The β-arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium

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Monasky MM, Taglieri DM, Henze M, Warren CM, Utter MS, Soergel DG, Violin JD, Solaro RJ. The β-arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium. Am J Physiol Heart Circ Physiol 305: H856–H866, 2013. First published July 19, 2013; doi:10.1152/ajpheart.00327.2013.—In the present study, we compared the cardioprotective effects of TRV120023, a novel angiotensin II (ANG II) type 1 receptor (AT1R) ligand, which blocks G protein coupling but stimulates β-arrestin signaling, against treatment with losartan, a conventional AT1R blocker in the treatment of cardiac hypertrophy and regulation of myofilament activity and phosphorylation. Rats were subjected to 3 wk of treatment with saline, ANG II, ANG II + losartan, ANG II + TRV120023, or TRV120023 alone. ANG II induced increased left ventricular mass compared with rats that received ANG II + losartan or ANG II + TRV120023. Compared with saline controls, ANG II induced a significant increase in pCαs0 and maximum Ca2+-activated myofilament tension but reduced the Hill coefficient (nH). TRV120023 increased maximum tension and pCαs0, although to lesser extent than ANG II. In contrast to ANG II, TRV120023 increased nH. Losartan blocked the effects of ANG II on pCαs0 and nH and reduced maximum tension below that of saline controls. ANG II + TRV120023 showed responses similar to those of TRV120023 alone; compared with ANG II + losartan, ANG II + TRV120023 preserved maximum tension and increased both pCαs0 and cooperativity. Tropomyosin phosphorylation was lower in myofilaments from saline-treated hearts compared with the other groups. Phosphorylation of cardiac troponin I was significantly reduced in ANG II + TRV120023 and TRV120023 groups versus saline controls, and myosin-binding protein C phosphorylation at Ser282 was unaffected by ANG II or losartan but significantly reduced with TRV120023 treatment compared with all other groups. Our data indicate that TRV120023-related promotion of β-arrestin signaling and enhanced contractility involves a mechanism promoting the myofilament response to Ca2+ via altered protein phosphorylation. Selective activation of β-arrestin-dependent pathways may provide advantages over conventional AT1R blockers.

β-arrestin; hypertrophy; angiotensin; myofilament calcium sensitivity; angiotensin II type 1 receptor

Therapies that act through the pharmacological inhibition of the renin-angiotensin-aldosterone system are routinely and effectively used, often as the first line of treatment of hypertension, chronic heart failure, cardiac remodeling after myocardial infarction, and diabetic nephropathy (6, 16). Three groups of pharmaceuticals, angiotensin-converting enzyme inhibitors, angiotensin II (ANG II) type 1 receptor (AT1R) blockers (ARBs), and mineralocorticoid antagonists, have been used for the past 35 yr to attenuate or reverse hypertension-induced myocardial hypertrophy and to reduce morbidity and mortality of cardiovascular disease (25, 37a, 37b).

Activation of the AT1R, a G protein-coupled receptor, plays a pivotal role in the regulation of cardiovascular physiology (32). Its sustained stimulation by ANG II is detrimental and leads to arterial hypertension, myocardial hypertrophy, and cardiac dysfunction (4). This effect is likely mediated by G protein signaling, as Goq activation generates fibrosis and can induce heart failure in transgenic mice (10, 42). Signaling through the AT1R receptor by ANG II binding occurs not only through heterotrimeric G proteins (primarily Goq) but also through β-arrestins, which are recruited to the activated receptor by G protein-coupled receptor kinases (8, 15). Consequently, G protein-dependent (20) and β-arrestin-dependent (27) mechanisms are amplified by the activation of downstream Ca2+-dependent PKC, MAPK signaling, and G protein-coupled receptor-mediated transactivation of receptor tyrosine kinases, among others (18, 23, 34). ARBs effectively block both G protein and β-arrestin pathways (43).

Recent discoveries have established approaches for selective blockade of G protein-coupled AT1R activation while preserving β-arrestin pathway signaling (35). Thus, these approaches have created a novel avenue of research with the goal of discovering “biased ligands” that may reduce or eliminate undesired on-target side effects while stimulating the beneficial effects of these pathways (9). The significance of the G protein-independent β-arrestin pathway has been established by the development of biased ligands with the ability to block G protein-coupled effects of ANG II on hypertension while promoting and preserving myocardial contractility.

Previous studies (13, 39) have demonstrated selective activation of β-arrestins by TRV120023 (Ser-Arg-Val-Tyr-Lys-His-Pro-Ala-OH) at the AT1R. TRV120023 antagonizes G protein signaling similarly to an ARB, but, unlike an ARB, it promotes β-arrestin recruitment and activation of kinase pathways. Moreover, unlike traditional ARBs, which either have no effect on cardiac performance or may even decrease it (9), TRV120023 increases cardiac contractility through a β-arrestin-2-dependent mechanism (13, 39). A recent study (13) found that TRV120023 does not lead to an accumulation of inositol monophosphate and diacylglycerol and suggested that the
β-arrestin-dependent inotropic effect of TRV120023 may be due to an increase in the myofilament Ca\(^{2+}\) response.

In the experiments reported here, we tested the hypothesis that TRV120023 is a modulator of myofilament Ca\(^{2+}\) responsiveness through activation of the β-arrestin-dependent pathway. We compared the effects of infusion of TRV120023 and the ARB losartan on the response of rat hearts to ANG II infusion, including myofilament posttranslational modifications and the myofilament response to Ca\(^{2+}\). Our data provide the first evidence showing that TRV120023 infusion in rats is able to block ANG II-mediated hypertrophy while modulating multiple myofilament posttranslational modifications and enhancing the myofilament Ca\(^{2+}\) response. These novel effects of TRV120023 indicate that selective activation of the β-arrestin-dependent pathway may provide beneficial advantages over conventional ARBs.

### MATERIALS AND METHODS

All protocols were in accordance with guidelines of and approved by the Animal Care and Use Committee of the University of Illinois and complied with the laws of the United States of America.

#### Generation of the hypertrophic rat model

Male Sprague-Dawley rats (age: 7 wk) were initially anesthetized with 3% isoflurane and 100% \(O_2\) inhaled in a closed anesthesia chamber. The plane of anesthesia for surgery was then regulated by delivery of 1% isoflurane administered through a nose cone with 100% \(O_2\). Alzet osmotic pumps (Cupertino, CA) delivered either saline, 1 mg/kg \(^{-1}\) day \(^{-1}\) ANG II, 1 mg/kg \(^{-1}\) day \(^{-1}\) ANG II + 10 mg/kg \(^{-1}\) day \(^{-1}\) losartan, 1 mg/kg \(^{-1}\) day \(^{-1}\) ANG II + 14.4 mg/kg \(^{-1}\) day \(^{-1}\) (10 \(\mu\)g/kg \(^{-1}\) min \(^{-1}\)) TRV120023, or 14.4 mg/kg \(^{-1}\) day \(^{-1}\) TRV120023 subcutaneously after being placed in a subcutaneous pouch and secured with surgical metal clips.

#### Transthoracic two-dimensional, M-mode, and pulsed Doppler echocardiography

Induction and maintenance of anesthesia were performed as described above. Rats were placed in the dorsal decubitus position on a warming pad to maintain normal body temperature. Transthoracic two-dimensional (2-D), M-mode, and pulsed Doppler images were acquired with a high-resolution echocardiographic system (VeVo 770, Visual Sonics, Toronto, ON, Canada) equipped with a 30-MHz mechanical transducer. Echocardiographic measurements were performed in all groups before and 3 wk after miniosmotic pump implantation. All measurements were taken in compliance with American Society of Echocardiography guidelines (26). Results were based on the average of at least three cardiac cycles.

#### Myosin heavy chain isoforms

The separation of cardiac myosin heavy chain isoforms has been previously described (41). The rat ventricle was homogenized in a 1:20 ratio of sample buffer [8 M urea, 2 M thiourea, 0.05 M Tris·HCl (pH 6.8), 75 mM DTT, 3% SDS, 0.5% CHAPS (UTC buffer), and the protein concentration was determined using a Bio-Rad protein assay]. The sample was loaded (2 \(\mu\)g/lane) onto a 1.0-mm-thick 16 × 18-cm gel. The gel was run using 20-mA constant current for 5.5 h with constant cooling to 8°C. After the electrophoresis finished, the gel was stained with Coomassie blue R-250 and destained in 10% methanol and 10% acetic acid.

#### Dissection of left ventricular papillary muscles and preparation of skinned fibers

After 3 wk of pharmacological treatment, rats were anesthetized with Nembutal (50 mg/kg) by an intraperitoneal injection, and the thoracic cavity was opened by a bilateral thoracotomy. Anesthetized with Nembutal (50 mg/kg) by an intraperitoneal injection.

After 3 wk of pharmacological treatment, rats were

Measurement of the myofilament Ca\(^{2+}\) response. Fiber bundles were mounted between a force transducer and a stationary rod using glue. The sarcomere length was set to 2.2 \(\mu\)m using He-Ne laser diffraction (7). The width and diameter were each measured at three points along the fiber bundle. Force per cross-sectional area was used to determine tension. The fiber was initially contracted at a saturating Ca\(^{2+}\) concentration (pCa 4.5, 50 \(\mu\)mol/l), and sarcomere length was again adjusted to 2.2 \(\mu\)m. The muscle was placed in solutions consisting of various ratios of relaxing solution and Ca\(^{2+}\) solution to create various Ca\(^{2+}\) concentrations at room temperature to measure myofilament Ca\(^{2+}\) responsiveness. Solutions again also contained 1 \(\mu\)g/ml leupeptin, 2.5 \(\mu\)g/ml pepstatin A, and 50 \(\mu\)M PMSF. Sarcomere length remained constant throughout the rest of the experiment.

Western immunoblot analysis. Western immunoblot analysis was performed using antibodies recognizing phospho-myosin-binding protein-C (MyBPC) at Ser282 (ALX-215-057, Enzo Life Sciences), total MyBPC (sc-137180, Santa Cruz Biotechnology), phospho-troponin I (TnI) at Ser\(^{2+}\)/Ser\(^{2+}\) (no. 40004, Cell Signaling Technology), and total TnI (no. 10R-T123K, Fitzgerald Industries). Incubation with primary antibodies diluted in Tris-buffered saline with 0.05% Tween 20 and BSA was performed overnight at 4°C. Incubation with secondary antibodies was performed with peroxidase-conjugated antibody anti-rabbit IgG (no. A0545, Sigma-Aldrich) or peroxidase-conjugated antibody anti-mouse IgG (no. A2304, Sigma-Aldrich) diluted in Tris-buffered saline with 0.05% Tween 20 and milk for 1 h at room temperature. Detection occurred by chemiluminescence (Amersham ECL Western Blotting System, GE Healthcare). Bands were quantified by dividing each “total protein” value by the highest total protein value, making the highest total protein value equal to 1. Each phosphorylation value was then divided by the corresponding total protein value. For MyBPC Ser\(^{2+}\), all of the bands except the very bottom, most faint band were used in the analysis.

#### Quantification of myofilament phosphorylation by the 2-D difference in gel electrophoresis

Details describing the investigation of myofilament proteins by the 2-D difference in gel electrophoresis (DIGE) method with spot identification and analysis have been previously described (14, 31, 40, 45). Myofilibrils were purified from liquid nitrogen frozen rat tissue and homogenized twice in standard relax buffer [10 mmol/l imidazole (pH 7.2), 75 mmol/l KCL, 2 mmol/l MgCl\(_2\), 2 mmol/l EDTA, and 1 mmol/l Na\(_3\)Cit] with 1% (vol/vol) Triton X-100. Myofilibrils were centrifuged, and the supernatant was removed and washed once in the above buffer without Triton X-100. The pellet was resuspended in 8 mol/l urea, 2 mol/l thiourea, and 4% CHAPS (UTC buffer), and the protein concentration was determined using an RCDC assay kit (Bio-Rad, Hercules, CA). Samples in UTC buffer (100 \(\mu\)g) were cleaned up with GE Healthcare’s 2-D clean-up kit (Piscataway, NJ) and then resuspended in UTC buffer. Since it is possible to separate up to three different samples within the same 2-D gel, two samples and an internal standard were included in every gel. The internal standard was a global standard composed of all samples in the data set equally mixed. Tissue samples were randomly labeled to receive either Cy3 or Cy5 to control for any dye differences, whereas the internal standard was labeled with Cy2 (CyDye DIGE Fluors were from GE Healthcare). Protein samples were labeled by adding 100 pmol CyDye to 50 \(\mu\)g protein and then quenched with 10 mmol/l \(\lambda\)-lysine. The gel’s first dimension was focused using the Protein IEF cell (Bio-Rad) with 18-cm pH 4–7 or 7–11 IPG strips. Samples were actively rehydrated at 50 V for 10–16 h with 90 \(\mu\)l IPG. Samples were then loaded and active focused in the first dimension. The gel was stained by the Protean IEF cell (Bio-Rad) with 18-cm pH 4–7 or 7–11 IPG strips. Samples were actively rehydrated at 50 V for 10–16 h with 90 \(\mu\)l IPG. Samples were then loaded and active focused in the first dimension.

#### PAGE gels

PAGE gels were imaged with a Typhoon 9410 scanned at 100

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µm and analyzed with PDQuest Advanced software (version 8.0.1, Bio-Rad). Spot density was computed by dividing the density of a particular posttranslationally modified protein spot by the total of all the spot densities for that protein.

Data analysis. Skinned fiber data were statistically analyzed by one-way ANOVA followed by a Newman-Keuls comparison test using GraphPad Prism software. Analysis of the relation between Ca\textsuperscript{2+} and tension was fitted using a modified Hill equation as previously described (17). The maximal tension, pCa\textsubscript{50}, and Hill coefficient for determination of myofilament Ca\textsuperscript{2+} responsiveness were calculated using the Hill equation (17). Hemodynamic parameters obtained by echocardiography were statistically analyzed by two-way ANOVA followed by Student’s t-test using JMP statistical software. Western blot data were statistically analyzed by one-way ANOVA followed by a Student’s t-test using OriginPro 8.5.1. P values of <0.05 were considered significantly different. Data are represented as means ± SE.

RESULTS

TRV120023 represses effects of ANG II on the heart. To compare the cardiac effects of ANG II versus TRV120023 as well as ANG II + losartan versus ANG II + TRV120023, we measured the dimensions of the LV anterior and posterior walls and LV chamber dimensions and calculated the mass of the LV by echocardiography. In addition, we measured the effects of TRV120023 on changes in global systolic function (i.e., fractional shortening, ejection fraction, and cardiac output) and diastolic function as measured by conventional pulse wave Doppler echocardiography (i.e., E-to-A ratio and transmitral early filling deceleration time).

The heart weight-to-tibia length ratio was increased in rats that received ANG II compared with saline. TRV120023 treatment alone had no effect on the heart weight-to-tibia length ratio compared with saline-treated rats (Fig. 1A). Co-

Fig. 1. TRV120023 (TRV) prevents the development of cardiac hypertrophy in rats. A: heart weight (HW)/tibial length (TL). B: dry lung weight (LWd)/body weight (BW). C: left ventricular (LV) mass. D: LV anterior wall thickness (LVAW). E: LV posterior wall thickness (LVPW). F: LV ejection fraction (EF). G: LV fractional shortening (FS). Angiotensin II (ANG II; ATII)-treated rats developed cardiac hypertrophy, which was attenuated by the administration of losartan (Los) and TRV, as assessed by measurement of HW/TL (A) and echocardiography (C). Changes in LV mass were associated with increase in LVAW and LVPW (D and E). ANG II, ANG II + Los, and ANG II + TRV did not significantly affect global systolic function (F and G). Finally, administration of ANG II for 3 wk induced a significant increase in LWd/BW compared with the saline-treated group, which was significantly blocked by Los and attenuated by TRV to a level that was not significantly different from the saline-treated group. Numbers of rats per group (N) were as follows: 6 for all groups (A and B); 9 for presaline and saline, 8 for presaline and saline and 5–6 for all other groups (D); 8 for presaline and saline and 5–6 for all other groups (E); 9 for presaline, 8 for saline, and 4–6 for all other groups (F); and 10 for presaline and saline and 5–6 all other groups (G). *P < 0.05.
administration of ANG II with either losartan or TRV120023 blocked the ANG II-dependent increase in the heart weight-to-tibia length ratio. We also observed an increase in dry lung weight-to-body weight ratio in the ANG II-treated group compared with the saline-treated group but no significant effect of TRV120023. The effect of ANG II was significantly attenuated by losartan and also attenuated by TRV120023 to a level no longer significantly different from saline treatment (Fig. 1B). There were no differences in the wet lung weight-to-body weight ratios between groups (data not shown).

Consistent with these findings, administration of ANG II for 3 wk resulted in significantly increased LV mass compared with both the LV mass of the same rats before treatment or the LV mass of rats that received saline solution. The LV mass of rats that received TRV120023 was not significantly different from saline-treated rats. The LV mass of the ANG II-treated group at 3 wk was also increased compared with rats that received a combination of ANG II and losartan or ANG II and TRV120023 (Fig. 1C). Therefore, losartan and TRV120023 coadministered with ANG II significantly attenuated the prohypertrophic effects of ANG II.

These findings were accompanied by significantly increased anterior and posterior LV wall thickness in the ANG II-treated group compared with the saline-treated group (Fig. 1, D and E), which were attenuated when losartan or TRV120023 were administered together with ANG II. Effects of TRV120023 alone on either of these parameters were not significantly different from the saline-treated group. No significant changes in body weight gain over the 3-wk treatment course were noted in any group compared with saline-treated rats (data not shown). Taken together, our results indicate that TRV120023 counteracts the prohypertrophic effects of ANG II in rats.

Analysis of LV fractional shortening (Fig. 1F) and LV ejection fraction (Fig. 1G) showed no changes in global systolic LV contractile performance after ANG II administration, both compared with measurements obtained before ANG II administration or compared with measurements in rats that received saline, ANG II + losartan, ANG II + TRV120023, or TRV120023 alone. Transmural early filling deceleration time was also not significantly different in any of the groups between the baseline and 3-wk time points within each treatment group (data not shown). Statistical analysis of the factors of treatment (administration of saline, ANG II, ANG II + losartan, ANG II + TRV120023, and TRV120023), time point (baseline and after 3 wk of treatment), and the interaction between these two factors is shown in Table 1.

TRV120023 blocks changes in MHC isoform expression. Previous studies (19, 28, 38) have well established that an increase in β-MHC isoform expression leads to slower contractile and relaxation kinetics. In the present study, the relative abundance of the MHC β-isoform increased with ANG II administration, an effect that was blocked by coadministration of TRV120023 and also blocked by the coadministration of losartan (Fig. 2). Administration of TRV120023 alone did not significantly alter the relative abundance of MHC isoforms.

TRV120023 administration affects the myofilament response to Ca2+. Although a previous study (13) has shown that TRV120023 positive inotropy is β-arrestin-dependent and that, unlike ANG II, TRV120023 does not mobilize Ca2+ in vitro, there have been no direct tests of the hypothesis that TRV120023 modulates the response of the myofilaments to Ca2+. We therefore determined the myofilament Ca2+ responsiveness of skinned fiber bundles from LV papillary muscles isolated from the same rats described above at the 3-wk time point after treatment with saline (N = 8 rats, 18 fibers), ANG II (N = 6 rats, 13 fibers), ANG II + losartan (N = 6 rats, 14 fibers), ANG II + TRV120023 (N = 5 rats, 11 fibers), or TRV120023 (N = 6 rats, 16 fibers) for 3 wk (Fig. 3A). As shown in Fig. 3B, maximum Ca2+-activated isometric tension (expressed in mN/mm2) was greater in papillary fibers obtained from ANG II-treated rats compared with fibers obtained from all other groups. TRV120023 also significantly increased maximal tension, but to a smaller extent. The ANG II response was blocked with TRV120023 treatment to approximately the level of TRV120023 alone, which was significantly higher than the maximal tension in the ANG II + losartan-treated group. Similar findings were noted for pCa50, which was significantly increased in fibers obtained from ANG II-treated rats compared with saline treatment (Fig. 1B).

### Table 1. Statistical results of echocardiography data analyzed by two-way ANOVA followed by Student's t-test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LV Mass, mg</th>
<th>LV Anterior Wall, mm diastole</th>
<th>LV Posterior Wall, mm diastole</th>
<th>Fractional Shortening, %</th>
<th>Ejection Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG II</td>
<td>0.0200*</td>
<td>0.1924</td>
<td>0.0833</td>
<td>0.3159</td>
<td>0.6064</td>
</tr>
<tr>
<td>ANG II + Losartan</td>
<td>&lt;0.0001*</td>
<td>0.0076*</td>
<td>0.0039*</td>
<td>0.7476</td>
<td>0.3169</td>
</tr>
<tr>
<td>ANG II + TRV120023</td>
<td>0.0208*</td>
<td>0.0390*</td>
<td>0.0280*</td>
<td>0.6438</td>
<td>0.5223</td>
</tr>
</tbody>
</table>

Data were analyzed using JMP statistical software. The factor “treatment” refers to the administration of saline, angiotensin II, angiotensin II + losartan, angiotensin II + TRV120023, and TRV120023. The factor “time point” refers to baseline and after 3 wk of treatment. P values of <0.05 were considered significant (*).
Fig. 3. Myofilament Ca\textsuperscript{2+} responsiveness assessment by permeabilized ventricular fiber bundles. A: representative isometric tension development tracings at various Ca\textsuperscript{2+} concentrations. B: maximum Ca\textsuperscript{2+}-activated isometric tension of permeabilized ventricular fiber bundles. C: pCa\textsubscript{50} of permeabilized ventricular fiber bundles. D: Hill coefficient of permeabilized ventricular fiber bundles. Data were statistically analyzed by one-way ANOVA followed by a Newman-Keuls comparison test using GraphPad Prism software. Analysis of the relation between Ca\textsuperscript{2+} and tension was fitted using a modified Hill equation. The maximal tension, pCa\textsubscript{50}, and Hill coefficient were calculated using the Hill equation. Values of $P < 0.05$ were considered significantly different. Data are represented as means ± SE; $N = 11–18$ fibers, 5–8 mice (see MATERIALS AND METHODS for further details). *$P < 0.05$. 

β-ARRESTRIN-BIASED AT\textsubscript{1}R LIGAND ENHANCES MYOFILAMENT RESPONSE

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with fibers obtained from all other groups (Fig. 3C). Compared with saline controls, pCa50 was also significantly increased in the TRV120023-treated group, although to a lesser extent than the ANG II-treated group. As with maximal tension, TRV120023 blocked ANG II effects, but pCa50 in the ANG II + TRV120023-treated group was significantly higher than pCa50 in the ANG II + losartan-treated group. Despite increased Ca2+ responsiveness in terms of maximal tension and pCa50, the Hill coefficient, which is an indicator of cooperative activation of the myofilaments, was significantly decreased in the ANG II-treated group compared with controls. In contrast, cooperativity was significantly increased in the group treated with TRV120023 alone, which had the highest value for this parameter (Fig. 3D). ANG II + losartan blocked

**Fig. 4. Analysis of myofibrillar proteins cardiac troponin I (cTnI) and tropomyosin (TM) by the two-dimensional (2-D) difference in gel electrophoresis (2-D DIGE). STD, internal standard; Px, species with posttranslational modifications (primarily phosphorylation with at least one site associated); U, unmodified protein; AU, arbitrary units. pH values indicate the pH range of the strip used for the first dimension. Samples were labeled separately with different CyDyes and mixed to run in the same 2-D gel. A and B: representative images of cTnI and Tm with the labeled spots used to determine posttranslational modifications. Images shown were separated and merged. C and D: histograms indicating relative posttranslational modifications expressed as means ± SE; n = 5. No significant differences were determined by ANOVA followed by a Fisher test. E–H: histograms indicating relative posttranslational modifications expressed as means ± SE; n = 4–5. Significance was determined by ANOVA followed by a Fisher test. *P < 0.05.**
the reduced cooperativity elicited by ANG II to values close to saline; in contrast, ANG II + TRV120023 not only blocked the ANG II effect but increased cooperativity to near the level of TRV120023 alone. Together, these data indicate that the overall effect of ANG II treatment is to increase the myofilament response to \(Ca^{2+}\) and that TRV120023 likewise increases myofilament \(Ca^{2+}\) responsiveness, but does so more modestly than ANG II. In addition, ANG II + TRV120023 preserves ANG II-enhanced myofilament \(Ca^{2+}\) responsiveness compared with ANG II + losartan. The opposite effects of ANG II and TRV120023 on \(Ca^{2+}\) cooperativity suggest that the two compounds engage different mechanisms of myofilament regulation.

**TRV120023 administration affects myofilament protein posttranslational modifications.** The changes we observed in myofilament \(Ca^{2+}\) responsiveness, including differences in maximum \(Ca^{2+}\)-activated isometric tension, \(pCa_{50}\), and the Hill coefficient, suggest possible posttranslational modifications of myofilament proteins. We investigated differences in posttranslational modifications of major sarcomeric proteins, including MyBPC, TnI, tropomysin (Tm), and myosin light chain 2 (MLC2). Posttranslational modifications of TnI, Tm, and MLC2 were assessed by 2-D DIGE analysis. Representative images are shown in Fig. 4, A and B. Less charged P1 and P2 cTnI phospho-spots did not significantly change between the groups (Fig. 4, C and D). Total cardiac TnI (cTnI) phosphorylation was not significantly changed by ANG II but was significantly reduced by TRV120023 compared with saline; consistent with this, ANG II + TRV120023, like TRV120023 alone, reduced total phosphorylation (Fig. 4G). ANG II + losartan was not significantly different from either ANG II alone or ANG II + TRV120023. These changes are driven by alterations in the highly charged variants reflecting multiple phosphorylation sites (P3 and P4; Fig. 4, E and F). Similar findings were observed for TnI phosphorylation at Ser23/Ser24 using Western immunoblot analysis, which revealed significantly decreased Ser23/Ser24 phosphorylation with TRV120023 compared with saline but no effect of ANG II (see Fig. 6A). Consistent with the 2-D DIGE results, ANG II + TRV120023 elicited a similar effect to TRV120023 alone, but was in this case not statistically significant. Together, these results indicate that TRV120023 causes dephosphorylation of cTnI; phosphorylation of these sites decreases \(Ca^{2+}\) responsiveness (36), suggesting that these sites may contribute to the increased response to \(Ca^{2+}\) seen with TRV120023.

In addition to effects on cTnI, Tm phosphorylation was significantly increased in ANG II, ANG II + TRV120023, and TRV120023 versus saline treatment (Fig. 4H), but the alterations were the same regardless of treatment. No significant differences in MLC2 phosphorylation were observed among any of the groups using 2-D DIGE (Fig. 5). MyBPC phosphorylation at Ser282, as determined by Western immunoblot analysis, was significantly reduced with TRV120023 treatment compared with all other groups (Fig. 6B). Thus, our data indicate that ANG II and TRV120023 infusion alter myofilament protein phosphorylation, consistent with the positive inotropy of these compounds. Also, in some cases, the alterations are most pronounced in the presence of TRV120023, consistent with the different mechanism of action for TRV120023 positive inotropy compared with ANG II (13, 39).

**DISCUSSION**

The use of biased ligands at the \(\alpha_{1}\)AR in heart failure holds great promise for a therapy that is beneficial in cardiorenal dysfunction while preserving cardiac contractility via a \(\beta\)-arrestin signaling pathway (1, 9). Speculation from indirect evidence on the mechanism for the preservation of contractility indicated a role for myofilament response to \(Ca^{2+}\) rather than altered \(Ca^{2+}\) fluxes (13). The results presented here are the first to directly test this hypothesis in a well-controlled chronic model of hearts responding to continuous infusion of ANG II or the \(\beta\)-arrestin-biased ligand TRV120023 and of hearts responding to coinfused ANG II and TRV120023 or coinfused ANG II and the ARB losartan. Our data demonstrate the ability of TRV120023 to significantly inhibit ANG II-induced LV hypertrophy and to increase the myofilament \(Ca^{2+}\) response compared with saline and when coadministered with ANG II. Our data also indicate unique effects of TRV120023 on changes in myofilament protein posttranslational modifications, illuminating a mechanistic basis for the unique actions of \(\beta\)-arrestin-biased ligands like TRV120023.

To the best of our knowledge, the results presented here are the first to report an increase in the myofilament response to \(Ca^{2+}\) in hearts stressed by a chronic infusion of ANG II. This

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**Fig. 5. Analysis of myofibrillar protein myosin light chain 2 (MLC2) by 2-D DIGE.** A: representative image of MLC2 with the labeled spots used to determine the phosphorylation status. Images shown were separated and merged. Only one phosphorylation spot was consistently observed and included in the analysis. B: histogram indicating relative posttranslational modifications expressed as means ± SE; \(n = 5\). No significant differences were determined by ANOVA followed by a Fisher test.
Fig. 6. Assessment of sarcomeric protein phosphorylation by Western immunoblot analysis. Each lane contained sample from one animal and was different from animals represented in other lanes. 

(A): phosphorylation of TnI at Ser23/Ser24 normalized to total TnI. 

(B): phosphorylation of myosin-binding protein-C (MyBPC) at Ser282 normalized to total MyBPC. 

N = 4–5 mice/group. *P < 0.05.
finding supports the idea that activation of the AT1R promotes a response associated with enhanced contractility and involving an altered myofilament Ca$^{2+}$ response. Analysis of the steady-state relation between Ca$^{2+}$ and tension (Fig. 3) revealed important differences between treatment with an unbiased ligand, losartan, and a biased ligand, TRV120023. Compared with losartan + ANG II, the myofilament response was increased in fibers from animals treated with either TRV120023 + ANG II or TRV120023 alone. Thus, with the biased ligand, there was preservation of the enhanced Ca$^{2+}$ responsiveness, especially tension and the Hill coefficient value for the activation of tension. While pCa$_{50}$ associated with ANG II infusion was increased, there was a similar effect to restore pCa$_{50}$ induced by losartan and TRV120023. Modifications in the Hill coefficient for myofilament activation are particularly significant. In this case, compared with saline controls, ANG II treatment induced a decrease in the Hill coefficient value, whereas treatment with TRV120023 significantly enhanced the Hill coefficient. Moreover, in the presence of ANG II, this increase with TRV120023 was greater than significantly enhanced the Hill coefficient. Moreover, in the presence of a single regulatory Ca$^{2+}$-troponin site occupied with Ca$^{2+}$, there is a homeostatic level of Tm phosphorylation and changes that are either too low or too high may be maladaptive. In any case, the increase of Tm phosphorylation with ANG II infusion was also elicited by TRV120023 and was not significantly affected by coadministered TRV120023 or losartan.

However, there were changes in cTnI and MyBPC phosphorylation between ANG II-stressed hearts treated with TRV120023 and losartan. It is generally recognized that the level of phosphorylation of cTnI is a significant regulator of the myofilament Ca$^{2+}$ response. There are multiple cTnI sites of phosphorylation, but sites that depress the Ca$^{2+}$ response (Ser$^{23}$, Ser$^{24}$, Ser$^{43}$, and Ser$^{45}$) (14) appear to predominate over sites (Ser$^{150}$) (22) that increase the Ca$^{2+}$ response. The results shown in Figs. 4, E–G, and 6A indicate that TRV120023 infusion induces a dephosphorylation of cTnI, thus providing a possible mechanism for the increase in the myofilament Ca$^{2+}$ response. Our results with a site-specific anti-phosphopeptide antibody probing Ser$^{23}$ and Ser$^{24}$ showed a decrease in phosphorylation in hearts treated with TRV120023 compared with saline- or ANG II-treated hearts. This was also the case with highly charged variants containing phosphorylation sites of cTnI, which decreased with TRV120023 treatment, suggesting a decrease in phosphorylation at sites that may differ from Ser$^{23}$ and Ser$^{24}$. We also found that TRV120023 alone induced a decrease in phosphorylation of MyBPC at Ser$^{282}$, a site viewed as critical for β-adrenergic responsiveness among the multiple sites of phosphorylation (29). Future studies, including dose-response testing, may help further explain these differences observed between ANG II and TRV120023. Additionally, future studies will further explore differences in iso-

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**Fig. 7. Regulation of cardiac myofilament contractility by pharmacological modulation of the ANG II type 1 receptor (AT1R).** ANG II stimulates G protein- and β-arrestin-2-mediated signaling at the AT1R, including Ca$^{2+}$ release via the G protein pathway, alterations in Tm phosphorylation, and relative β- and α-MHC expression. The net effect of these signals after chronic ANG II stimulation is cardiac hypertrophy, increased Ca$^{2+}$ sensitivity, increased maximum tension, and decreased Ca$^{2+}$ cooperativity of cardiac myofilaments. Angiotensin receptor blockers (ARBs), such as Los, antagonize both G protein and β-arrestin pathways, blocking the cardiac hypertrophy and changes in myofilament contractility caused by ANG II. β-Arrestin-biased ligands, such as TRV, block ANG II-mediated cardiac hypertrophy but preserve or enhance the inotropic effects of altered myofilament phosphorylation. Orange shading indicates effects that may be adverse, whereas green shading indicates effects that may be beneficial in the setting of cardiovascular disease. GRK, G protein-coupled receptor kinases.
form expression of other sarcomeric proteins and other post-translational modifications and identify other sarcomeric protein targets of this signaling pathway to more fully explain the novel findings of this first study on the chronic effects of TRV120023.

An important issue is the relation between these changes in myofilament phosphorylation and contractility. One study (5) tested acute administration of TRV120027, a β-arrestin-biased agonist closely related to TRV120023, in dogs with pacing-induced heart failure and demonstrated cardiac unloading and a reduction in end-diastolic volume. In addition, TRV120027 as well as TRV120023 have previously been shown to acutely increase fractional shortening in isolated mouse cardiomyocytes, unlike unbiased ARBs (39). Despite these changes in protein phosphorylation in the present study, with a protocol involving chronic administration of TRV120023, there were no significant differences in fractional shortening or ejection fraction, as assessed by echocardiography, among any of the groups after 3 wk of in vivo continuous infusion treatment. This may be due to effects in our chronic model in which there are hemodynamic compensations and myocardial compensatory remodeling. This result may also be due to the techniques used to measure in situ cardiac contractility.

An increase in the relative abundance of the β1 isoform of MHC is a marker for pathological hypertrophy (11, 24) and is associated with slower contractile and relaxation kinetics (19, 28, 38). The ability of TRV120023 to prevent the change in the relative abundance of MHC isoforms agrees with our findings that TRV120023 also prevents the hypertrophic response to ANG II.

Our data provide the first insights into a TRV120023-related promotion of β-arrestin signaling to a signaling pathway affecting myofilament posttranslational modifications and the response to Ca2+. A diagram showing the regulation of cardiac myofilament contractility by pharmacological modulation of the AT1,R is shown in Fig. 7. Our results support and significantly extend results of previous studies that focused on acute effects of biased ligands acting at the AT1,R.

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DISCLOSURES

D. G. Soergel and J. D. Violin are employees of Trevena, a company that discovers and develops G protein-coupled receptor-targeted drugs, including TRV120023. R. J. Solaro is a member of the Scientific Advisory Board of Cytokinetics, Incorporated.

AUTHOR CONTRIBUTIONS


REFERENCES


