Novel Role of Transient Receptor Potential Vanilloid 2 in the Regulation of Cardiac Performance

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Abstract

Transient receptor potential cation channels have been implicated in the regulation of cardiovascular function, but only recently has our laboratory described the vanilloid 2 subtype (TRPV2) in the cardiomyocyte, though its exact mechanism of action has not yet been established. This study tests the hypothesis that TRPV2 plays an important role in regulating myocyte contractility under physiological conditions. Therefore, we measured cardiac and vascular function in WT and TRPV2^{−/−} mice in vitro and in vivo and found that TRPV2 deletion resulted in a decrease in basal systolic and diastolic function without affecting loading conditions or vascular tone. TRPV2 stimulation with probenecid, a relatively selective TRPV2 agonist, caused an increase in both inotropy and lusitropy in WT mice which was blunted in TRPV2^{−/−} mice. We examined the mechanism of TRPV2 inotropy/lusitropy in isolated myocytes and found that it modulates Ca^{2+} transients and sarcoplasmic reticulum Ca^{2+} loading. We show that the activity of this channel is necessary for normal cardiac function, and that there is increased contractility in response to agonism of TRPV2 with probenecid.

Key Words: ion channels, cardiac myocytes, contractility, calcium, blood flow, blood pressure, hemodynamics, vascular resistance, probenecid.
Introduction

The transient receptor potential (TRP) family is comprised of Ca\(^{2+}\) selective channels (18) that have been primarily described in regard to their many roles in the nervous (2, 24), immune (23, 24) and renal systems (22), but information regarding their potential role in the cardiovascular system is less well defined. These cation channels have been implicated in the regulation of cardiovascular function (3, 5, 19), and our laboratory has found that the Vanilloid 2 subtype (TRPV2) is present and active in the cardiomyocyte (9, 10). The TRPV2 subtype has also been described in the vascular system, where it appears to be stimulated by osmotic and stretch stimuli (13), and investigations via quantitative real time PCR determined that expression levels of TRPV2 were second only to TRPV4 in deendothelialized rat aorta (25). Interestingly, Iwata et al found that overexpression of TRPV2 in the heart resulted in cardiomyopathy due to Ca\(^{2+}\) overload (6); however, this study did not evaluate the role or mechanism of endogenous channel expression under baseline conditions. Therefore, the present study was performed to explore the mechanism of action of this receptor and the role that it plays in regulating Ca\(^{2+}\) within the cardiomyocyte. Specifically, we examined the effects of TRPV2 activation in WT and TRPV2 null mice at the whole animal, organ and cellular level.

The uricosuric drug probenecid has been described as a potent, relatively selective, TRPV2 agonist in sensory neurons (1) and more recently as a positive inotrope in live mice (9). In previous studies, we showed that inotropic responses to probenecid were absent in TRPV2 null mice, suggesting an important role of endogenously expressed channels in modulating myocyte contractile function (9, 10). Therefore, in this study, we sought to elucidate the cardiovascular sequellae of TRPV2 gene deletion both at baseline and in response to activation by probenecid, and to examine the calcium handling mechanisms that may participate in the observed responses.
Materials and Methods.

Animals and reagents. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati and in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, revised 1996). All wild type (WT) and TRPV2−/− littermates (breeding pairs on a B6129SF2/J background were provided by Dr. M. Caterina, John’s Hopkins, Baltimore, MD) were males 12-16 weeks of age (17). A water soluble preparation of probenecid (Molecular Probes, Life Technologies, Eugene, Oregon) was used for all of the myocyte experiments and was administered in vivo at doses of 30 mg/kg and 100 mg/kg (the lower dose being similar with the highest human dose of 2gr/daily) and in vitro at concentrations of 10⁻⁷ to 10⁻² mol/L.

Echocardiography. Echocardiographic studies were performed with a Vevo 2100 Ultrasound system (Visualsonics, Toronto CA) using a MS400 probe (30 MHz centerline frequency) and were post-processed with Vevostrain software (Visualsonic, Vevo 2100, v1.1.1 B1455). Mice were anesthetized with isoflurane (1.5-2%), and images obtained from a parasternal long axis view between 2 and 10 mm in depth in both M-mode and B-mode and measured as previously described (10).

Cardiovascular function in vivo. Measurements of left ventricular performance and carotid artery blood flow were determined for WT and TRPV2−/− mice as previously described (12). Mice were anesthetized with i.p. ketamine (50 µg/gBW) and thiobutabarbtial (100 µg/gBW, Sigma, MA), a tracheotomy was performed (PE-90), and body temperature was monitored and maintained with a feedback-controlled heating table. The right femoral artery and vein were cannulated with polyethylene tubing for measurement of blood pressure (COBE Cardiovascular, Arvada, CO) and for delivery of drugs. A high fidelity, 1.2F Seisense pressure catheter
(Scisense, London, ON, Canada) was inserted into the right carotid artery and advanced into the left ventricle to measure pressure. For carotid blood flow measurements, the left carotid artery was isolated and fitted with a 0.5-PSB perivascular flow probe connected to a TS420 flowmeter (Transonic Systems, Ithaca, NY). Cerebral blood flow (CBF) was chosen as it represents a highly regulated and auto-regulated vasculature that can be readily assessed with minimal invasiveness. Pressure and flow signals were recorded and analyzed using a PowerLab system (ADInstruments). Hemodynamic measurements were taken at baseline and after the administration of 30 and 100 µg/gBW i.v. doses of probenecid, with 5 minutes between each dose. Measurements were taken from the final 30-40 seconds of each dosage period. Maximum dP/dt (dP/dt\(_{max}\)) and dP/dt at 40 mmHg of developed pressure (dP/dt\(_{40}\)) were calculated from the first derivative of the pressure waveforms. Cerebral vascular resistance (CVR) was calculated from recorded channels of mean arterial pressure (MAP) and mean CBF.

**Vascular smooth muscle reactivity in isolated aorta.** Analyses of contractile properties of isolated vascular smooth muscle from WT and TRPV2\(^{-/-}\) mice, and the vasoactive effects of probenecid were performed in both intact (+E) and endothelium-denuded (-E) thoracic aortae, using a DMT myograph (Danish Myo technology, Marietta GA), as previously described (12). The bath solution contained (in mmol/L) NaCl 118, KCl 4.73, MgCl\(_2\) 1.2, EDTA 0.026, KH\(_2\)PO\(_4\) 1.2, CaCl\(_2\) 2.5, and glucose 5.5, buffered with 25 NaH\(_2\)CO\(_3\); pH, when bubbled with 95%O\(_2\)/5% CO\(_2\), was 7.4 at 37°C. Data were collected and analyzed using a PowerLab system (ADInstruments). Resting length of each aorta was set to 90% of the estimated circumference at an estimated transmural pressure of 100 mmHg using the AD Instruments DMT normalization module. Before the start of the experiment, each aortic segment was challenged with 100 mM KCl and 10 µM phenylephrine (PE) to ensure reproducible forces. Cumulative concentration-
force relationships for increasing doses of probenecid, from $10^{-7}$ M to $10^{-2}$ M, were first tested in order to examine whether probenecid can induce contraction. In separate experiments to examine the relaxing effects of probenecid, vessel rings were first contracted with 3 μM PE, and then exposed to increasing concentrations of probenecid from $10^{-7}$ M to $10^{-2}$ mol/L. The EC$_{50}$ for the relaxing effects of probenecid were determined using a logistic non-linear curve-fitting routine on mean data (OriginLab, Northampton MA).

**Histology.** Following euthanasia, body weights were taken, whole hearts dissected out and weighed to estimate heart weight/body weight ratio (HW/BW). Hearts were then rinsed with PBS to remove excess blood prior to fixation. Hearts (n=4) were fixed with 3.7% buffered formaldehyde and delivered to the CCHMC Department of Pathology Research Core (Cincinnati, Ohio), where they were routinely processed and embedded in paraffin, cut into 6μm sections and individually stained with hemotoxylin and eosin (H&E) and Masson’s trichrome (MT). H&E-stained slides were evaluated by a board-certified (ACVP) veterinary pathologist to identify potential endpoints of cardiomyopathy.

**Histomorphometry.** H&E-stained slides were imaged at 40x magnification with a Zeiss Axio Scope.A1 equipped with an AxioCamERc5s and Zeiss’ Zen Blue Edition software. Myocyte size was determined by measuring the circumference of 30 myocytes, from 10 nonoverlapping left ventricle images for each heart, by using the NIH ImageJ 1.47 software. Measurements were taken in triplicate by a blinded researcher, averaged for each mouse, then averaged for the 2 groups. MT-stained sections were visually accessed for the degree of collagen deposition.

**Quantitative RT-PCR.** Smooth muscle (aorta) was obtained for RNA isolation and qRT-PCR was performed from WT and TRPV2$^{-/-}$ mice as previously described (9). Tissues were flash frozen in liquid nitrogen and stored at -80°C until processed for RNA isolation (Qiazol, Qiagen,
Maryland) and cDNA synthesis (high capacity RNA-to-cDNA kit; Applied Biosystems, Carlsbad, CA) per manufacturer’s instructions. Assessment of TRPV2 transcript levels was done using C-terminal located primers 5’-CTACTGCTCAACATGCTC-3’ (sense) and 5’-CTCATCAGGTATACCATCC-3’ (antisense) which generate a 198 base pair product. qRT-PCR amplification and product detection was performed using SYBR Green with ROX reference dye (Fisher Scientific) on a Stratagene Mx3000p qPCR instrument. All samples were performed in triplicate, with a minimum of 3 independent replicates, and expression differences calculated using the delta-delta Ct approximation method with 18S mRNA as a loading control.

Isolated cardiomyocytes. Cardiac myocytes from WT and TRPV2−/− mice were isolated according to a protocol described previously (15). Briefly, adult mice were anesthetized with sodium pentobarbital (50 mg/kg); hearts were excised and perfused on a Langendorff apparatus with oxygenated solution containing 0.65 units/ml Liberase TH (Roche, Indianapolis, IN). Following digestion, the left ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240-μm screen. The cell suspension was then sequentially washed in 25, 100, 200 μm and 1 mM Ca-Tyrode and resuspended in 1.8 mM Ca-Tyrode for further analysis. All experiments were carried out at room temperature (22-25°C) in standard Tyrode solution containing (mM): 140 NaCl, 5 KCl, 1 MgCl2, 10 glucose, 10 HEPES, 1.8 CaCl2 and pH 7.4 adjusted with NaOH.

Cell mechanics and intracellular calcium measurements \([Ca^{2+}]_i\). Isolated myocytes were paced with a stimulation frequency of 0.5 Hz and myocyte contractions were measured using a video edge detector (Crescent Electronics model VED-105, UT, USA) as previously described (16). For Ca²⁺ signal measurements, myocytes were loaded with the membrane-permeable fluorescent Ca²⁺ indicator Fura-2 (Fura-2/AM; 2μM) and alternately excited at 340 and 380 nm.
by a Delta Scan dual-beam spectrophotofluorometer (Photon Technology International, Birmingham, NJ) at baseline conditions and after application of 10 mmol/L caffeine to induce release of sarcoplasmic reticulum (SR) Ca\(^{2+}\) and assess of SR Ca\(^{2+}\) load. Ca\(^{2+}\) transients were expressed as the 340/380 nm ratio of the resulting 510 nm emissions. The decline of [Ca\(_i\)] during a caffeine-induced Ca\(^{2+}\) transient was taken as a measure of Na/Ca exchange. Data were analyzed by Felix software (Photon Technology International, Birmingham, NJ).

**Western Blot Analysis.** Total protein lysate was prepared from flash-frozen hearts and blotting was performed as previously described (9, 11). Aliquots of total protein were separated on Novex gels (Life Technologies, NY), transferred to nitrocellulose membrane (Life Technologies, NY) and blocked with 5% milk-TBS-Tween. 20\(\mu\)g of protein was loaded on 12% gels for the phospholamban antibodies: PLNt (Thermo Scientific, KY), p-PLN S16 (Millipore, MA) and p-PLN T17 (Badrilla). 50\(\mu\)g of protein was loaded on 4-12% gradient gels for the ryanodine receptor antibodies: RyR2a (Thermo Scientific, KY), p-RyR S2808 (Badrilla) and p-RyR S2814 (gift of Dr. Arnold Schwartz, University of Cincinnati). 50\(\mu\)g of protein was loaded on a 10% gel for the Sodium/Calcium exchanger (NCX, Swant) and sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2, Thermo Scientific, KY) antibodies. Proteins bands were visualized using Western Lightning reagents (Perkin Elmer, KY) and the FluorChemE (ProteinSimple, CA). The densitometry of the bands was determined using AlphaEase software (ProteinSimple, CA) and normalized to GAPDH for loading control.

**Data Analysis.** Statistical analysis was performed by analysis of variance (ANOVA) using either a single factor within-subjects design, or a two-factor mixed design with repeated measures as appropriate. Where significance was indicated, post-hoc testing was performed using the Holm-Sidak method for comparing individual means and correcting for family-wise
error (SigmaPlot v.11.0, Systat Software, Inc., San Jose, CA). Data are presented as means ± S.E.M., and differences were regarded as significant at $P \leq 0.05$. 

Results

**TRPV2-deficiency results in depressed myocardial function in vivo.** We first used echocardiography to examine baseline cardiac function and geometry non-invasively in male, age-matched WT (n=8) and TRPV2−/− mice (n=12), under light anesthesia. Representative images are shown in Figure 1A. All indices of cardiac function were significantly lower in the TRPV2−/− compared to the WT mice and summary data are shown in Figure 1B. Histological analysis in Masson’s trichome-stained hearts (Figure 1C), indicated no overt differences in myocardial collagen content or distribution, and there was no microscopic evidence of myocardial pathology such as inflammation, myofiber degeneration, or necrosis. Measurements of myocyte area in H&E-stained sections (representative image, Figure 1D) revealed no evidence of hypertrophy (273±28 μm² in WT vs. 290±20 μm² in TRPV2−/−; n=4). Heart weight normalized to body weight was also not different between the two groups (HW/BW: 5.78±0.17 mg/g in WT vs. 5.74±0.19 mg/g in TRPV2−/−; n=7 and 8, respectively).

Using the closed-chest, anesthetized mouse model to further evaluate hemodynamic function in these mice, we confirmed that baseline cardiac contractile function, as assessed by LV dP/dt\(_{\text{max}}\) and dP/dt\(_{40}\), was significantly reduced in TRPV2−/− mice compared to WT (Figures 2A-B). Furthermore, whereas administration of probenecid resulted in a robust, dose-dependent stimulation of contractile function in WT mice, this response was blunted in the TRPV2−/− mice (significant group × dose interaction, P<0.004 and P<0.01, respectively). These highly sensitive pressure-phase indices of cardiac function, confirm and extend the non-invasive ejection-phase echo measurements, and illustrate important deficits in the TRPV2−/− mice in terms of both baseline function and the response to probenecid. The observed effects of probenecid on LV dP/dt were generally maximal within one minute of bolus administration, and were stable for at
least 5 minutes (at the time of reported measurement). A similar pattern of responses was observed for diastolic function, with slower rates of relaxation in the TRPV2⁻⁄⁻, as assessed by dP/dt_min (Figure 2C).

Cardiac contractile effects of TRPV2-deficiency are not dependent on changes in preload or afterload. Mean arterial pressure (MAP) and LV systolic pressure (LVP_sys) were not significantly different between WT and TRPV2⁺⁄⁺ mice under baseline conditions or during administration of probenecid, and probenecid did not alter these pressures in either group (Figures 3A-B). Likewise, LV end diastolic pressure (LVEDP) was not different between the two genotypes at baseline or during treatment with probenecid (Figure 3C), though there was a significant decrease (P<0.01) in LVEDP in WT mice at the highest dose of probenecid which was not present in the TRPV2⁺⁄⁻ mice (group × dose interaction, P<0.004). Overall, these data indicate that changes in afterload and preload were not responsible for the observed differences in myocardial function between the WT and TRPV2⁺⁄⁻ mice. Finally, in contrast to our echocardiographic results, we found no differences in HR between the two genotypes at baseline, and probenecid administration modestly increased HR in both cohorts. Though the overall differences were small, a modest chronotropic effect of probenecid in WT mice was blunted in TRPV2⁺⁄⁻ (group × dose interaction, p=0.05; Figure 3D).

TRPV2-deficiency does not alter vascular responsiveness. As an initial examination of vascular reactivity, we assessed carotid artery blood flow at baseline and during administration of probenecid. Although carotid blood flow appeared to be somewhat lower in TRPV2⁺⁄⁻ mice compared to WT (Figure 4A), the difference was not significant (p=0.09). This level of uncertainty is likely due to a high level of variability in these measurements, which in our experience is not unusual, and likely reflects individual differences in relative blood flow.
through the carotid and vertebral arteries from mouse to mouse. Nonetheless, a meaningful
difference cannot be ruled out. Calculated values for cerebral vascular resistance (CVR) were of
a similar nature (WT=94±12% vs TRPV2−/−=124±5%; P=0.09). Due to this high level of
variability, changes in CVR in response to probenecid were calculated as a percentage of
baseline levels, as shown in Figure 4B. It is apparent from these data that administration of
probenecid at these doses had no effect on the cerebral vasculature in WT and TRPV2−/− mice.

To further examine vascular responsiveness ex vivo, we examine force production in
isolated aortic segments from the two groups of mice. We first determined whether there were
any vasoconstrictor effects of probenecid over a wide concentration range (10^{-6} to 10^{-2} mM), and
we found no evidence of increased force production, with or without intact endothelium (data not
shown). We then examined the relaxing effects of increasing concentrations of probenecid after
pre-contracting the vessels segments with 3 µM phenylephrine. In endothelium-denuded vessels
(-E, Figure 5 A-B), there were no differences in maximal developed tension in response to
phenylephrine between WT (n=8) and TRPV2−/− (n=8) mice (5.99 ± 0.74 vs 6.07 ± 0.55 mN,
respectively). In response to increasing probenecid in these pre-contracted vessels, there were no
significant dilatory responses until the concentration reached 1mM. At the highest concentration
of 10 mM, the developed force was reduced to about 25% of maximum in both groups of mice
(Figure 5B). These concentrations are several orders greater than the relevant pharmacological
dose of 1 gram (orally administered) which results in an approximate serum concentration of 246
µmol/L in humans (21). Finally, there were no differences in the concentration-response
characteristics between WT and TRPV2−/− mice without endothelium (EC_{50}: 4.56 ± 0.34 vs 4.59
± 0.52 mM, respectively).
In WT and TRPV2⁻⁻ vessels with intact endothelium (+E, Figures 5C-D), maximal developed tension in response to phenylephrine was substantially less than in -E vessels, but was not different between the two genotypes (1.45 ± 0.12 vs 1.75 ± 0.25 mN in WT and TRPV2⁻⁻, respectively, Figure 5C). Vessels with intact endothelium were somewhat more sensitive to probenecid than -E vessels, but again there were no differences in the concentration-response characteristics between the two genotypes (EC₅₀: 0.67 ± 0.21 vs 0.52 ± 0.11 mM, respectively, Figure 5D). The increased responsiveness of +E vessels to probenecid suggests some participation of the endothelium in the dilatory response to probenecid, but through a TRPV2⁻⁻ independent mechanism. This is confirmed by qRT PCR of smooth muscle from WT and TRPV2⁻⁻ mice which demonstrated no significant expression of TRPV2 (data not shown).

**TRPV2-deficiency is associated with compromised contractile function and Ca²⁺ signaling in isolated cardiomyocytes.** In order to further examine the mechanisms associated with decreased contractility in TRPV2⁻⁻ mice in vivo, we evaluated the mechanical properties in isolated cardiomyocytes, representing a load-independent system. Compared to WT, TRPV2⁻⁻ myocytes had significantly reduced fractional shortening (FS, P<0.001), shortening rate (+dL/dt, P<0.001) and relengthening rate (-dL/dt, P<0.001) (Figure 6A, representative trace in 6B). Treatment with probenecid significantly increased fractional shortening in WT cells (P<0.001) but had no effect in TRPV2⁻⁻ myocytes (Figure 6C). Similarly, after exposure to probenecid the WT myocytes increased their shortening rate by 25.4% (+dL/dt, P<0.001), and relengthening rate by 24.5%(-dL/dt, P<0.001) while the TRPV2⁻⁻ myocytes exhibited no significant change. Calcium handling and kinetics were also measured in these isolated cardiomyocytes. The peak of the Ca²⁺ transient was significantly reduced in TRPV2⁻⁻ cardiomyocytes (P<0.001; representative trace in Figure 7A, summary data in Figure 7B). Critically, the time to 50% decay
of the Ca\(^{2+}\) peak (T\(_{50}\), Figure 7B) and the Tau were prolonged in TRPV2\(^{-/-}\) myocytes relative to WT (P<0.001), indicating impaired SR Ca\(^{2+}\) cycling. Furthermore, the caffeine-induced Ca\(^{2+}\) transient peak, as an index of SR load, was reduced in TRPV2\(^{-/-}\) myocytes (P<0.001; representative trace in Figure 7C; summary data in Figure 7D). Assessment of NCX function by measuring the time constant (\(\tau\)) of Ca\(^{2+}\) decline during caffeine-induced Ca\(^{2+}\) transients indicated no difference between WT and TRPV2\(^{-/-}\) myocytes (P=0.49; Figure 7D) (11). There were no significant differences in resting Ca\(^{2+}\) concentration between WT and TRPV2\(^{-/-}\) myocytes.

**TRPV2\(^{-/-}\) mice exhibit no changes in expression of traditional calcium handling proteins.**

We investigated various proteins known to be involved in the regulation of physiologic cardiac function and which have been shown to change expression levels and/or phosphorylation status in heart failure. These included phospholamban (phosphorylated and total, pPLN and tPLN, respectively), ryanodine receptor 2 (phosphorylated and total, pRyR2 and tRyR2, respectively), the sodium/calcium exchanger (NCX) and the cardiac isoform of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2). Western blot analysis of typical Ca\(^{2+}\) handling proteins showed no significant changes in protein levels or phosphorylation status between the WT and TRPV2\(^{-/-}\) mouse hearts (Figure 8A-C).
Discussion

Data presented in this study implicate TRPV2 channels in the regulation of myocardial function under physiologic conditions. TRPV2-deficient mice consistently showed depressed myocardial performance, both in vivo and ex vivo, and inotropic and lusitropic responses to the TRPV2 agonist probenecid were blunted in TRPV2 knockout mice. Despite previous evidence that TRPV2 channels might play a role in regulating vascular tone (13, 25), our results indicated that the myocardial effects of TRPV2 activation were not secondary to differences in loading conditions between the WT and TRPV2−/− mice. Data from isolated myocytes show that Ca²⁺ handling is altered in TRPV2-deficient myocytes and suggest that the inotropic effects of TRPV2 activation are primarily intrinsic to cardiomyocytes. Overall, our findings confirm TRPV2 as an important component of the Ca²⁺ handling machinery in cardiomyocytes in vivo.

Initial examination of the TRPV2−/− phenotype using echocardiography revealed depressed systolic function (i.e SV, EF and CO) as estimated from M-mode-derived measurements. These data were supported and confirmed by invasive measurements of left ventricular systolic and diastolic function, which showed depressed values of positive and negative dP/dt in TRPV2-deficient mice as well as blunted responsiveness to probenecid. Although the data from these different approaches yielded consistent results with respect to contractility, we found that HR was slightly lower in the TRPV2-deficient mice using echocardiography under light anesthesia and not different under the heavier anesthetic load required for invasive catheterization. Thus, a chronotropic influence of TRPV2 channels cannot be ruled out. Consistent with this notion, probenecid increased HR mildly in the WT mice, and this effect was blunted in TRPV2 knockouts. This TRPV2-dependent positive chronotropic effect would be consistent with data shown in Fig. 1B. The lack of a difference in HR between the groups at baseline may be due to
the deeper level of anesthesia in this set of experiments (a bottom effect). Finally, morphological studies indicate that there are no significant differences in cardiac histopathology or cardiomyocyte size to account for or reflect upon the observed alterations in function.

We examined the participation of TRPV2 in the regulation of vascular tone, in vivo and ex vivo, and we found little evidence to support a substantial role. In particular, data from isolated aortae revealed no differences in force production between genotypes, and there were no apparent vasoactive effects of probenecid in either intact or deendothelialized vessels until superpharmacologic concentrations were administered (i.e. ≥1 mmol/L). These ex vivo data are supported by intact measurements which showed that cerebral blood flow and vascular resistance were unaffected by administration of probenecid in both WT and TRPV2−/− mice. We also saw no differences in MAP or LVPsys between the two groups, indicating that there were no primary disparities in afterload. LVEDP was not different between the WT and TRPV2−/− mice at baseline, despite the apparent tendency for lower baseline values in the TRPV2−/− mice (Figure 3C). Moreover, whereas probenecid caused a significant decrease in LVEDP in WT mice at the higher dose, this effect was abolished in the TRPV2−/− mice. Thus, the aforementioned increase in cardiac performance in response to probenecid (Figures 2A-B) occurred in spite of rather than because of a decrease in preload.

The effects of TRPV2-deficiency on cerebral blood flow might be interpreted in light of other roles that TRPV2 has been shown to play in the nervous system (14), as well as with possible effects in the microvasculature that were not studied in these experiments. TRPV2 channels are highly expressed in both the forebrain and hindbrain, including areas that have important roles in regulating body fluid homeostasis, autonomic function, and metabolism (14). Furthermore, though the brains of TRPV2−/− mice are evidently not grossly abnormal, a detailed
analysis has not been reported, and an influence of TRPV2 gene deletion on brain development and morphology cannot be ruled out. We did not measure brain weight, and we were therefore unable to express blood flow normalized to tissue weight, as would be the convention.

Additional studies will focus on these possibilities and will also attempt to put in perspective the findings by Muraki et al. that previously described TRPV2 as an active player in vascular tone with a series of experiments using murine aortic myocytes (13). Based on experiments that stretched and swelled mouse aortic myocytes in a number of different manners and in transiently transfected cells, they concluded that TRPV functions as an important stretch sensor in vascular smooth muscles. Though our studies did not reveal a difference in vascular function between WT and TRPV2\(^{-/-}\), we did not examine the stretch-dependent myogenic responses in these mice. Therefore, our data neither support nor refute the earlier findings described, under stress conditions, by Muraki and colleagues and future studies will be performed to ascertain the role that TRPV2 plays in the vasculature under physiologic and stress conditions.

The isolated myocyte studies provide additional information regarding the role that TRPV2 plays in cardiomyocyte contractility and relaxation. We show with a load independent system that intrinsic myocyte function is reduced in the TRPV2\(^{-/-}\) cells. This confirms our in vivo findings of the importance of TRPV2 at the cardiac level as this unloaded system allows the differences in cardiomyocyte function to become more apparent. The studies of Ca\(^{2+}\) dynamics further clarify the role of TRPV2 by demonstrating lower Ca\(^{2+}\) transients and Ca\(^{2+}\) SR load in the TRPV2 knockout mice, and this occurs without affecting NCX function. This implicates, though does not prove, a role of TRPV2 in SR Ca\(^{2+}\) cycling. We speculate that this is an indirect role, due to moderate uptake of Ca\(^{2+}\) through these sarcolemmal channels, and that small increases in cytosolic Ca\(^{2+}\) would lead to increased SR load and release. This conclusion is supported by the
protein expression analysis of Ca^{2+} handling proteins in WT and TRPV2^{-/-} mice, where we found no difference for PLN, p-PLN (S16 and T17), RyR2, p-RyR2 (S2808 and S2814), NCX and SERCA2.

With respect to the mechanism of TRPV2 activation, we previously reported that probenecid administration, putatively through TRPV2 stimulation, results in increased cytosolic Ca^{2+} concentrations in isolated WT cardiomyocytes (9). It has also been shown that TRPV2 (described as GRC) translocates to the cytosolic membrane (8), and that cardiac-specific overexpression results in cardiomyopathy due to Ca^{2+} overload (6). This latter finding led the authors to suggest that GRC (i.e TRPV2) is a “key player” in myocyte degeneration, since it was elevated in the sarcolemma of humans with muscular dystrophy as well as in the dilated heart samples of transgenic mice. Based on our findings, we would extend this argument to suggest that under physiologic conditions, TRPV2 plays a clinically relevant and readily assessed role in myocyte contractility through small changes in Ca^{2+} transients, and that under experimental conditions of marked overexpression of TRPV2, the exaggerated influx of Ca^{2+} is associated with the development of cardiomyopathy. A future direction of study will evaluate the role that TRPV2 plays under stress conditions such as pressure overload as its activity may be modulated under tonic stress.

Probenecid, an FDA approved drug with a very safe clinical profile (20), has been previously demonstrated to be a TRPV2 agonist (1). Based on this information, we recently showed that probenecid has inotropic properties, which for decades have been overlooked (9), and that this effect was secondary to transient increases in cytosolic Ca^{2+} through SR release and not through the traditional inotropic pathway of β-adrenergic stimulation (10). This is a crucial finding, since clinically available positive inotropes generally increase metabolic demand,
activate pro-apoptotic signaling pathways and promote malignant arrhythmias which result in increased mortality (7). In this study, we not only confirm our previous findings of probenecid as an inotrope with invasive measurements, but we also demonstrate inotropic and lusitropic effects at both high (100 mg/kg) and low (30 mg/kg) doses. This latter dose likely reflects the higher end of the dose range that can be safely administered to humans, as it represents approximately 2 to 3 grams/day dosage in an average person (4).

In conclusion, our data provide evidence that the TRPV2 channel participates in the Ca\(^{2+}\) handling machinery of the cardiomyocyte, and contributes to normal baseline function. Activation of this channel increased function though modulation of the Ca\(^{2+}\) transient, and perhaps through an indirect effect on SR Ca\(^{2+}\) load. This mechanism is quite different from that of clinically used inotropes and opens the possibility for clinical applications for patients with heart failure.
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Disclosures

J.R and W.K.J are founding members of TRP Therapeutics with the goal of commercializing TRP channel modulators. Neither has received fees, honoraria or any income from this venture.
References


Figure Legends

Figure 1. Echocardiographic and morphological indices of cardiac phenotype in WT and TRPV2<sup>−/−</sup> mice. A. Representative echocardiographic M-mode images for WT and TRPV2<sup>−/−</sup> mice are shown. B. M-mode measurements of stroke volume (SV), ejection fraction (EF), cardiac output (CO) and heart rate demonstrated diminished contractile function in TRPV2<sup>−/−</sup> mice compared to WT (n=8 and 12, respectively, *P<0.05). C. Histological analysis of heart sections did not show evidence of increased fibrosis in TRPV2<sup>−/−</sup> mice, Masson’s Trichrome stain, 40x; and D. There was no histological evidence of cardiac pathology and no change in cardiomyocyte cell area (see text), Hematoxylin and eosin (H&E), 40x.

Figure 2. Invasive measurements of cardiac function in WT vs. TRPV2<sup>−/−</sup> mice at baseline and in response to low (30µg/gBW) and high (100 µg/gBW) i.v. injections of probenecid. A. The maximum rate of contraction, dP/dt<sub>max</sub>, and B. the rate of contraction at 40 mmHg of developed pressure, dP/dt<sub>40</sub>, show that TRPV2<sup>−/−</sup> mice have lower baseline function and blunted responses to probenecid. C. Measurements of the maximum rate of relaxation, dP/dt<sub>min</sub>, also show blunted responses. *P<0.01 compared to baseline; †P<0.05 compared to corresponding value in WT.

Figure 3. Invasive hemodynamic measurements in WT vs. TRPV2<sup>−/−</sup> mice at baseline and in response to probenecid. A. mean arterial pressure (MAP) and B. left ventricular systolic pressure (LVPsys) revealed no differences between the two genotypes as indices of afterload. C. LV end diastolic pressure (LVEDP) was not significantly different between the genotypes; though it was decreased in WT mice, but not in TRPV2<sup>−/−</sup>, in response to the high dose of probenecid (100µg/gBW i.v.). D. Heart rate (HR) was not different at baseline, but increased to a greater extent in response to probenecid in WT compared to TRPV2<sup>−/−</sup> mice (significant group × dose interaction). *P<0.01 in comparison to baseline.
Figure 4. Invasive blood flow measurements in WT and TRPV2^-/- mice at baseline and in response to probenecid. A. There were no significant differences in carotid blood flow (CBF) between WT and TRPV2^-/- mice at baseline or during treatment with probenecid. B. Relative changes in cerebral vascular resistance, ΔCVR, indicated that probenecid had no effect on cerebral vascular tone.

Figure 5. Measurements of force production in isolated aortic ring preparations from WT (thin line, open symbol) and TRPV2^-/- mice (thick line, closed symbol; n=8/group), after pre-contraction with phenylephrine (PE) and in response to escalating doses of probenecid. Measurements were made in vessels in which the endothelium had been removed (-E, panels A and B) and intact vessels (+E, panels C and D). Top panels: developed force following pre-contraction with 3 µM PE and cumulative addition of probenecid; bottom panels: percent relaxation in response to probenecid with logistic curve fit ($y = \frac{\left((A_1 - A_2)\right)}{\left(1 + \left(\frac{x}{x_0}\right)^p\right)} + A_2$). In both -E vessels and +E vessels, there were no observed differences in vascular smooth muscle function between the two genotypes. Very high concentrations of probenecid (1mM and above) caused a significant vasodilation that was not different between WT and TRPV2^-/- mice.

Figure 6. Isolated cardiomyocyte studies of single cell contractile function in WT and TRPV2^-/- mice. A. Fractional shortening (FS), rate of contraction (+dL/dt), and rate of relengthening (-dL/dt) were each significantly decreased in the TRPV2^-/- myocytes (n=15 cells/3 hearts) compared to WT myocytes (n=14 cell/3 hearts). B. Representative tracings of cell length changes. *P<0.01 versus WT. C. Fractional shortening (FS) increased in WT myocytes after exposure to probenecid but not in TRPV2^-/- myocytes (n=15 cells/3 hearts). *P<0.01 versus WT.
Figure 7. Calcium handling in isolated myocyte from TRPV2−/− and WT mice. A. Representative tracings of Ca^{2+} transients in myocytes paced at 0.5 Hz. B. Summary data for Ca^{2+} transient peak amplitude and time to 50% decay (T50) of Ca^{2+} transient in myocytes from WT (n=22 cells/3 hearts) and TRPV2−/− mice (n=24 cells/3 hearts), as indicated by the 340:380 fura-2 ratio. C. Representative tracings of caffeine-induced Ca^{2+} release in isolated myocytes. D. The amplitude of the caffeine-induced Ca^{2+} transient was measured as an index of SR Ca^{2+} load, and the time constant of the pulse decay (τ, tau) was used as an index of NCX activity in myocytes from WT (n=15 cells/3 hearts) and TRPV2−/− mice (n=16 cells/3 hearts). *P<0.05 versus WT.

Figure 8. Western blot analysis Ca^{2+} handling proteins from hearts of WT (n=4) and TRPV2−/− (n=5) mice. Left panels show representative blots, and right panels show summary data for densitometry readings normalized to GAPDH. A. Protein expression levels for phospholamban (PLN) and phosphorylated phospholamban at Serine 16 (p-PLN S16) and Threonine 17 (p-PLN T17). B. Protein expression levels for ryanodine receptor 2A (RyR2A) and phosphorylated ryanodine receptor 2A at Serine 2808 (p-RyR2 S2808) or Serine 2814 (p-RyR2 S2814). C. Protein expression level for sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2A) and the sodium/calcium exchanger (NCX). No differences were observed between WT and TRPV2−/− mice.
**Figure 1**

- **A**
  - WT
  - TRPV2-/-
  - Heart Rate
  - Cardiac Output
  - Ejection Fraction
  - Stroke Volume

- **B**
  - Stroke Volume
  - Ejection Fraction
  - Cardiac Output
  - Heart Rate

- **C**
  - WT
  - TRPV2-/-

- **D**
  - WT
  - TRPV2-/-
Figure 2

A

- Wild type (n=6)
- TRPV2−/− (n=5)

Grp: p=0.01
Dose: p<0.001
Grp X Dose: p=0.004

B

Grp: p=0.005
Dose: p<0.001
Grp X Dose: p=0.01

C

Grp: p=0.09
Dose: p<0.001
Grp X Dose: p=0.003

Control 30 100

Probenecid Dose (µg/gBW)
Wild type (n=6) vs. TRPV2⁻/⁻ (n=5)

MAP (mmHg)

Grp: p=0.63
Dose: p=0.50
Grp X Dose: p=0.08

LVPsys (mmHg)

Grp: p=0.59
Dose: p=0.36
Grp X Dose: p=0.10

LVEDP (mmHg)

Grp: p=0.21
Dose: p<0.001
Grp X Dose: p=0.004

HR (bpm)

Grp: p=0.26
Dose: p<0.001
Grp X Dose: p=0.05

Figure 3
Figure 4

(A) CBF (ml/min)

- Wild type (n=6)
- TRPV2−/− (n=5)

(B) ΔCVR (%)

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Figure 4
Figure 5
Figure 6

A

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<tr>
<td>WT</td>
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<tr>
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B

Isolated Myocytes

Cell length (µm)

WT

KO

C

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* Indicates significant difference.
Figure 7
Figure 8

A

- tPLN
- pPLN S16
- pPLN T17
- GAPDH

B

- tRyR2
- pRYR2 S2808
- pRYR2 S2814
- GAPDH

C

- SERCA2a
- NCX
- GAPDH

Relative Density/GAPDH

WT
TRPV2−/−