様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　２ 年　 ５ 月 ２８ 日現在

機関番号：３７１１１
研究種目：若手研究(B)
研究期間：2017～2019
課題番号：１７Ｋ１５５６６

研究課題名（和文）Quantitative investigation of CaMKII-mediated TRPM4 regulation in atrial remodeling-associated arrhythmias

研究課題名（英文）Quantitative investigation of CaMKII-mediated TRPM4 regulation in atrial remodeling-associated arrhythmias

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交付決定額（研究期間全体）：（直接経費）　　2,100,000円

研究成果の概要（和文）：心筋細胞がCa負荷を受けるとCaMKIIの異常な活性化が生じる。これが嵩じると、EADやDADなどの異常興奮が起こりやすくなり、重篤な心不整脈が発生する可能性がある。CaMKIIを介したTRPM4チャネル過剰活性化の分子基盤を調べるため、免疫染色とDuolinkシグナル増幅技術によって、新規蛋白質間相互作用のスクリーニングを行った。その機能的な意義を明らかにするため、電気生理学実験と数値シミュレーションを行った。その結果、TRPM4チャンネルのゲーティングがCaMKIIの活性によって効果的に制御されていることを見出し、更にシミュレーションを行うことで、不整脈の基質となりうることを明らかにした。

研究成果の学術的意義や社会的意義
In this study, an important mechanism underlying CaMKII-mediated TRPM4 regulation and their kinetic relationships under altered Ca2+-handling was displayed. The simulation described here could facilitate our understanding about electrophysiological changes induced in remodelled cardiomyocytes.

研究成果の概要（英文）：Recent studies indicate that increased activity of TRPM4 contributes to acquired arrhythmic changes under stressed conditions. TRPM4 channel activity is strongly modified by Ca2+/calmodulin. We therefore investigated it by noting a potential significance of CaMKII-mediated TRPM4 channel overactivation under disrupted Ca2+ homeostasis.
The possible protein-protein interaction between TRPM4 and CaMKII was tested by the Duolink immunoassay. In order to quantitatively evaluate how CaMKII modifies TRPM4 activation, the gating kinetics of TRPM4 channel on both voltage-dependence and Ca2+ sensitivity were reconstructed. In HL-1 cells, the incidence of early afterdepolarizations (EADs) was increased after incubation with AngII which also activated the CaMKII signalling. Both TRPM4 channel blocker and CaMKII inhibitor could suppress this arrhythmic change. Mathematical simulation also indicated that inhibition of CaMKII may exert anti-arrhythmic effects via suppression of TRPM4 activities.

研究分野：Cardiac electrophysiology

キーワード：TRPM4　CaMKII　Arryhthmia

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Although considerable information has been accumulating for the pathogenic mechanisms of cardiac arrhythmia, it is still an intractable arrhythmia having a high morbidity with serious complications in Japan as well as in the world. Thus, more comprehensive understanding of its patho-mechanisms is a matter of high priority. It is well accepted that electrical and structural remodeling is a key process underlying the initiation and progression of cardiac arrhythmia, where abnormal calcium signaling and its downstream mechanisms play critical roles.

Recently, transient receptor potential (TRP) channels attract growing attention because of their involvement in many stress-induced dysfunctions including arrhythmias.(Simard C. et al. *J Mol Cell Cardiol*. 2013) A notable example is TRPM4 channel, a melastatin subfamily member of TRP that acts as a Ca^{2+}-activated non-selective cation channel. TRPM4 is expressed ubiquitously in the heart, but most abundantly in left and right aria and conduction system. It has been suggested that, in addition to some forms of hereditary arrhythmias (familial AV block, Brugada syndrome), TRPM4 contributes to acquired arrhythmic changes in remodeled or injured heart, e.g. action potential (AP) prolongation and early afterdepolarization (EAD).(Simard C.et al. *Br J Pharmacol*. 2012) These changes are thought to result from the predominance of inward vs. outward currents due to reduced K^+ currents (e.g. impaired KCNQ1 expression/activity), increased inward currents (e.g. sustained Na^+ currents, reactivation of Ca^{2+} currents) or both. TRPM4 may act as an additional Na^+-loading pathway into the cell during AP cycle.

Cardiac arrhythmia-related remodeling occurs concurrently with hypertension and heart failure which accompany over-activation of the renin-AngiotensinII (AngII) system.(Wakili R. et al. *J Clin Invest*. 2011) Sustained activation of CaMKII occurs in response to abnormal cytosolic Ca^{2+} rise, but also independently of Ca^{2+}/calmodulin through auto-phosphorylation, AngII-induced oxidation, glycosylation in diabetic conditions, and S-nitrosylation by NO during β-adrenergic stimulation. CaMKII phosphorylation has been shown to be central to delayed afterdepolarization (DAD)-related atrial fibrillation.(Sag CM. et al. *J Mol Cell Cardiol*. 2014) In our experiments with an atrial myocyte cell line HL-1, AngII treatment resulted in upregulation of TRPM4 activity, causing AP prolongation, diastolic depolarization and spontaneous AP activities. It is thus possible that under neurohormonal stresses, e.g. by AngII, increased TRPM4 activities may contribute to the pathogenesis of cardiac arrhythmia via enhanced CaMKII activity.

２．研究の目的

In the present study, based on the above considerations, we aim to seek the mechanisms underlying underlying CaMKII–mediated TRPM4 regulation and their kinetic relationships under altered Ca^{2+}-handling, and pursue its contribution to the pathogenesis of cardiac arrhythmia.

３．研究の方法

Immuno-fluoresence and Duolink proximity experiment

HL-1 cells were seeded on pre-coated cover-slips and cultured in Claycomb medium supplemented with 10% fetal bovine serum (FBS). Cells were incubated with AngII for 3 days. After washing once with phosphate buffered saline (PBS), cells were subsequently fixed with 4% para-formaldehyde. Cells were permeabilized and blocked with blocking solution, then immunolabelled. Confocal laser scanning microscopy was performed using a Zeiss LSM 710 Confocal Microscope.

The Duolink proximity ligation assay (PLA) was performed according to manufacturer's recommendations on both HL-1 cells and expressed TSA-201 cells. Briefly, cells were fixed by blocking solution then incubated with primary antibodies and washed with PBS. Duolink secondary antibodies conjugated to PLA probes were added on the cells. The PLA probes consist of oligonucleotides which would subsequently link together in a circle by ligase enzyme. After rolling circle amplification, Cell samples on cover-slips were then mounted with mounting medium with DAPI and viewed with a confocal microscope. Samples without primary antibodies were taken as negative controls. Images were analyzed with ImageJ software (National Institutes of Health, USA).

Electrophysiology

For patch clamp experiments, borosilicate glass electrodes (4-6MΩ) were used in conjunction with a high impedance, low noise patch clamp amplifier, which was manipulated by a

commercial software 'Patchmaster'. The data analysis and illustration were performed by using commercial data analysis softwares such as Origin 9 and Clampfit v.10.

Numerical model simulation

For numerical model simulation models, an open simulation platform Cor1.1 was used. The Luo-Rudy 2000 AP model was taken from the library of Cor (written in CellML), and modified by writing the parameters and codes for TRPM4 gating kinetics. During simulation ordinary differential equations of numerical models were solved by the 4th-order Runge-Kutta algorithm, and APs and currents were iterated every 0.005ms. Finally, simulated results were graphed with 1ms resolution.

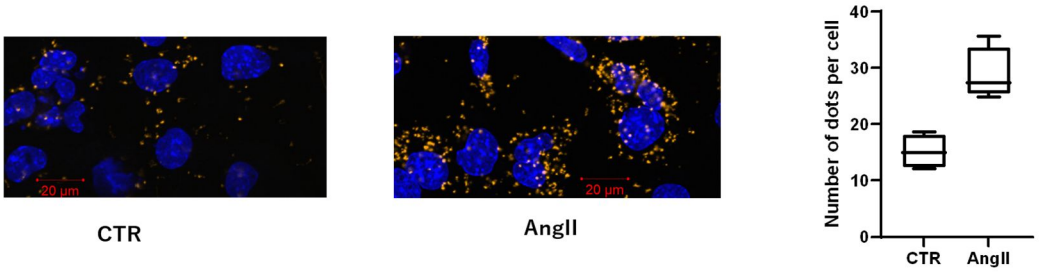
4．研究成果

Close localization of TRPM4 and CaMKIIδ proteins in cardiomyocytes

Co-localization of CaMKII and TRPM4 proteins was tested by the confocal immunofluorescence microscopy in HL-1 cardiomyocytes. The merged images indicated co-localization of the two fluorescent dye-labelled TRPM4 and CaMKII proteins. The area of co-localization sites in HL-1 cells is greatly increased after incubation with AngII.

To get more accurate information about the two-protein interaction, Duolink PLA assay was performed. Protein targets can be readily detected in location with single molecule resolution in cells and tissues. Duolink dot signals denote that TRPM4 and CaMKII δ protein are localized in close proximity of less than 40 nm. When TRPM4 and CaMKII δ were co-expressed in TSA201 dells, much more dot signals were generated than either CaMKII δ or TRPM4 alone. Fluorescent images from HL-1 cells demonstrate the close proximity of endogenous TRPM4 and CaMKII δ proteins.

Interestingly, incubation with AngII increased these orange dot signals in HL-1 cells displaying enhanced TRPM4-CaMKII δ interaction, the result being consistent with that of confocal immunofluorescence microscopy.

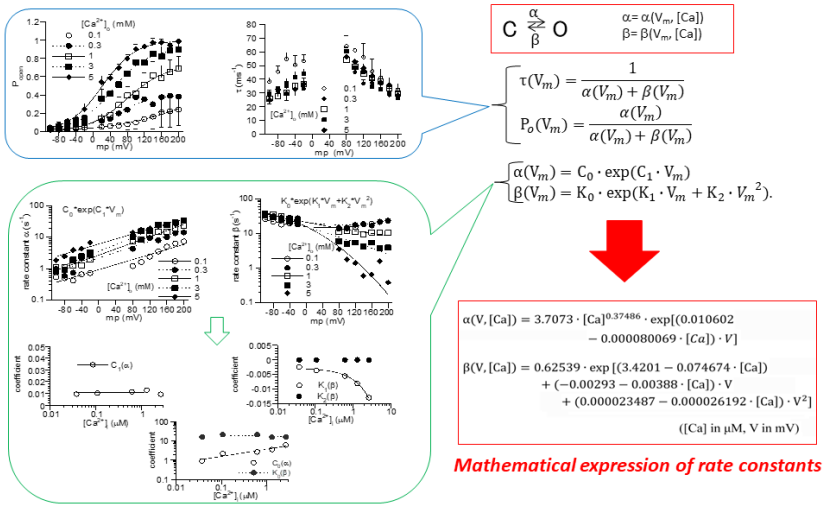


Quantitative description of TRPM4 gating kinetics

In order to explore the functional implications of the TRPM4-CaMKII δ interaction in physiological and pathophysiological contexts, we applied voltage jump experiments to evaluate TRPM4 channel gating kinetics under different Ca^{2+} concentration by ionomycin-based cell attached recording. Here, the desensitization and rundown of TRPM4 current was minimized. Each current trace is fitted to a mono-exponential time course, from which the rate constants for the open-closed state transitions α and β were calculated. And the mathematical formulae describing the voltage and Ca^{2+}-dependence of these rate constants were obtained to quantify the electrophysiological impact of TRPM4 upregulation on AP morphology of cardiomyocytes.

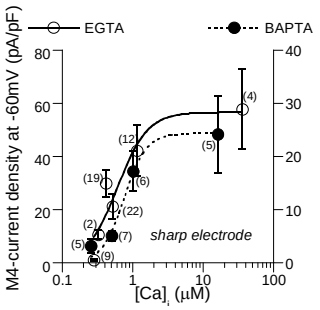
The figure below summarizes the results of experiments. Averaged steady state open probability (Po) and time constant (τ) are plotted against a wide range of membrane potential (Vm) at five different $[Ca^{2+}]o$ values, respectively. $[Ca^{2+}]i$ values corresponding to respective $[Ca^{2+}]o$ were separately assessed by Ca^{2+} imaging experiments. We adopted an empirical Hodgkin-Huxley-type formalism to describe TRPM4 channel gating as a simple two-state transition model consisting of the rate constants of opening and closing (α and β) that can be expressed as the complex functions of Vm and Ca^{2+}. Right bottom are the mathematical expressions finally obtained for α and β. The complex functions of membrane potential (Vm) and intracellular calcium concentration $[Ca^{2+}]_I$ are shown as follows, publicated in Hu, et al. *Cardiovascular research* 2017.

Quantitative assessment of Ca^{2+} - and voltage-dependent TRPM4 gating kinetics with Iono-C/A recording, Mathematical description of rate constants by HH-type formulations

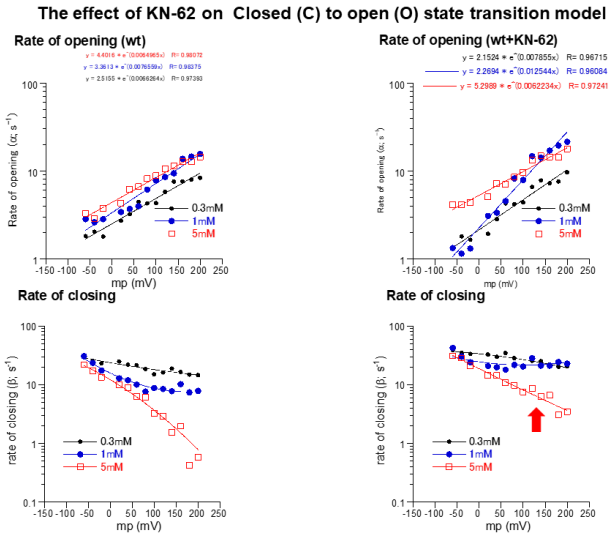


Suppression of TRPM4 activity by CaMKII inhibition probably through acceleration of its open-to-closed state transition

To confirm the existence of Ca^{2+} dependent facilitation (CDF) in TRPM4 channel, firstly we recorded macroscopic I_{TRPM4} by directly dialyzing various $[Ca^{2+}]_i$ into the cell with different Ca^{2+} chelators BAPTA and EGTA in the patch pipette solution. To minimize the severe run down of I_{TRPM4} due to washout, we adopted sharp patch electrodes. As shown in the right figure, the magnitudes were dependent on $[Ca^{2+}]_i$ in the patch pipette with apparent activation threshold lower than 0.3 μ M. Even though the threshold did not essentially change, the amplitudes of was greatly suppressed in the presence of fast Ca^{2+} chelator BAPTA, which may underline the contribution of CaMKII to the CDF.

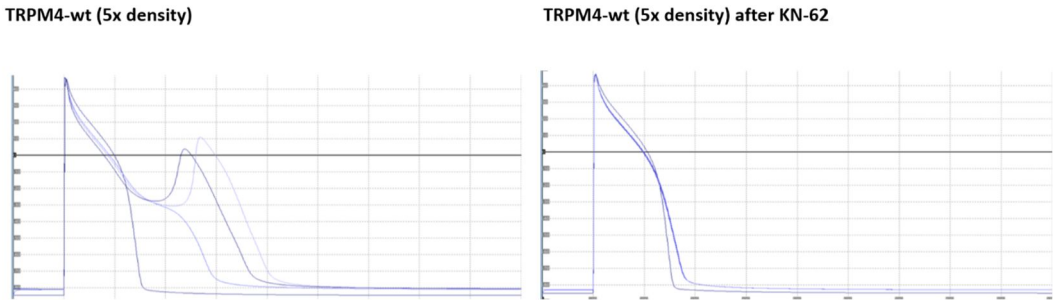


When TRPM4 was overexpressed in HEK293 cells, and under ionomycin-permeabilized cell-attached mode, introduction of Ca into the bath evoked cation currents via $[Ca^{2+}]_i$ elevation. To examine whether this $[Ca^{2+}]_i$ elevation is enough to activate CaMKII as well, a CaMKII-specific inhibitor KN-62 was pretreated and the fluorescence of a FRET-based CaMKII biosensor Camui was recorded. The inward cation currents induced by Ca^{2+} introduction was suppressed by the presence of KN-62. The currents immediately after the voltage step pulses suggested that the deactivation of tail currents representing TRPM4 activities was markedly accelerated by the CaMKII inhibition as displayed in the right upper panel. Changes in the rate constants α and β estimated from this altered kinetics indicate that the transition from the open to close state is markedly accelerated after the CaMKII inhibition. (show in the figure below)

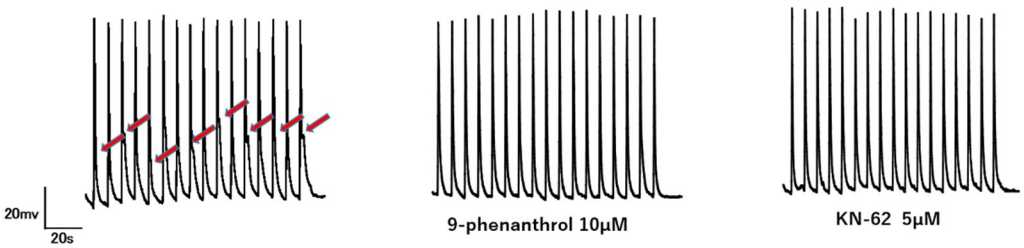


Inhibition of CaMKII activity produces an anti-arrhythmic effect via reduced TRPM4 activities, classified by numerical AP models and remodeled HL-1 cardiomyocytes

To decipher this altered gating after CaMKII inhibition in the physiological context, we incorporated α and β values into the LR2000 AP model. As displayed in the figure below, increased TRPM4 expression prolongs AP duration, and more than 5-fold increase triggers EADs. However, when CaMKII is inhibited, even 5-fold increase in TRPM4 density cannot induce EADs.



Experiments with HL-1 cells showed a similar tendency. In an AngII treated HL-1 cell which is expected to upregulate TRPM4 as well as CaMKII activities, EADs were frequently recorded. However, these arrhythmic activities could be abolished by either TRPM4 blocker 9-phenanthrol or a CaMKII inhibitor KN-62 alone (show in the figure below).



From these results, we could speculate that inhibition of CaMKII may exert anti-arrhythmic effects via suppression of TRPM4 activities.

５．主な発表論文等

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掲載論文のDOI（デジタルオブジェクト識別子） 10.14814/phy2.13796	査読の有無 有
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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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