Thrombospondin-4 induces prolongation of action potential duration in rat isolated ventricular myocytes

Keisuke IMOTO, Momoko ARATANI, Takahiro KOYAMA, Muneyoshi OKADA and Hideyuki YAMAWAKI

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Higashi 23 bancho 35-1, Towada city, Aomori 034-8628, Japan.

Short running title: TSP-4 PROLONGS APD IN CARDIOMYOCYTES

*Corresponding author: Muneyoshi Okada, DVM, PhD.

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Higashi 23 bancho 35-1, Towada city, Aomori 034-8628, Japan.

Phone: +81-176-23-4371; FAX: +81-176-23-8703

E-mail: mokada@vmas.kitasato-u.ac.jp

¶ These authors contributed equally to this work.
ABSTRACT

Expression of thrombospondin-4 (TSP-4), a matricellular protein, is increased in the heart tissue of various cardiac disease models. In dorsal root ganglion neurons, TSP-4 inhibits L-type Ca\(^{2+}\) channel (LTCC) activity. Although TSP-4 might be related to the electrophysiological properties in heart, it remains to be clarified. The present study aimed to clarify the effects of TSP-4 on action potential (AP), LTCC current (\(I_{\text{CaL}}\)) and voltage-dependent K\(^+\) (K\(v\)) channel current (\(I_{\text{Kv}}\)) in rat isolated ventricular myocytes by a patch clamp technique. Ventricular myocytes were isolated from the heart of adult male Wistar rats. The ventricular myocytes were treated with TSP-4 (5 nM) or its vehicle for 4 hr. Then, whole-cell patch clamp technique was performed to measure AP (current-clamp mode) and \(I_{\text{CaL}}\) and \(I_{\text{Kv}}\) (voltage-clamp mode). The mRNA expression of Kv channels was examined by reverse transcription-polymerase chain reaction. TSP-4 had no effect on the resting membrane potential and peak amplitude of AP. On the other hand, TSP-4 significantly prolonged AP duration (APD) at 50% and 90% repolarization. TSP-4 significantly inhibited the peak amplitudes of \(I_{\text{CaL}}\) and \(I_{\text{Kv}}\). TSP-4 had no effect on mRNA expression of Kv channels (Kcna4, Kcna5, Kcnb1, Kcnd2 and Kcnd3). The present study for the first time demonstrated that TSP-4 prolongs APD in rat ventricular myocytes, which is possibly mediated through the suppression of Kv channel activity.

KEY WORDS: action potential duration; arrhythmia; L-type calcium channel; thrombospondin-4; voltage-dependent potassium channel
INTRODUCTION

Lethal ventricular arrhythmia, such as persistent ventricular tachycardia, ventricular fibrillation and torsade de pointe, requires immediate treatment [2]. QT interval prolongation accompanied with early afterdepolarization (EAD) is one of the causes of lethal ventricular arrhythmia [20]. Long QT syndromes (LQTS) is divided into a congenital and a secondary LQTS [20]. The genetic mutation in \textit{LQT1-13} is a major cause of congenital LQTS [31]. On the other hand, drugs (antiarrhythmic agents (I, III), psychotropic drugs, antihypertensive agents, antihistaminic drugs, antimycotic agents and antibacterial agents), electrolyte disorders and basal cardiac diseases (myocardial infarction, cardiac hypertrophy and heart failure) are the triggers for secondary LQTS [8, 14, 18, 19, 28].

Matricellular proteins are a family of non-structural extracellular matrix (ECM) proteins, which mediate cell-cell and cell-matrix interaction by binding to other structural ECM, growth factors, cytokines, proteases and cell-surface receptors [5]. Thrombospondin-4 (TSP-4), a matricellular protein, is composed of 961 amino acid residues in human (Accession number: CAA79635.1). TSP-4 is expressed in whole body organs, especially high in heart and skeletal muscles [21, 33]. The expression of TSP-4 is known to increase in the heart tissue of patients with coronary artery disease and various heart disease model animals, such as pressure overload-induced hypertrophied mice, spontaneously-hypertensive rats and myocardial infarcted rats [12, 13, 23, 29]. It has been reported that TSP-4 exerts a cardioprotective effect through the inhibition of interstitial fibrosis via regulation of cardiac fibroblasts [12, 30]. TSP-4 gene knock-out inhibited the increase of stroke volume in transaortic-constriction (TAC) model mice. Thus, TSP-4
might play a pivotal role in systolic function of the hearts [7]. On the other hand, in dorsal root ganglionic neurons, TSP-4 inhibits L-type Ca\(^{2+}\) channel (LTCC) activity via binding to the \(\alpha_2\delta_1\) subunit [25]. Although TSP-4 might be related to the electrophysiological properties in heart, it remains to be clarified. The present study aimed to clarify the effects of TSP-4 on action potential (AP), LTCC current (\(I_{\text{CaL}}\)) and voltage-dependent K\(^+\) (\(K_v\)) channel current (\(I_{K_v}\)) in rat ventricular myocytes by a patch clamp technique.
MATERIALS AND METHODS

Animals

All animal studies were approved by Institutional Animal Care and Use Committee of Kitasato University (Approved No. 17-085, 18-022 and 19-127) and conducted in accordance with the guidelines of the Kitasato University. Five to seven-week-old male Wistar rats (CLEA Japan, Tokyo, Japan) were used to isolate ventricular myocytes. The animals were fed with a standard chow diet and tap water, and maintained in a 12 hr /12 hr light-dark cycle.

Reagent

Recombinant mouse TSP-4 (R&D Systems, Minneapolis, MN, USA) and Verapamil (Sigma-Aldrich, St. Louis, MO, USA).

Isolation of rat ventricular myocytes

Rat ventricular myocytes were isolated by an enzymatic digestion of perfused heart as described previously [16]. Briefly, rats were anesthetized by an intraperitoneal injection of urethane (1.5 g/kg). The heart was excised and connected to a modified Langendorff apparatus via aorta. HEPES-Tyrode solution [(in mM): NaCl 143, KCl 5.4, NaH₂PO₄ • 2H₂O 0.33, MgCl₂ • 6H₂O 0.5, glucose 5.5, HEPES 5 and CaCl₂ 1.8 adjusted to pH 7.4 with NaOH] was perfused for 5 min at 37 ºC. After changing the perfusate into Ca²⁺-free normal HEPES-Tyrode solution for 5 min, 0.02% collagenase (Wako, Osaka, Japan) was treated for 30-40 min. Then, the heart was washed with modified Kraft-Bruhe (KB) solution [(in mM): KOH 70, L-glutamic acid 50, KCl 40,
taurine 20, MgCl₂ · 6H₂O 3, glucose 10, HEPES 10, EGTA 1 adjusted to pH 7.4 with KOH]. The ventricular myocytes isolated from digested heart were seeded and attached on 1% laminin (Discovery Labware, Billerica, MA, USA)-coating coverslips in KB solution for 1 hr (37 ºC, 5% CO₂). The ventricular myocytes were treated with TSP-4 (5 nM) or equal volume of solvent (phosphate buffered saline; Vehicle) for 4 hr (37 ºC, 5% CO₂) in Medium 199 (Sigma-Aldrich) supplemented with taurine (5 mM), creatine (5 mM) and L-carnitine (2 mM).

**Electrical recording**

Patch clamp technique was performed using a Patch/Whole Cell Clamp Amplifier CEZ-2400 (Nihon Kohden, Tokyo, Japan) with a pCLAMP 10 software (Clampex 10, Molecular Devices/Axon Instruments, Union City, CA, USA) as described previously [16]. Patch pipettes were pulled from glass capillary (1.5 × 90 mm, MODEL G-1.5, Narishige, Tokyo, Japan) using a PC-10 Needle Puller (Narishige) and filled with pipette solution. Ventricular myocytes on laminin-coating coverslip were placed in a recording chamber equipped with an inverted microscope (IMT-2 or CKX53, Olympus, Tokyo, Japan) and superfused with a bath solution at a rate of 3 ml/min at 34 ± 1 ºC. AP was recorded by a current-clamp mode. The bath solution for AP recording was composed of the followings (mM): NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 1, glucose 10, HEPES 5 and L-arginine 1 adjusted to pH 7.4 with NaOH. The pipette solution for AP recording was composed of the followings (mM): NaCl 8, KCl 10, potassium aspartate 140, HEPES 5 and Mg-ATP 2 adjusted to pH 7.2 with KOH. AP was elicited by a 0.8−1 nA rectangular pulse for 5−8 msec. I_cal and I_Kv were recorded by a voltage-clamp mode. Normal HEPES-Tyrode solution was used as the bath
solution for $I_{CaL}$ recording. The pipette solution for $I_{CaL}$ recording was composed of the followings (mM): CsCl 120, MgCl$_2$ 6, EGTA 10, HEPES 10 and ATP-Mg 2 adjusted to pH 7.2 with CsOH. $I_{CaL}$ was elicited by 0.4 sec depolarization pulse to the test potentials ranging -50–40 mV in 5 mV increments from a holding potential of -40 mV (0.2 sec after elevation from -80 mV) every 5 sec. The bath solution for $I_{Kv}$ recording was composed of the followings (mM): NaCl 136, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1, glucose 10, HEPES 5 and NaH$_2$PO$_4$ 0.33 adjusted to pH 7.35 with NaOH. Verapamil (10 μM) was added in the bath solution to block $I_{CaL}$. The pipette solution for $I_{Kv}$ recording was composed of the followings (mM): KCl 20, MgCl$_2$ 1, potassium aspartate 110, HEPES 10, Mg-ATP 5, GTP 0.1, creatine phosphate dipotassium salt 5 and EGTA 10 adjusted to pH 7.3 with KOH. $I_{Kv}$ was elicited by 0.4 sec depolarization pulse to the test potentials ranging -60–60 mV in 5 mV increments from a holding potential of -70 mV every 10 sec. The peak amplitudes of $I_{CaL}$ relative to the end of depolarization pulse and of $I_{Kv}$ relative to zero current level were measured to each test potentials by using pCLAMP 10 software (Clampfit 10, Molecular Devices/Axon Instruments). The peak amplitudes (pA) were normalized by cellular membrane capacitance (pF) and the current-voltage curves were depicted.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed using Quick Taq HS DyeMix (TOYOBO, Osaka, Japan) as described previously [16]. Total RNA was isolated from ventricular myocytes treated with TSP-4 (5 nM, 4 hr) or Vehicle using TRI REAGENT (Molecular Research Center, Montgomery, OH, U S A.). The cDNA was obtained from the total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). After initial
activation at 94°C (2 min), the amplification reaction was run for 35 cycles in Kv channel (Kcna4, Kcna5, Kcnb1, Kcnd2 and Kcnd3) and 27 cycles in glyceraldehyde 3-phosphate dehydrogenase (Gapdh) at 94°C (30 sec), 62°C (30 sec), and 68°C (1 min). The primer sequences were shown in Table 1. Agarose gel electrophoresis was performed to separate the PCR products. The bands were detected by an ATTO light capture system (AE-6972, ATTO Corporation, Tokyo, Japan).

Statistical analysis

All data are shown as means ± standard error of the mean. Statistical analyses were performed using Student’s t-test (Figs. 1B, 1C and 4B) or two-way ANOVA followed by Bonferroni’s post hoc test (Figs. 2B and 3B). A value of $p<0.05$ was considered statistically significant.
RESULTS

*TSP-4 prolongs AP duration (APD) in rat ventricular myocytes*

We examined the effects of TSP-4 (5 nM, 4 hr) on AP in rat ventricular myocytes. There is no difference in the stimulating pulse between vehicle- and TSP-4-treated ventricular myocytes (Vehicle: 1.00 ± 0.00 nA, 5.09 ± 0.34 msec, n=11; TSP-4: 0.98 ± 0.02 nA, 5.44 ± 0.31 msec, n=9). In the TSP-4-treated group, repolarization phase was prolonged compared with the Vehicle-treated group (Fig. 1A). TSP-4 had no effect on the resting membrane potential (RMP) and peak amplitude of AP (Fig. 1B). On the other hand, TSP-4 significantly prolonged the APD at 50% and 90% repolarization (APD$_{50}$ and APD$_{90}$) (Fig. 1C, $p<0.05$, Vehicle: n=11; TSP-4: n=9).

*TSP-4 decreases $I_{\text{Ca,L}}$ in rat ventricular myocytes*

The increase of inward $I_{\text{Ca,L}}$ is one of the causes of APD prolongation [1, 10]. Thus, we examined the effects of TSP-4 (5 nM, 4 hr) on the peak amplitude of $I_{\text{Ca,L}}$ in rat ventricular myocytes (Fig. 2A). TSP-4 significantly decreased $I_{\text{Ca,L}}$ in the range of -10–5 mV (Fig. 2B, $p<0.05$, Vehicle: n=16; TSP-4: n=11).

*TSP-4 decreases $I_{\text{Kv}}$ without influencing mRNA expression of Kv channels in rat ventricular myocytes*

The decrease of outward $I_{\text{Kv}}$ is a major cause of APD prolongation [1, 10]. Thus, we examined the effects of TSP-4 (5 nM, 4 hr) on the peak amplitude of $I_{\text{Kv}}$ in rat ventricular myocytes. TSP-4 significantly decreased $I_{\text{Kv}}$ (Fig. 3A and 3B, $p<0.05$, Vehicle: n=10; TSP-4: n=9). On the other hand, TSP-4 (5 nM, 4 hr) had no effect on
mRNA expression of Kv channels (Kcna4, Kcna5, Kcnb1, Kcnd2 and Kcnd3), which are responsible for the $I_{Kv}$ in repolarization phase of AP, in rat ventricular myocytes (Fig. 4A and 4B)
DISCUSSION

The present study for the first time demonstrated that TSP-4 prolongs APD in rat isolated ventricular myocytes possibly through the inhibition of $I_{Kv}$ but not $I_{CaL}$.

It has been reported that serum concentration of TSP-4 in healthy human volunteers was in the range of 300-600 ng/ml [17]. In addition, the expression of TSP-4 in heart tissue is increased in patients with coronary arterial disease and animal models with cardiac hypertrophy and myocardial infarction [12, 13, 23, 29]. Thus, 5 nM of TSP-4 (approximately 700 ng/ml) used in this study might be within the pathophysiological range. Pan et al. reported that TSP-4 (5 nM) inhibited $I_{CaL}$ by 20% in dorsal root ganglionic nerve cells [25]. In addition, the acute bath application (10 min) of TSP-4 had no effect on $I_{Ca,L}$ while treatment of the cells with TSP-4 for 4 hr reduced it [25]. From these observations, we stimulated the ventricular myocytes with recombinant TSP-4 (5 nM) for 4 hr, although a possibility that the acute application of TSP-4 affects ion channel activity in ventricular myocytes could not be excluded.

Typical AP waveform was obtained in Vehicle-treated ventricular myocytes (Fig. 1A) [34]. Na⁺/K⁺ ATPase and inward-rectifier K⁺ channel current form the RMP, whereas Na⁺ channel current is a main component of the peak amplitude of AP [3, 24, 32]. Because the RMP and peak amplitude of AP were not changed by the TSP-4 treatment (Fig. 1B), it is suggested that TSP-4 did not influence the activity of Na⁺/K⁺ ATPase, inward-rectifier K⁺ channel and Na⁺ channel in rat ventricular myocytes. On the other hand, APD₅₀ and APD₉₀ in TSP-4-treated myocytes were prolonged during repolarization phase compared with Vehicle-treated myocytes (Fig. 1C). Repolarization phase of AP is mainly composed of inward $I_{Ca,L}$ and outward $I_{Kv}$. The increase of $I_{Ca,L}$ prolongs the AP duration
In the present study, contrary to expectation, TSP-4 decreased $I_{\text{CaL}}$ in rat ventricular myocytes (Fig. 2A and 2B). Thus, we next examined $I_{\text{Kv}}$, the decrease of which induces prolongation of APD. TSP-4 significantly inhibited $I_{\text{Kv}}$ in rat ventricular myocytes (Fig. 3B). TSP-4 did not affect mRNA expression of Kv channels responsible for $I_{\text{Kv}}$ (Fig. 4). From these results, it is suggested that TSP-4 inhibits the activation but not expression of Kv channels. Chae et al. reported that sevoflurane, which decreases both $I_{\text{Kv}}$ and $I_{\text{CaL}}$, prolonged APD without changing the amplitude and RMP in rat ventricular myocytes [6]. In addition, the prolongation of APD concomitant with the decrease of both $I_{\text{Kv}}$ and $I_{\text{CaL}}$ was observed in ventricular myocytes isolated from myocardial infarction model rats [1]. These observations support our results. The repolarization phase of AP in rat ventricular myocytes lacked a clear plateau phase, which is mainly composed of $I_{\text{CaL}}$, unlike in the case of large animals, such as guinea pig and rabbit [34]. Thus, the inhibition of outward $I_{\text{Kv}}$ might have a greater effect on the prolongation of APD than the decrease of inward $I_{\text{CaL}}$ in rat ventricular myocytes.

It was reported that TSP-4 inhibited LTCC activity via binding to the $\alpha_2\delta_1$ subunit in dorsal root ganglionic neurons [25]. Thus, it is proposed that TSP-4 might inhibit $I_{\text{CaL}}$ by the same mechanism. Integrins, a family of adhesion molecules, mediate signal transduction from ECM proteins [15]. Kv channels bind to certain integrins which regulate the gating property [4]. TSP-4 is known to interact with $\alpha_M\beta_2$, $\alpha_V\beta_3$ and $\alpha_2$ integrins [11, 22, 26]. Thus, it might be possible that TSP-4 inhibits the activity of Kv channels via changing the binding property to integrins. Interestingly, prolongation of APD with the decrease of outward $I_{\text{Kv}}$ was observed in left ventricular myocytes isolated from integrin-linked kinase (ILK) knock-out mice [27]. ECM components, such as collagen and fibronectin, activate ILK by binding to integrins [9]. Therefore, it is
proposed that TSP-4 might decrease $I_{Kv}$ by inhibiting ILK activity through the interaction with integrins. Further study is needed to clarify the detailed mechanisms for the inhibition of LTCC and Kv channel by TSP-4 treatment in rat ventricular myocytes.

The expression of TSP-4 in heart tissue was increased in various cardiac disease model animals [12, 22, 29, 30]. Several studies reported that TSP-4 exerted a cardioprotective effect in cardiac diseases [12, 30]. In the present study, TSP-4 prolonged APD with suppressing the activation of calcium and potassium channels. Both calcium and potassium channel blockers are well known as antiarrhythmic drugs, which are used for treatment of ventricular tachycardia and ventricular fibrillation. Thus, TSP-4 might be applicable as an antiarrhythmic agent. On the other hand, QT prolongation is a side effect of potassium channel inhibition, indicating that TSP-4 might be an onset factor for arrhythmia by prolonging APD and QT interval. Further in vivo study is needed to clarify the actual impact of TSP-4 on the electrophysiological properties of heart.
ACKNOWLEDGMENTS

This research was funded by Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant-in-Aid for JSPS Research Fellow), grant number 18J11540.
REFERENCES

1. Aimond, F., Alvarez, J. L., Rauzier, J.-M., Lorente, P. and Vassort, G. 1999. Ionic basis of ventricular arrhythmias in remodeled rat heart during long-term myocardial infarction. Cardiovasc. Res. 42: 402–415.

2. Al-Khatib, S. M., Stevenson, W. G., Ackerman, M. J., Bryant, W. J., Callans, D. J., Curtis, A. B., Deal, B. J., Dickfeld, T., Field, M. E., Fonarow, G. C., Gillis, A. M., Granger, C. B., Hammill, S. C., Hlatky, M. A., Joglar, J. A., Kay, G. N., Matlock, D. D., Myerburg, R. J. and Page, R. L. 2018. 2017 AHA/ACC/HRS Guideline for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: A report of the American college of cardiology/American heart association task force on clinical practice guidelines and the heart rhythm society. J. Am. Coll. Cardiol. 72: e91–e220.

3. Anumonwo, J. M. and Lopatin, A. N. 2009. Cardiac strong inward rectifier potassium channels. J. Mol. Cell. Cardiol. 48: 45–54.

4. Becchetti, A., Petroni, G. and Arcangeli, A. 2019. Ion channel conformations regulate integrin-dependent signaling. Trends Cell Biol. 29: 298–307.

5. Bornstein, P. and Sage, E. H. 2002. Matricellular proteins: Extracellular modulators of cell function. Curr. Opin. Cell Biol. 14: 608–616.
6. Chae, J. E., Ahn, D. S., Kim, M. H., Lynch, C. and Park, W. K. 2007. Electrophysiologic mechanism underlying action potential prolongation by sevoflurane in rat ventricular myocytes. *Anesthesiology*. **107**: 67–74.

7. Cingolani, O. H., Kirk, J. A., Seo, K., Koitabashi, N., Lee, D. I., Ramirez-Correa, G., Bedja, D., Barth, A. S., Moens, A. L. and Kass, D. A. 2011. Thrombospondin-4 is required for stretch-mediated contractility augmentation in cardiac muscle short communication. *Circ. Res*. **109**: 1410–1414.

8. Davey, P. P., Bateman, J., Mulligan, I. P., Forfar, C., Barlow, C. and Hart, G. 1994. QT interval dispersion in chronic heart failure and left ventricular hypertrophy: Relation to autonomic nervous system and Holter tape abnormalities. *Br. Heart J*. **71**: 268–273.

9. Dedhar, S. 2000. Cell-substrate interactions and signaling through ILK. *Curr. Opin. Cell Biol.* **12**: 250–256.

10. Dehghani-Samani, A., Madreseh-Ghahfarokhi, S. and Dehghani-Samani, A. 2019. Mutations of voltage-gated ionic channels and risk of severe cardiac arrhythmias. *Acta Cardiol. Sin.* **35**: 99–110.

11. Frolova, E. G., Pluskota, E., Krukovets, I., Burke, T., Drumm, C., Smith, J. D., Blech, L., Febbraio, M., Bornstein, P., Plow, E. F. and Stenina, O. I. 2010. Thrombospondin-4 regulates vascular inflammation and atherogenesis. *Circ. Res*. **107**: 1313–1325.
12. Frolova, E. G., Sopko, N., Blech, L., Popović, Z. B., Li, J., Vasanji, A., Drumm, C., Krukovets, I., Jain, M. K., Penn, M. S., Plow, E. F. and Stenina, O. I. 2012. Thrombospondin-4 regulates fibrosis and remodeling of the myocardium in response to pressure overload. *FASEB J.* 26: 2363–2373.

13. Gabrielsen, A., Lawler, P. R., Yongzhong, W., Steinbrüchel, D., Blagoja, D., Paulsson-Berne, G., Kastrup, J. and Hansson, G. K. 2007. Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium. *J. Mol. Cell. Cardiol.* 42: 870–883.

14. Halkin, A., Roth, A., Lurie, I., Fish, R., Belhassen, B. and Viskin, S. 2001. Pause-dependent torsade de pointes following acute myocardial infarction a variant of the acquired long QT syndrome. *J. Am. Coll. Cardiol.* 38: 1168–1174.

15. Harburger, D. S. and Calderwood, D. A. 2009. Integrin signalling at a glance. *J. Cell Sci.* 122: 1472.

16. Imoto, K., Kumatani, S., Okada, M. and Yamawaki, H. 2016. Endostatin is protective against monocrotaline-induced right heart disease through the inhibition of T-type Ca^{2+} channel. *Pflugers Arch.* 468: 1259–1270.

17. Jeschke, A., Bonitz, M., Simon, M., Peters, S., Baum, W., Schett, G., Ruether, W., Niemeier, A., Schinke, T. and Amling, M. 2015. Deficiency of Thrombospondin-4 in Mice Does Not Affect Skeletal Growth or Bone Mass Acquisition, but Causes a Transient Reduction of Articular Cartilage Thickness. *PLoS One.* 10: 1–18.
18. Kang, Y. Cardiac hypertrophy: A risk factor for QT-prolongation and cardiac sudden death. 2006. *Toxicol. Pathol.* **34**: 58–66.

19. Kojo, K., Yoshinaga, M., Kucho, Y., Hazeki, D., Tanaka, Y. and Mizota, M. 2012. A 6-year-old boy with secondary long QT syndrome. *J. Arrhythmia.* **28**: 61–64.

20. Lankipalli, R. S., Zhu, T., Guo, D. and Yan, G. X. 2005. Mechanisms underlying arrhythmogenesis in long QT syndrome. *in: J. Electrocardiol.* **38**: 69–73.

21. Lawler, J., Duquette, M., Whittaker, C. A., Adams, J. C., Mchenry, K. and Desimone, D. W. 1993. Identification and characterization of thrombospondin-4, a new member of the thrombospondin gene family. *J. Cell Biol.* **120**: 1059–1067.

22. Muppala, S., Frolova, E., Xiao, R., Krukovets, I., Yoon, S., Hoppe, G., Vasanji, A., Plow, E. and Stenina-Adognravi, O. 2015. Proangiogenic properties of thrombospondin-4. *Arterioscler. Thromb. Vasc. Biol.* **35**: 1975–1986.

23. Mustonen, E., Aro, J., Puhakka, J., Ilves, M., Soini, Y., Leskinen, H., Ruskoaho, H. and Rysä, J. 2008. Thrombospondin-4 expression is rapidly upregulated by cardiac overload. *Biochem. Biophys. Res. Commun.* **373**: 186–191.

24. Nerbonne, J. M. and Kass, R. S. 2005. Molecular physiology of cardiac repolarization. *Physiol. Rev.* **85**: 1205–1253.

25. Pan, B., Guo, Y., Wu, H.-E., Park, J., Trinh, V. N., Luo, Z. D. and Hogan, Q. H. 2016. Thrombospondin-4 divergently regulates voltage-gated Ca^{2+} channel subtypes in sensory neurons after nerve injury. *Pain.* **157**: 2068–2080.
26. Pluskota, E., Stenina, O. I., Krukovets, I., Szpak, D., Topol, E. J. and Plow, E. F. 2005. Mechanism and effect of thrombospondin-4 polymorphisms on neutrophil function. *Blood.*** **106**: 3970–3978.

27. Quang, K. Le, Maguy, A., Qi, X.-Y., Naud, P., Xiong, F., Tadevosyan, A., Shi, Y.-F., Chartier, D., Tardif, J.-C., Dobrev, D. and Nattel, S. 2015. Loss of cardiomyocyte integrin-linked kinase produces an arrhythmogenic cardiomyopathy in mice. *Circ. Arrhythm. Electrophysiol.* **8**: 921–932.

28. Roden, D. M. 2016. Predicting drug-induced QT prolongation and torsades de pointes. *J. Physiol.* **594**: 2459–2468.

29. Rysä, J., Leskinen, H., Ilves, M. and Ruskoaho, H. 2005. Distinct upregulation of extracellular matrix genes in transition from hypertrophy to hypertensive heart failure. *Hypertens.* **45**: 927–933.

30. Sawaki, D., Hou, L., Tomida, S., Sun, J., Zhan, H., Aizawa, K., Son, B.-K., Kariya, T., Takimoto, E., Otsu, K., Conway, S. J., Manabe, I., Komuro, I., Friedman, S. L., Nagai, R. and Suzuki, T. 2015. Modulation of cardiac fibrosis by Krüppel-like factor 6 through transcriptional control of thrombospondin 4 in cardiomyocytes. *Cardiovasc. Res.* **107**: 420–430.

31. Schwartz, P. J., Crotti, L. and Insolia, R. 2012. Long-QT syndrome: from genetics to management. *Circ. Arrhythm. Electrophysiol.* **5**: 868–877.

32. Shatock, M. J., Ottolia, M., Bers, D. M., Blaustein, M. P., Boguslavskyi, A., Bossuyt, J., Bridge, J. H. B., Chen-Izu, Y., Clancy, C. E., Edwards, A.,
Goldhaber, J., Kaplan, J., Lingrel, J. B., Pavlovic, D., Philipson, K., Sipido, K. R. and Xie, Z. J. 2015. Na⁺/Ca²⁺ exchange and Na⁺/K⁺-ATPase in the heart. J. Physiol. 593: 1361–1382.

33. Stenina-Adognravi, O. and Plow, E. F. 2019. Thrombospondin-4 in tissue remodeling. Matrix Biol. 75–76: 300–313.

34. Varró, A., Lathrop, D. A., Hester, S. B., Nánási, P. P. and Papp, J. G. 1993. Ionic currents and action potentials in rabbit, rat, and guinea pig ventricular myocytes. Basic Res. Cardiol. 88: 93–102.
FIGURE LEGENDS

Fig. 1. Thrombospondin-4 (TSP-4) prolongs action potential (AP) duration (APD) in rat ventricular myocytes

Rat ventricular myocytes were treated with TSP-4 (5 nM) or equal volume of phosphate buffered saline (Vehicle) for 4 hr. Patch clamp technique (current-clamp mode) was performed to measure AP, which was elicited by a 0.8–1 nA rectangular pulse for 5–8 msec. (A) Representative AP waveform in the rat ventricular myocytes treated with TSP-4 (red) or Vehicle (black) was shown. (B) The resting membrane potential (RMP) and peak amplitude of AP were measured and shown as mean ± standard error of the mean (S.E.M.) (Vehicle; n=11, TSP-4; n=9). (C) The APD at 50% and 90% repolarization (APD$_{50}$ and APD$_{90}$) were measured and shown as means ± S.E.M. (Vehicle; n=11, TSP-4; n=9). *p<0.05 vs. Vehicle.

Fig. 2. Thrombospondin-4 (TSP-4) inhibits L-type Ca$^{2+}$ channel current ($I_{CaL}$) in rat ventricular myocytes

Rat ventricular myocytes were treated with TSP-4 (5 nM) or Vehicle for 4 hr. Patch clamp technique (voltage-clamp mode) was performed to measure $I_{CaL}$. $I_{CaL}$ was elicited by 0.4 sec depolarization pulse to the test potentials ranging -50–40 mV in 5 mV increments from a holding potential of -40 mV (0.2 sec after elevation from -80 mV) every 5 sec. (A) Representative trance of $I_{CaL}$ in the rat ventricular myocytes treated with TSP-4 (lower) or Vehicle (upper) was shown. Inset: depolarization pulse protocol. (B) Current-voltage curve for the peak amplitude of $I_{CaL}$ was shown as means ± S.E.M. The peak current (pA) was normalized by cellular membrane capacitance (pF). (Vehicle:
n=16; TSP-4: n=11).

*p<0.05 vs. Vehicle.

Fig. 3. Thrombospondin-4 (TSP-4) inhibits voltage-dependent K⁺ (Kv) channel current ($I_{Kv}$) in rat ventricular myocytes

Rat ventricular myocytes were treated with TSP-4 (5 nM) or Vehicle for 4 hr. Patch clamp technique (voltage-clamp mode) was performed to measure $I_{Kv}$, which was elicited by 0.4 sec depolarization pulse to the test potentials ranging -60–60 mV in 5 mV increments from a holding potential of -70 mV every 10 sec. (A) Representative trace of total outward $I_{Kv}$ in the rat ventricular myocytes treated with TSP-4 (lower) or Vehicle (upper) was shown. Inset: depolarization pulse protocol. (B) Current-voltage curve for the peak amplitude of $I_{Kv}$ was shown as means ± S.E.M. The peak current (pA) was normalized by cellular membrane capacitance (pF). (Vehicle: n=10; TSP-4: n=9).

* *p<0.05 vs. Vehicle.

Fig. 4. Thrombospondin-4 (TSP-4) has no effect on mRNA expression of Kv channels in rat ventricular myocytes

Total RNA was isolated from the rat ventricular myocytes treated with TSP-4 (5 nM) or Vehicle for 4 hr, and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed. (A) Representative electrophoretic images of PCR products for *Kcna4*, *Kcna5*, *Kcnb1*, *Kcnd2*, *Kcnd3* and *Gapdh* (genes for Kv1.4, Kv1.5, Kv2.1, Kv4.2, Kv4.3, and glyceraldehyde 3-phosphate dehydrogenase, respectively) were shown. (B) The mRNA expression of Kv channels was corrected by *Gapdh*, and the normalized expression relative to Vehicle was shown as mean ± S.E.M. (Vehicle: n=4;
TSP-4: $n=3$).
Table 1. Primer sequences for reverse transcription-polymerase chain reaction analysis

| Gene   | Primer sequences              | Accession number |
|--------|------------------------------|------------------|
| Kcna4  | Forward 5’-GCCATTGCGGGGTGTCTTACC-3’ | NM_012971.2      |
|        | Reverse 5’-AGGTATGGGCAACTGCATGC-3’ |                 |
| Kcna5  | Forward 5’-AGCGTCTCTGGAGCACTTTTC-3’ | NM_012972.1      |
|        | Reverse 5’-CACACATGTGGTCTCCACGA-3’ |                 |
| Kcnb1  | Forward 5’-CTGGGAGAAGCCCAACTCGTC-3’ | NM_013186.1      |
|        | Reverse 5’-CGAAACTCGTCTAGGCTCTG-3’ |                 |
| Kcnd2  | Forward 5’-CGCTACGGTTATGTTCTACGCA-3’ | NM_031730.2      |
|        | Reverse 5’-GTCGCCATACCCCAGTGTTG-3’ |                 |
| Kcnd3  | Forward 5’-GGCAAGACCACGTCACTCAT-3’ | AB003587.1       |
|        | Reverse 5’-CGTGGTTCTTGATGGTGGG-3’ |                 |
| Gapdh  | Forward 5’-GAGAATGGGAAGCTGGTCAT-3’ | NM_017008.4      |
|        | Reverse 5’-GAAGACGCCAGTAGACTCCA-3’ |                 |

*Kcna4, Kcna5, Kcnb1, Kcnd2, Kcnd3 and Gapdh* are genes for Kv1.4, Kv1.5, Kv2.1, Kv4.2, Kv4.3 and glyceraldehyde 3-phosphate dehydrogenase, respectively.
Fig. 1.

A. Electrical activity recordings for vehicle (black) and TSP-4 (red).

B. Comparison of resting membrane potential (RMP) and peak amplitude.

C. Graph illustrating the effects on APD_{50} and APD_{90}.
Fig. 3.

A

Vehicle

TSP-4

B

-60  -40  -20  0  20  40  60

-10  -5  0  5  10  15  20  25

Vehicle

TSP-4

pA/pF vs mV
Fig. 4.

A

| Gene  | Band Size (bp) |
|-------|---------------|
| Kcna4 | 134           |
| Kcna5 | 96            |
| Kcnb1 | 127           |
| Kcnd2 | 112           |
| Kcnd3 | 130           |
| Gapdh | 120           |

B

![Graph showing mRNA expression](image)

- mRNA expression (Relative to Vehicle)
- Kcna4, Kcna5, Kcnb1, Kcnd2, Kcnd3
- Vehicle and TSP-4 conditions