin. Homozygous mice lacking functional gravin protein (designated gravin-t/t; *where t refers to truncation*) do not express the critical region required for β_2-AR, PKA or PKC binding that are encoded by exon 3. WT mice and homozygous mutant gravin mice (gravin-t/t) are bred on a C57bl/6 background. For experimental studies, male gravin mutant mice aged 10–12 weeks were compared with WT mice.

Research animals were generated and used in compliance with federal, state and local laws and institutional regulations. Specifically, the Institutional Animal Care and Use Committee (IACUC) and ethics committee at the University of Houston (UH) and the Johns Hopkins University (JHU) approved all animal studies. Also, animal care was provided for in *Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)* accredited animal barrier facilities at UH and JHU. Animals were maintained and these experiments were performed in accordance with the guidelines and ethical standards laid down in the 1996 National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* and in the 1964 Declaration of Helsinki and its later amendments. All authors of this report gave their informed consent prior to their inclusion in the study.

2.2. Isolation of intact trabecular muscles

Trabeculae from the right ventricle of the hearts were dissected and mounted between a force transducer and a motor arm in a bath with a volume of 300 µl. The muscles were superfused with Krebs-Henseleit solution [K-H, (in mM) NaCl 120, $NaHCO_3$ 20, KCl 5, $MgCl_2$ 1.2, glucose 10, 0.5 $CaCl_2$ (gassed with 95% O_2 and 5% CO_2 mixture, pH 7.35–7.45)] at a rate of ~10 ml/min and stimulated at 0.5 Hz at room temperature (21–22 °C). Force was measured with a force transducer system (SI, Germany) and expressed in millinewtons per square millimeter (mN/mm^2). The muscles were stretched to a length at which unstimulated force reached ~15% of total twitch force. This resting length corresponded to a sarcomere length of 2.2–2.3 µm (Gao et al., 1995) and was maintained throughout the experiments. Extracellular Ca ($[Ca^{2+}]_o$) was maintained at 0.5 mM during dissection and fura-2 loading (see below) and was increased to 1 mM during the execution of the experimental protocols.

2.3. Measurements of intracellular Ca^{2+}($[Ca^{2+}]_i$) from trabecular muscles

$[Ca^{2+}]_i$ was measured with the free acid form of fura-2 as described in our previous studies (Gao et al., 1998, 1999; Tan et al., 2009). Fura-2 potassium salt was microinjected iontophoretically into one cell and allowed to spread throughout the whole muscle (via gap junctions). The tip of the electrode (~0.2 µm in diameter) was filled with fura-2 salt (1 mM), and the remainder of the electrode was filled with 150 mM KCl. After a successful impalement into a superficial cell in the unstimulated muscle, a hyperpolarizing current of 5–10 nA was passed continuously for less than 10 min. As previously established, the loading does not affect force development. The epifluorescence of fura-2 was measured by exciting at 380 and 340 nm. The fluorescent light collected at 510 nm by a photomultiplier tube (R1527, Hamamatsu). $[Ca^{2+}]_i$ was given by the following equation (after subtraction of the autofluorescence):

$$[Ca^{2+}]_i = K_d^{'}(R - R_{min})/(R_{max} - R) \quad (1)$$

where R is the observed ratio of fluorescence (340/380), $K_d^{'}$ is the apparent dissociation constant, R_{max} is the ratio of 340 nm/380 nm at saturating $[Ca^{2+}]$, and R_{min} is the ratio of 340 nm/380 nm at zero $[Ca^{2+}]_i$. The values of $K_d^{'}$ (=4.0), R_{max} (=8.45), and R_{min} (0.32) were determined by in vivo calibrations, as described previously (Gao et al., 1998).

2.4. Immunoblotting

Immunoblot analysis was carried out as previously described using antibodies for GAPDH (Cell Signaling), SERCA2A (Cell Signaling), protein phosphatase inhibitor 1 (Santa Cruz Biotechnology), calsequestrin 2 (Pierce antibodies), L-type calcium channel (Santa Cruz Biotechnology) and sodium / calcium exchanger (Thermo Fisher Scientific) (McConnell et al., 2009). Crude heart homogenates or cytosolic fractions were resolved by SDS-PAGE gradient (4–12% Bis-