様式 C-19

# 科学研究費補助金研究成果報告書

平成２３年　６月　１４日現在

機関番号：32696
研究種目：基盤研究（C）
研究期間：2008　～2010
課題番号：20590206
研究課題名（和文）幹細胞における陰イオンチャンネルの機能と分化に果たす役割の解明

研究課題名（英文）Investigation of the physiological functions of anion channels during the differentiation in stem cells.

研究代表者
川野 誠子　（KAWANO SEIKO）
駒沢女子大学・人間健康学部・教授
研究者番号：00177718

研究成果の概要（和文）：
幹細胞における陰イオンチャネルの役割と生理機能を検討した。マウス胚性幹細胞及びヒト間葉系幹細胞では容量依存性クロライドチャネル（ClC-3、ClC-4)及び ClCA $Ca^{2+}$ 活性化クロライドチャネル、Bestrophin（ベスト病原因遺伝子)の遺伝子の発現がＲＴ－ＰＣＲによって検出された。一方、ＣＦＴＲは未分化幹細胞では検出されなかった。ヒト間葉系幹細胞ではパッチクランプによる細胞膜電流記録で容量依存性クロライド電流を記録することができたが、Ca 活性化クロライド電流は非常に小さく記録が困難であった。細胞内 Ca 活性化Ｋ電流はクロライド電流は大きな外向き電流として機能していた。ヒト間葉系幹細胞では細胞内 Ca 濃度が経時的に変動しオシレーションすることより、Ca 動態が分化に影響を及ぼす可能性を脂肪細胞へ分化誘導して検討した。細胞内 Ca のハンドリングにより幹細胞から脂肪細胞への分化が調節されていることが示され、その詳細な調節機序の解明は更なる今後の研究が必要であると考えられる。

研究成果の概要（英文）：
We investigated the physiological functions of anion channels in mouse embryonic stem cells (mES) and human bone marrow-derived mesenchymal stem cells (hMSCs) during the differentiation to cardiac myocytes or adiposities. Using RT-PCR, the expression of mRNA for ClC-3, ClC-4 and Bestrophin could be detected in both undifferentiated mES cells and hMSCs. In the patch clamp experiments, $Ca^{2+}$ activated outward $K^{+}$ currents ($I_{KCa}$) could be recorded, however, $Ca^{2+}$ activated chloride currents were too small to analyze. Volume sensitive Cl currents were could be recorded in the hypotonic solutions. We concluded that anion channels exist in mES cells and hMSCs and Cl currents coded by ClC-3 have a function in undifferentiated hMSCs. We have demonstrated Cai oscillations in hMSCS previously (2003, 2004, 2005, in Cell Calcium) , therefore, we hypothesize that Cai might affect the differentiation processes. When Ca channel blockers were added in the culture medium, adiposeness were inhibited, indicating the contribution of Cai to the differentiating processes from mesenchymal stem cell to adiposities.

交付決定額

（金額単位：円）

| | 直接経費 | 間接経費 | 合　計 |
|---|---|---|---|
| 2008年度 | 1,300,000 | 390,000 | 1,690,000 |
| 2009 年度 | 1,400,000 | 420,000 | 1,820,000 |
| 2010 年度 | 800,000 | 240,000 | 1,040,000 |
| 年度 | | | |
| 年度 | | | |
| 総　計 | 3,500,000 | 1,050,000 | 4,550,000 |

研究分野：医学生理学、電気生理学、循環器病

科研費の分科・細目：基礎医学・生理学一般

キーワード：幹細胞、分化誘導、イオンチャネル、心筋細胞、脂肪細胞

１．研究開始当初の背景

近年、幹細胞を用いた再生医療の研究は盛んで、新しい治療法開発や臨床応用へと発展しつつある。しかし、幹細胞の生理機能や特定の細胞へ分化するメカニズムやそれらの調節機構に関しては未だ充分に解明されていない。特に、細胞の生理機能の主役であるイオンチャネルやトランスポーターの幹細胞における機能に関する研究は非常に少ない。未分化時の増殖機構における役割や特定の細胞へ分化する過程で細胞膜や内膜に発現し、細胞機能を獲得していくメカニズム及びその調節機構に関しては全く不明である。

２．研究の目的

本研究では、幹細胞におけるイオンチャネルやトランスポーター、特に陰イオンチャネルの生理機能、及び増殖・分化機構における役割を明らかにすることを目的とした。具体的には、幹細胞に発現しているクロライドチャネル遺伝子の同定、その生理機能の検討、そして分化過程での役割解明の研究を行う。また細胞内 Ca 動態に関係するイオンチャネル及びトランスポーターと分化の関係を明らかにすることを目的とした。

３．研究の方法

実験用の細胞としては市販されているマウス胚性幹細胞（mES）の cell line（D3, BL6)、ヒト間葉系幹細胞（hMSC)を培養して用いた。hMSC、mES 細胞のクロライドチャネル遺伝子発現の解析には RT-PCR で検討した。検討するクロライドチャネルは CFTR クロライドチャネル、ClC ファミリー電位依存性クロライドチャネル,容量依存性クロライドチャネル(ClC-3,), CLCA Ca2+活性化クロライドチャネル,Bestrophin (ベスト病原因遺伝子)に特定して検討する。生理機能検査としてのパッチクランプ法を用いた細胞膜電流解析、及び、細胞内 Ca の動態の測定にコンフォーカル蛍光顕微鏡等を用いて検討した。

４．研究成果

I、mES 細胞及び hMSC に発現しているクロライドチャネルを RT-PCR にて検討した結果、mES 細胞と hMSC では ClC-3 と ClC-4、及び Bestrophin の発現が認められたが、CFTR は検出されなかった（図１）。一方、細胞内 Ca により活性化される Ca 活性化クロライドチャネルと考えられている Bestrophin は未分化幹細胞で既に発現していた。そのサブタイプでは Bestrophin-4 の発現が確認された（図２）。

図 1

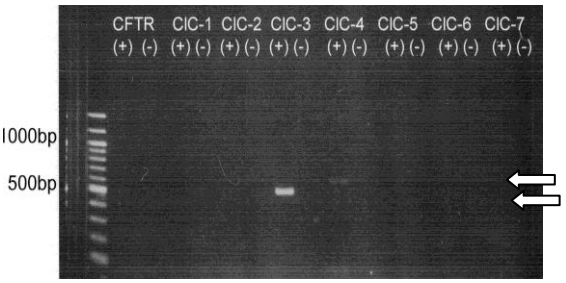



図１；552 bp と 660bp に認められるバンドは各々ClC3 と ClC4 の mRNA に相当する。

図 2

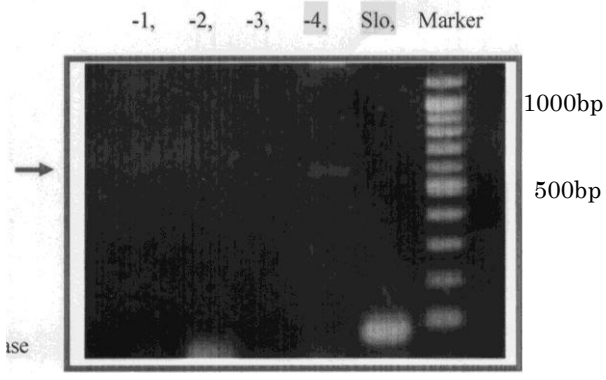



図２；Bestrophin 1,2,3,4, につき検討した。500bph 付近に Bestrophin-4 のバンドを認める。また、hSlo（ヒト BK チャネル、Ca 活性化 K チャンネル）の発現も確認された。

以上の結果より、ヒト間葉系幹細胞では未分化時に既に、ClC-3, 4,及び Ca 活性化クロライドチャネルの発現していることが明らかになった。

II、幹細胞の電気生理学的検討では全細胞膜電流の記録を行った。細胞内 Ca 活性化K電流はヒト間葉系幹細胞では大きな外向き電流として機能していたが、Ca 活性化クロライド電流は非常に小さく記録が困難であった。一方、容量感受性 Cl 電流は細胞外液の浸透圧を下げて活性化させると膜電流として記録でき、生理学的に機能していることが判明した(図３)。

図３

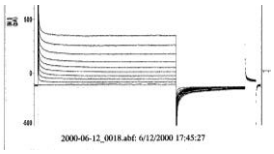
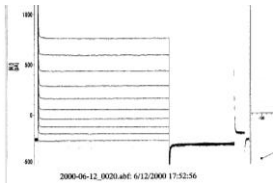
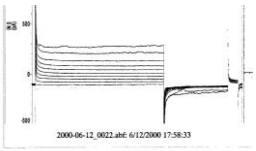
| | |
|---|---|
| Control<br>(300mOsmo) |  |
| Hypo<br>(240mOsmo) |  |
| W.O.<br>(300mOsmo) |  |

図３；細胞内外の K イオンは NMDG 置換し Cl 電流をパッチクランプ法で細胞膜電流として記録した。

III,
我々は今までに未分化間葉系幹細胞の細胞内 Ca はオシレーションする（図４）ことを既に報告しているが、本研究ではこの細胞内 Ca 動態が細胞の分化に及ぼす影響を検討した。

図４

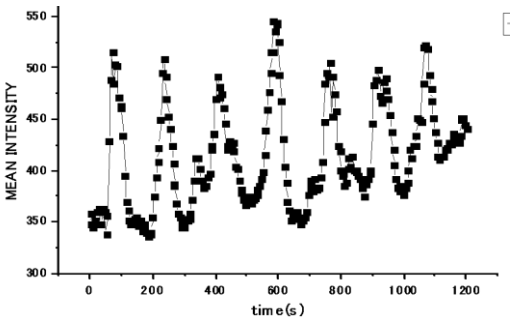
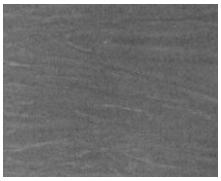



図４；細胞内 Ca を Fluo-3 で染色して共焦点顕微鏡で観察したものである。一個の細胞の細胞内 Ca 濃度の変動を経時的にプロットしている。細胞内 Ca 濃度がオシレーションすることが解る。

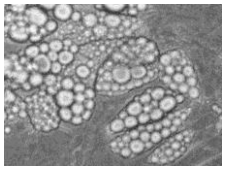
次に、細胞内 Ca のハンドリングが幹細胞の分化にどのような影響を及ぼすかを hMSC を用いて検討した。各種 Ca チャネルブロッカーを添加した培地で hMSC を脂肪細胞へ分化させた場合の影響を検討した。細胞外からの Ca 流入経路として電位依存性 Ca チャネルブロッカー、小胞体からの Ca 放出や、取り込みをブロックした場合においても、脂肪細胞への分化は抑制されなかった。一方、Ni を添加した培地で培養した hMSC は脂肪細胞への分化が完全に抑制された。Cl チャネルブロッカーも試したが、hMS から脂肪細胞への分化過程に明らかな影響は認められなかった(図５)。

図５;hMSCs を脂肪細胞へ分化誘導

（１） 間葉系幹細胞を confluent に培養。

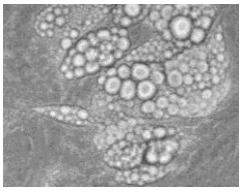


（２） 間葉系幹細胞を脂肪細胞へ分化誘導

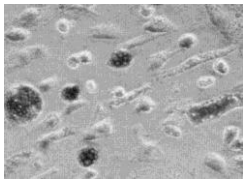


（３） Dehydropirydine Bolocker を培地に添加した条件でも脂肪細胞へ分化誘導されたことより、DHPR は幹細胞の分化には重要な役割を果たしていな

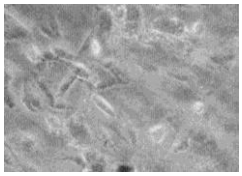
いことが推測された。



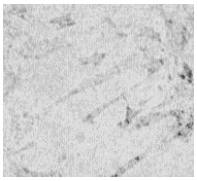
（４） Ryanodine
細胞内 Ca 遊離機構抑制により脂肪細胞への分化が抑制された。



（５） ２APB 投与により脂肪細胞への分化は抑制された



（６） Co 添加により脂肪細胞への分化は完全にブロックされた。



以上の結果より、細胞内 Ca のハンドリングにより幹細胞から脂肪細胞への分化が調節されていることが示された。
その詳細な調節機序の解明は更なる今後の研究が必要であると考えられる。

IV、その他の研究として

１、CFTR クロライドチャネルのブロッキングキネチィクスの詳細を検討した。
[Title]
“Blocking kinetics of cftr channel by aromatic carboxylate positional isomers characterised using a novel amplitude distribution analysis method.”
[Abstract]
To investigate the pore structure of the cystic fibrosis transmembrane conductance regulator (CFTR) channel, we performed a systematic pore probing on CFTR channel pore with a series of small aromatic carboxylic acids, including their positional isomers, e.g., 9-anthracene carboxylic acid (9-AC) and 1-anthracene carboxylic acid (1-AC).

Small compounds presumably interacting the channel protein with a few points are sensitive to structural changes of the binding site. However such low affinity blockers show fast – intermediate blocking kinetics which give us the overall affinity, but not on- and off- rates separately. To overcome this problem, we developed an iterative simulation method to estimate the on- and off- rate constants in the 9-AC or 1-AC block from the single channel amplitude distribution.

The newly developed Amplitude Distribution Analysis (ADA) program first generated a single-channel current according to the given kinetic scheme and added a Gaussian noise to the currents for mimicking the background noise. The simulated currents were low-pass filtered and digitized at the same frequencies as those in the experiments and binned into an amplitude histogram. Then the program repeats a direct likelihood comparison between the simulated and experimental current amplitude distributions to find the best fitted values for the blocking kinetic parameters.

The ADA program showed that the off-rate of 1-AC block is 3-fold slower than that of 9-AC and the on-rate of 1-AC is ~3-fold faster than that of 9-AC. The voltage-dependences of on- and off-rates of 1-AC are similar to those of 9-AC, respectively. These suggest that 1-AC and 9-AC block CFTR channel by binding to a common binding site which should be modeled by a combination of a positive charge tightly surrounded by hydrophobic residues.
in 54th Biophysical Society (USA), at San Francisco

２、心臓に発現している human *ether- a-go- go* -related gene (*hERG*)がコードする外向き K 電流が女性ホルモンと抗生物質の相乗効果で強く抑制され、これが女性に致死的不整脈を誘発する原因であることを初めて明らかにした。published in (2011) Journal of Membrane Biology May;241(1):31-8.

[Title]

Synergic effects of β-estradiol and erythromycin on hERG currents

Fumiaki Ando, Akinori Kuruma, Seiko Kawano

[Abstract]

The incidences of long QT syndrome (LQTS) and drug-induced Torsades de Pointes (TDPs) are higher in women than men. Although gonadal steroids are assumed to play an important role for gender-based differences in cardiac electrophysiological properties, the underlying mechanisms by gender-based differences are not fully understood. Especially, $I_{Kr}$, which composes the repolarization phase of action potential, has not been well understood in its modulation by sex hormones. To asses this, we examined the effects of female sex hormone, β-estradiol, on the human *ether-a-go-go*-related gene (*hERG*)-encoded potassium current stably expressed in human embryonic kidney-293 (HEK) cells. We demonstrated that hERG currents were inhibited by β-estradiol maximally to 62 % of control with an $IC_{50}$ of 1.3 μM and Hill coefficient of 0.87, which might account for sex-related differences in LQTS. We also examined whether estrogen modulated drug-induced blocking effects on hERG currents or not. With simultaneous application of 10 μM erythromycin, which is known to block hERG currents but not in low doses, the blocking effects of β-estradiol on hERG currents were enhanced. Namely, hERG currents were inhibited maximally to 45.8 % of control with an $IC_{50}$ of 59 nM ($P$<0.02) by β-estradiol with 10 μM erythromycin. We conclude here that a significant block of hERG currents by β-estradiol may account for sex-related differences in LQTS. And the synergic effects of β-estradiol and erythromycin imply the higher risk of drug-induced TDPs in women than men.

**Key words**:

hERG current, β-estradiol, sex hormone, $I_{kr}$, patch clamp, erythromycin,
QT prolongation,

Gender-based differences are familiar in cardiac electrophysiology, such as the rate-corrected QT (QTc) intervals in normal cardiac repolarization, the incidences of congenital long-QT syndrome and drug-induced Torsades de Pointes (TDPs) (Pham and Rosen 2002; Makkar et al, 1993; Sanguinetti and Tristani-Firouzi 2006; James et al, 2007). Earlier studies suggest that the estrogen receptor–mediated effects play a major role in the gender-based differences in the incidence of ventricular tachyarrhythmia after myocardial infarction in humans (Cupples et al, 1992). Female sex hormone, estrogen (β-estradiol), is supposed to play an important role for the expression and function of ion channels in cardiac myocytes (Du et al, 2006; Saba et al, 2002). The animal experiments prove that early afterdepolarizations (EADs) induced by the $I_{Kr}$ blocker, E4031, are more frequently induced in 17β-estradiol–treated rabbits than with 5α-dihydrotestosterone treatment rabbits (Hara et al, 1998). On the other hand, testosterone has been reported to diminish the pro-arrhythmic effects of dofetilide in female rabbits (Pham et al, 2002). Recent studies also indicate that testosterone shortens APD by modulating both $I_{KS}$ and $I_{Ca,L}$ in guinea pig hearts (Bai et al, 2005), indicating that sex hormones affect ion channels and modulate the repolarization phase of action potentials. However, the precise mechanisms by gender-based differences in cardiac electrophysiology have not been fully understood. Especially, $I_{Kr}$, which composes the repolarization phase of action potential, has not been well understood in its modulation by sex hormones (Trepanier-Boulay et al, 2001, Kurokawa, et al 2008).

To prove this, we investigated whether estrogen affected $I_{Kr}$ or modulated the drug-induced blocking effects using human embryonic kidney-293 (HEK) cells stably expressed hERG. We found that hERG currents were significantly inhibited by 17β-estradiol in a dose dependent manner and its blocking effects were increased while co-application of low dose of erhtyromycin.

## Materials and Methods

*Cell Preparation and Chemicals.*

HEK-293 cells stably expressing hERG potassium channels (gift from Dr. Craig T. January, (Zhou et al, 1998) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum and antibiotics at 37 °C in a humidified atmosphere of 95 % and 5 % $CO_2$. On the day of the experiment, cells were gently dissociated by a pipette and stored at room temperature.

*Electrophysiology*

Patch clamp experiments were performed as reported previously (Kawano et al, 2003). Briefly, using a patch clamp amplifier (Axopatch 2A and pCLAMP8, Axon Instruments, Foster City, CA, USA), whole cell membrane currents were recorded. Recording electrodes were made from

borosilicate glass, coated with Sylgard (Dow Corning Corp., Midland, MI) and fire polished to a resistance of 3–7 MΩ, when filled with internal pipette solutions. Data were stored on hard disk digitized at 10 KHz and low-pass filtered at 1 KHz by a filter with Bessel characteristics (octave attenuation, 48 dB) and analyzed off-line on a computer (Dell VZ-6000, Epson, Tokyo, Japan).
All experiments were performed at temperature of 35±5 ◦ C, which was maintained with a TC2 temperature controller (Cell Micro Controls, Virginia Beach, VA). The input resistance and membrane capacity were always checked at the beginning and end of experiments. We have omitted the data where the clamp was inadequate and membrane resistance or capacity changed during experiments.

hERG currents were recorded by applying step pulses or ramp pulses. We usually started to measure the currents at 5 minutes after achievement of whole cell clamp mode because of waiting the complete replacement of the internal solution. In the series of experiments, hERG currents decreased by about 22 % at 5 minutes after starting the experiments. In pooled data, the amplitudes of hERG currents became about 78 %±19 of control, n= 22, maen ± SE). We compensated the control values by the so called natural run down rate (78%), in each experiment. By using this value, we estimated the true effects of drugs on hERG currents. All experiments were analyzed by using this method in this study.

*Solution and Drugs*
For patch clamp experiments to record membrane currents, we used HEPES buffer bath solution containing (in mM) NaCl, 137; KCl, 4; $CaCl_2$, 1.8; $MgCl_2$, 1 and HEPES, 10. pH was adjusted to 7.4 with NaOH. Internal pipette solution contains (in mM) KCl, 130; $MgCl_2$, 1; EGTA, 5; MgATP, 5 and HEPES 10. pH was adjusted to 7.2 with KOH. β-estradiol-water soluble (E4389), (2-hydroxypropyl)- β-cyclodextrin solution (H5784) and erythromycin were purchased from Sigma-Aldrich (St. Louis, MO). Various concentrations of β-estradiol such as 300 nM, 3 μM, 30 μM, 300 μM and 3 mM were used for experiments. Erythromycin was dissolved to 10 μM. E-4031 was generously donated by Eisai, Japan.

***Statistics***
The data are expressed as mean ± S.D. or S.E. as indicated in the text. Student's paired *t*-test or unpaired *t*-test was used to assess the statistical significance. *P*-values of <0.05 was considered significant.

**Results**

**# 1, Effects of β-estradiol on hERG currents**
We investigated the effects of β-estradiol on hERG current, $I_{Kr}$, using the patch clamp methods. hERG currents were elicited by repolarizing ramp pulses (0.5 V/s) from -10 mV to -80 mV at 0.25 Hz, as reported previously (Wu et al 2003, Hiramatsu et al 2004, Sasano et al, 2004). We have confirmed hERG currents by the application of E-4031 (data not shown). We examined the Effects of β-estradiol on hERG currents. As shown in Fig.1, with 300 nM β-estradiol, hERG currents were slightly inhibited (Fig. 1a). When the higher concentration of β-estradiol (30 μM) was applied to the bath solution, the amplitudes of tail currents were clearly inhibited (Fig. 1b). Therefore, we examined the β-estradiol effects on hERG currents at various membrane potentials. The steady-state currents and tail currents were recorded. A series of 4-second depolarizing pulses were applied to voltages between -60 mV to +50 mV with 10 mV increments from -80 mV holding potentials and then repolarized to -50 mV at 0.1 Hz. By the application of 30 μM β-estradiol, hERG currents were blocked at almost all membrane potentials (Fig. 2a, b). Next, we studied the concentration-response relationships of β-estradiol among 300 M, 3 μM, 30 μM, 300 μM and 3 mM. The results showed the higher concentrations of β-estradiol, the stronger blocking of hERG currents (Fig. 2c and d). By analyzing the tail currents from the pooled data, we concluded that hERG currents were inhibited by β-estradiol maximally to 62 % of control in a dose dependent manner (Fig. 2c). The value of $IC_{50}$ was 1.3 μM and Hill coefficient was 0.87 (Fig.2d). Since it is reported that E2 is poorly soluble in aqueous buffers (Himmel, 2007), we tested whether cyclodextrin-encapsulated solution affected hERG currents or not by using (2-hydroxypropyl)- β-cyclodextrin solution. In our experiments hERG currents were not significantly affected by the application of 300 μM (2-hydroxypropyl)- β-cyclodextrin (data not shown).

**# 2, Blocking Properties of β-estradiol**
The blocking properties of β-estradiol were analyzed. As shown in Fig.3a, the amplitudes of normalized tail currents in the presence of various concentrations of β-estradiol (300 nM, 3 μM, 30 μM, 300 μM and 3 mM) were blocked in a dose dependent manner, but not significantly different depended on membrane potentials between -60 mV and +50 mV, indicating the voltage-independent block. The half maximally activations and slope factors were analyzed. The normalized tail currents were plotted as function of voltages (Fig.3b) and data were fitted with a Boltzmann function: $I/I$max - 1/{1 - exp[($V_{1/2}$ -

*V*m)/*S*]}, where *I* represents the tail current, *V*m is the test membrane potential, $V_{1/2}$ is the half-maximal activation voltage, and *S* is the slope factor, which reflects the steepness of the voltage dependence. The voltages of half maximally activation and slope factors were not significant different among these concentrations of β-estradiol.

In addition, we evaluated whether these blocking effects were use-dependent or not by applying the continuous stimulations at 0.25 Hz. In the presence of 30 μM β-estradiol, as shown in Fig.3C, hERG currents elicited by the ramp pluses (0.5 V/s) from -10 mV to -80 mV at 0.25 Hz were gradually decreased Thus, the amplitude of hERG current at 90th pulse was reduced to about 50 % of control by the application of 30 μM β-estradiol, (Fig. 3d). Without the continuous stimulations, the reductions of hERG currents were 50 ± 4 % (n=7 cells) at 6 minutes after application of β-estradiol, which is almost identical to those with stimulations (Fig. 3d). From these results, we concluded that blocking effects of β-estradiol were use-independent.

**# 3, Effects of β-estradiol and Erythromycin.**

It is well known that several macrolides cause QT prolongation and ventricular arrhythmias (Abriel et al, 2004). Previous reports have shown that hERG currents are inhibited by various macrolides in a concentration-dependent manner (Volberg et al, 2002). It is also suggested that antibiotics induce ventricular tachycardia more frequently in female than male (Coker 2008; PharmD et al, 2008 ). Therefore, we hypothesized that β-estradiol might modulate the blocking effects of macrolides on hERG currents. To test this, we tested the combination effects of erythromycin and β-estradiol together on hERG currents. Since the low dose of β-estradiol or 10 μM erythromycin itself do not affect hERG currents significantly (Fig.2) , we used 10 μM erythromycin and various concentrations of β-estradiol. We found that simultaneous application of 10 μM erythromycin and 300 nM β-estradiol markedly blocked hERG currents (Fig.4b). In Fig. 4c, the hERG currents traces were superimposed in the presence of 300 nM β-estradiol and 10μM erythromycin. When both drugs were applied simultaneously, hERG currents were markedly blocked. In the presence of 10 μM erythromycin, the amplitude of tail current became to 65.5 ± 9.4 % of control (Fig. 4a) and in the presence of 300 nM β-estradiol, it became to 69.27 ± 6.7 % of control, which was not much different from those in the absence of drugs (76.2 ± 16.7 %). When both 10 μM erythromycin and 300 nM β-estradiol were applied together, hERG current were markedly depressed to 45.8 ± 9.8 % (Fig. 4d, e) (n=4～7cells). These results clearly indicated that simultaneous application of both β-estradiol and erythromycin markedly enhanced the blocking effects of each drug (Fig. 4e). Furthermore, the examinations with various concentration of β-estradiol in the presence of 10 μM erythromycin showed a concentration-dependent block (between 30 nM and 3 μM β-estradiol, n=4～7cells) (Fig. 4d).

The dose response curves were compared between in the presence and absence of erythromycin. In the presence of erythromycin, the half blocking concentration was markedly shifted to left. $IC_{50}$ was 1.31 μM in the absence of erythromycin and 59 nM in the presence of 10 μM erythromycin (Fig.5). The maximal inhibition of β-estradiol was increased to 45.8 ± 9.8 % (n= 7 cells), in the presence of erythromycin. We summarized these results in Fig.5c. Low dose of erythromycin ( 10 μM) or low dose of β-estradiol (300 nM) does not affect hERG currents, however, both application these drugs simultaneously significantly blocked hERG currents, indicating the synergic effects.

**Discussion**

In the present study, we clearly demonstrated that β-estradiol blocked hERG currents and this blocking effect was enhanced with the simultaneous application of erythromycin.

#1; Blocking effects of β-estradiol on hERG currents

Sex steroid hormones are known to regulate signaling pathways in cardiovasucular system (Pham et al, 2002; Du et al, 2006). Gender differences in electrophysiological properties suggest that sex hormones may directly affect membrane currents in heart, however, the precise hormonal mechanisms is not fully understood (Makkar et al, 1993 ; James et al, 2007; Coker 2008).

In electrophysiological studies, it has been reported that estrogen prolongs the QT interval and testosterone plays an important role in a shortened QT interval (Pham et al, 2002; Saba et al, 2002). The cellular examinations also demonstrate that myocytes from females in mice show a prolonged action potential repolarization compared with myocytes from males (Trepanier-Boulay et al, 2001; Wu and Anderson 2002; Pham et al, 2002; Saba et al, 2002; Brouillette et al, 2005;). Previously, it is reported that testosterone regulate $I_{Ks}$ and $I_{CaL}$ to contribute to the QTCs interval (Bai et al, 2005).

Although hERG current is well known to involve in the repolarization of the cardiac action potential and contribute to QT intervals (Sanguinetti and Mitcheson 2005; Sanguinetti and

Tristani-Firouzi 2006), the modulations of hERG currents by sex hormones have not been fully evaluated. It is reported that estradiol may modulate E4031-induced prolongation of APD and magnitude of early afterdepolarizations (Hara et al, 1998). In isolated guinea pig ventricular myocytes, 17β-estradiol prolongs APD mainly by inhibiting the $I_K$ components $I_{Kr}$ and $I_{Ks}$, suggesting the blockage of hERG currents (Tanabe et al, 1999). Recently it is reported that physiological concentrations of E2 partially suppressed I(Kr) (Kurokawa et a, 2008). In this study, we prove the inhibition of hERG currents by β-estradiol using human embryonic kidney-293 (HEK) cells expressed with hERG, and also showed the synergic effects with erythromycin, for the first time.

#2; β-estradiol modulates drug-induced and erythromycin.

It is well recognized that many kinds of drugs, not only antiarrhythmic drugs such as class IA, IC and II, but also varieties of antibiotics, neurotropic, antifungal and antimalarial durgs block hERG channels and prolong the repolarizing phase of the cardiac action potential to lengthen the QT interval (Volberg et al, 2002; Abriel et al, 2004; Finlaysona et al, 2004; Thomas et al, 2004; Sanguinetti1 and Mitcheson 2005; PharmD et al, 2008; Hancox et al, 2008). A recent paper demonstrates that flavonoid compounds in grapefruit juice block cardiac hERG channels and may cause a prolongation of the QTc interval as a consequence (Zitron et al, 2005). These findings provide a rational basis for potential effects of flavonoids on cardiac electrophysiology (Scholz et al, 2005). Furthermore, the drug-induced LQTS and the risk of TdP are more frequent in females than males (Cupples et al, 1992; Lehmann et al, 1996; James et al, 2007; Coker 2008; PharmD et al, 2008). It is unclear whether sex-based differences in repolarization and responsiveness to $I_K$ blockers are due entirely to gonadal steroids or are associated with other sex-related factor. In the present study, we confirmed the synergic effects of β-estradiol and erythromycin on hERG currents with simultaneous application of both drugs (Fig.4,5). Erythromycin is a widely used antibiotic that infrequently causes QT-prolongation and torsades de pointes cardiac arrhythmias (Nattel et al, 1990). For antiarrhythmic drugs, it is reported that women are at a higher risk for these cardiac arrhythmias (Drici et al, 1998; PharmD et al, 2008). Our evidences in the present study clearly proved the underlying mechanisms in which erythromycin causes a higher risk for TDPs in women.

#3; Clinical implications

Since many of $I_{Kr}$ blocking drugs induce cardiac arrhythmias, it is very important to know the modulation of ion channel function and how this modulation influences the response to these drugs. Our evidences in this study clarify one of the mechanisms by gender-based differences in cardiac electrophysiology. Therefore, the medication of drugs which block $I_{Kr}$ should be paid greater attentions to women. It is reported that sex-specific changes in drug transport and metabolism will result in different plasma and intracellular levels acting along a dose-response effect on IKr block. Consequently, important hormone-dependent factors such as metabolic enzymes and membrane transporters need to be investigated in new basic research studies. (Hreiche et al,2008)

In this study we have not studied the effects of progesterone or other hormones on hERG currents. Furthermore, the underlying molecular mechanisms of β-estradiol effects on hERG currents have not clarified, yet. We need the further studies.

**References**


Abriel H, Schlapfera J, Keller DI et al (2004) Molecular and clinical determinants of drug-induced long QT syndrome: an iatrogenic channelopathy. SWISS MED WKLY 134: 685–694

Bai CX, Kurokawa J, Tamagawa M et al (2005) Nontranscriptional Regulation of Cardiac Repolarization Currents by Testosterone. Circulation 112: 1701- 1710.

Brouillette J, Rivard K, Lizotte E et al (2005) Sex and strain differences in adult mouse cardiac repolarization: importance of androgens. Cardiovasc Res 65: 148-57.

Coker S, (2008) Drugs for men and women-How important is gender as a risk factor for TdP? Pharmacology & Therapeutics 119:186–194

Cupples AL, Gagnon DR, Kannel WB (1992) Long- and short-term risk of sudden coronary death. Circulation 85:11–18.

Drici MD, Knollmann BC, Wang WX et al (1998) Cardiac actions of erythromycin: influence of female sex. JAMA 280(20):1774-6

Du XJ, Fang L, Kiriazis H (2006) Sex dimorphism in cardiac pathophysiology: Experimental findings, hormonal mechanisms, and molecular mechanisms. Pharmacology & Therapeutics 111: 434 – 475

Finlaysona K, Witchelb HJ, McCullocha J et al (2004) Acquired QT interval prolongation and hERG: implications for drug discovery and development. European Journal of Pharmacology (Review) 500: 29-142

Hancox JC, McPate MJ, Harchi AE et al (2008) The hERG potassium channel and hERG screening for drug-induced torsades de pointes. Pharmacology & Therapeutics 119: 118–132

Hara M, Danilo PJr, Rosen MR (1998) Effects of gonadal steroids on ventricular repolarization and on the response to E4031. J Pharmacol Exp Ther 285:1068 –1072.

Himmel HM (2007) Suitability of commonly used excipients for electrophysiological in-vitro safety pharmacology assessment of effects on hERG potassium current and on rabbit Purkinje fiber action potential. J Pharmacol Toxicol Methods. 56:145-58.

Hreiche R, Morissette P, Turgeon J (2008) Drug-induced long QT syndrome in women: review of current evidence and remaining gaps. Gend Med. 5:124-35.

Hiramatsu M, Wu LM, Hirano Y, Kawano S, Furukawa T, Hiraoka M (2004) Block of Herg current expressed in HEK293 cells by the Na-channel blocker cibenzoline. Heart Vessels 19:137-143

James AF, Choisy SCM, Hancox JC (2007) Recent advances in understanding sex differences in cardiac repolarization. Progress in Biophysics and Molecular Biology 94: 265–319

Kawano S, Otsu K, Shoji S et al (2003) $Ca^{2+}$ oscillations regulated by $Na^{+}$–$Ca^{2+}$ exchanger and plasma membrane $Ca^{2+}$ pump induce fluctuations of membrane currents and potentials in human mesenchymal stem cells. Cell Calcium 34 :145–156

Kurokawa J, Tamagawa M, Harada N, Honda S, Bai CX, Nakaya H, Furukawa T (2008) Acute effects of oestrogen on the guinea pig and human IKr channels and drug-induced prolongation of cardiac repolarization. J Physiol. 586:2961-73.

Lehmann MH, Hardy S, Archibald D (1996) Sex difference in risk of torsade de pointes with d,l-sotalol. Circulation 94:2535–2541

Makkar RR, Fromm BS, Steinman RT et al (1993) Female gender as a risk factor for torsades de pointes associated with ardiovascular drugs. JAMA 270:2590-:2597.

Milnes JT, Witchel HJ, Leaney JL (2006) hERG K+ channel blockade by the antipsychotic drug thioridazine: An obligatory role for the S6 helix residue F656. Biochemical and Biophysical Research Communications 351:273–280

Pham TV, Sosunov EA, Anyukhovsky EP et al (2002) Testosterone diminishes the proarrhythmic effects of dofetilide in normal female rabbits. Circulation 106:2132–2136

Pham TV, Rosen MR. Review (2002) Sex, hormones, and repolarization. Cardiovascular Research.; 53: 740–751

Pharm DHR, Morissette P, BPharm JT (2008) Drug-Induced Long QT Syndrome in Women: Review of Current Evidence and Remaining Gaps. GENDER MEDICINE 5: 124-135

Nattel S, Ranger S, Talajic MA (1990) Erythromycin-induced long QT syndrome: concordance with quinidine and underlying cellular electrophysiologic mechanism. J Med 89(2): 235-8.

Saba S, Zhu W, Aronovitz MJ, et al (2002) Effects of estrogen on cardiac electrophysiology in female mice. J Cardiovasc Electrophysiol 13:276 –280

Sanguinetti1 MC, Tristani-Firouzi M (2006) hERG potassium channels and cardiac arrhythmia. Nature 440: 463-469

Sanguinetti1 MC, Mitcheson JS (2005) Predicting drug–hERG channel interactions that cause acquired long QT syndrome. TRENDS in Pharmacological Sciences 26:119-124.

Sasano T, Ueda K, Orikabe M, Hirano Y, Kawano S, Yasunami M, Isobe M, Kimura A, Hiraoka M (2004) Novel C-terminus frameshift mutation, 1122fs/147, of HERG in LQT2: additional amino acids generated by frameshift cause accelerated inactivation. J.Mol.Cell.Cardiol. 37:1205-1211

Scholz EP, Zitron E, Kiesecker C (2005) Inhibition of cardiac hERG channels by grapefruit flavonoid naringenin: implications for the influence of dietary compounds on cardiac repolarisation.; Naunyn Schmiedebergs Arch Pharmacol 371(6): 516-25.

Thomas D, Hammerling BC, Wu K (2004) Inhibition of cardiac hERG currents by the DNA topoisomerase II inhibitor amsacrine: mode of action. Br J Pharmacol 142: 485-494

Tanabe S, Hata T, Hiraoka M (**1999**) Effects of estrogen on action potential and membrane currents in guinea pig ventricular myocytes. Am J Physiol Heart Circ Physiol **277**: **826** - 833.

Trepanier-Boulay V, St-Michel C, Tremblay A et al (2001) Gender-based differences in cardiac repolarization in mouse ventricle. Circ Res 89:437- 444.

Volberg WA, Koci B J., Su W et al (2002) Blockade of Human Cardiac Potassium Channel Human Ether-a-go-go-Related Gene (hERG) by Macrolide Antibiotics. JPET 302:320–327.

Wu LM, Orikabe M, Hirano Y, Kawano S, Hiraoka M (2003) Effects of Na+ channel blocker, pilsicainide, on HERG current expressed in HEK-293 cells. Journal of Cardiovasc Pharmacology. 42: 410-418.

Wu Y, Anderson ME (2002) Reduced repolarization reserve in ventricular myocytes from female mice. Cardiovasc Res 53:763-9.

Zitron E, Scholz E, Owen RWet al (2005) QTc prolongation by grapefruit juice and its potential pharmacological basis: hERG channel blockade by flavonoids. Circulation.; 111(7):835-8.

Zhou Z, Gong Q, Ye B et al (1998) Properties of hERG channels stably expressed in HEK293 cells studied at physiological temperature. Biophys J 74:230–241

Figure 1

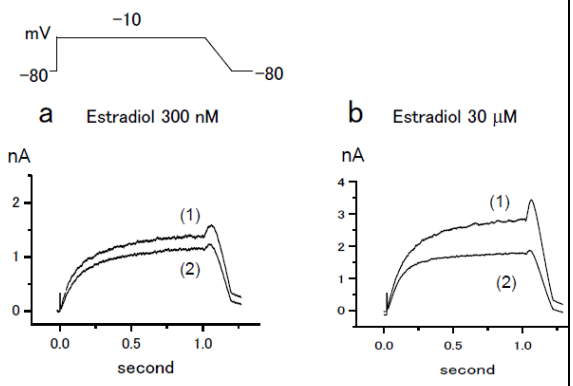



Fig.1
The effects of β-estradil on hERG currents.
Superimposed current traces in an experiment with 300 nM (a) and 30 μM (b) β-estradiol. The whole cell membrane currents were elicited by a 1 second depolarizing pulse to -10 mV from a holding potential of -80 mV, followed by a repolarizing ramp pulse (0/5V/s) to -80 mV. Stimulations were applied at 0.25 Hz before and after drug. Each current showed before (***1***) and 6 minutes after application of β-straddle (***2***).

Figure 2

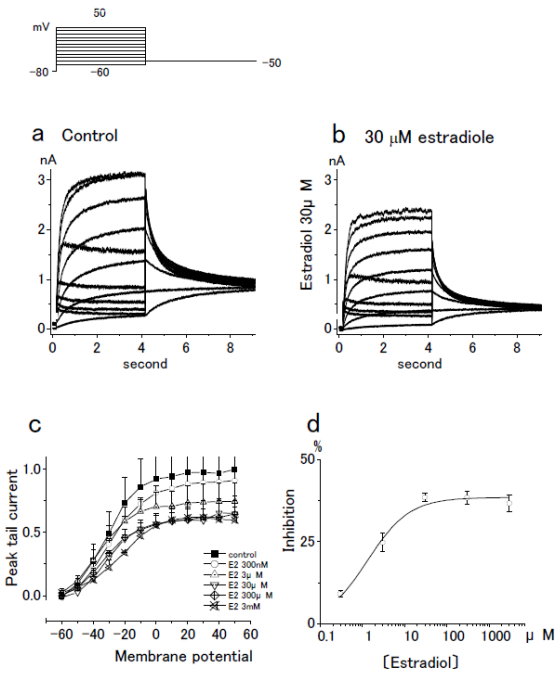



Figure 2
β- estradiol effects on the steady-state and tail hERG currents.
Currents were recorded by applying a series of 4-second depolarizing pulses to voltages between -60 mV to +50 mV with 10 mV increments from -80 mV holding potentials and then repolarized to -50 mV at 0.1 Hz. Currents in **a** and **b** show in control and 6 minutes after application of 30 μM β-estradiol, respectively. In **c**, the tail currents were analyzed in the presence of various concentrations of β-estradiol. Each symbol indicates control ■, 300 nM ○, 3 μM △, 30 μM ∇ , 300 μM ◊ and 3 mM, respectively. The data were normalized from 5-8 experiments in each condition. In **d**, the dose response curve showed that the value of $IC_{50}$ was 1.3 μM and Hill coefficient was 0.87.

Figure 3

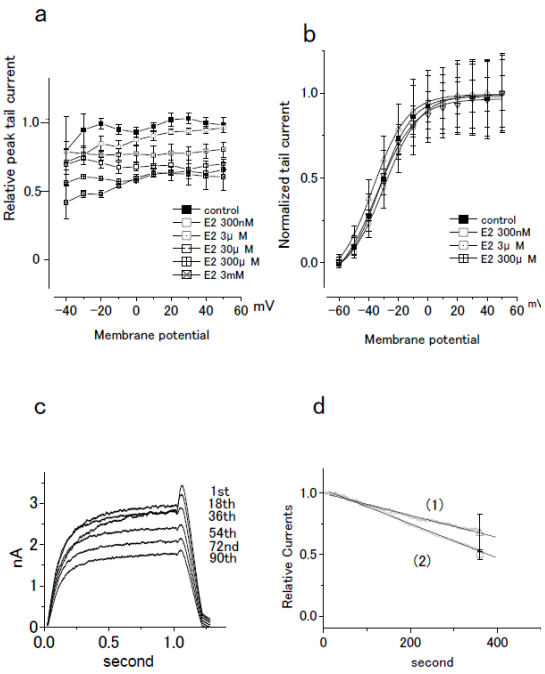



Figure 3

The blocking properties of β-estradiol.

*a*, The peak late currents at various concentrations of β-estradiol were plotted. The data were obtained from 5-8 experiments in each condition and normalized to the control currents at +20 mV test potential. At membrane potentials between -60 mV and +50 mV, the blocking by β-estradiol were not much different.

*b*, The voltage-dependent activations were analyzed in the presence of various concentrations of β-estradiol. Data were obtained by measuring normalized tail currents at the voltage of +20 mV and fitted with a Boltzmann function. The voltages of half maximally activations and slope factors were not significant different. Stimulations were applied at 0.25 Hz before and after drug. Each current showed before (1) and 6 minutes after application of β-estradiol (2).

*c*, The superimposed currents traces in the presence of 30 μM β-estradiol. The 1 second depolarizing pulse to -10 mV from a holding potential of -80 mV, followed by a depolarizing ramp pulse (0/5V/s) to -80 mV were applied at 0.25 hertz. The 1st trace indicates a control current and the 90th current indicates one 6 minutes after application of β-estradiol.

*d*, The time courses of hERG currents were plotted and compared with and without continuous stimulations. In the presence of 300 nM β-estradiol (***1***) and 30 μMβ-estradiol (2) amplitudes of tail currents were plotted, while applying ramp pulses at 0.25 Hz continuously. Without applying the continuous pulses, the mean amplitudes of tail currents were recorded at 6 minutes after the applications of 300 nM β-estradiol (△, triangle) and 30 μM (□, square) (mean ± S.E, n= 7 cells). Data in a, b, and d were normalized.

Figure 4

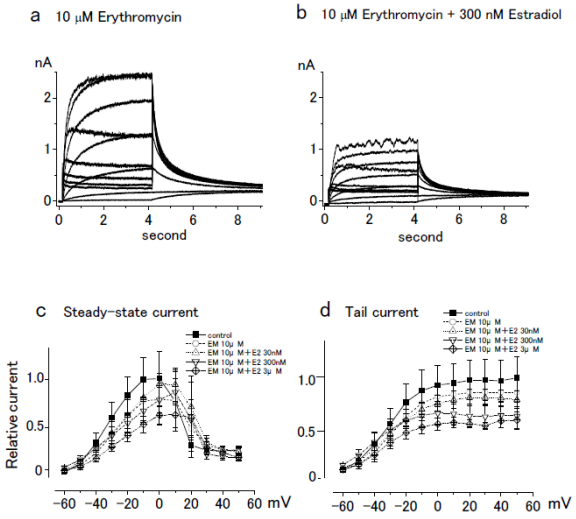



Figure 4.

*a*, *b*, The superimposed hERG currents when 10 μM erythromycin was applied (a) and both 10 μM erythromycin and 300 nM β-estradiol were applied (b).

*c*, *d*, The steady-state currents (*c*) and the tail currents (*d*) were plotted in the presence of 10 μM erythromycin, and various concentration of β-estradiol. (1) Control (■), (2) 10 μM erythromycin (○), (3) both 10 μM erythromycin and 30 nM (△), (4) both 10 μM erythromycin and 300 nM (▽), and both (5) 10 μM erythromycin and 3 μM β-estradiol (◊) (mean ±S.E, n= 7-8 cells).

Data in c and d were normalized.

Figure 5

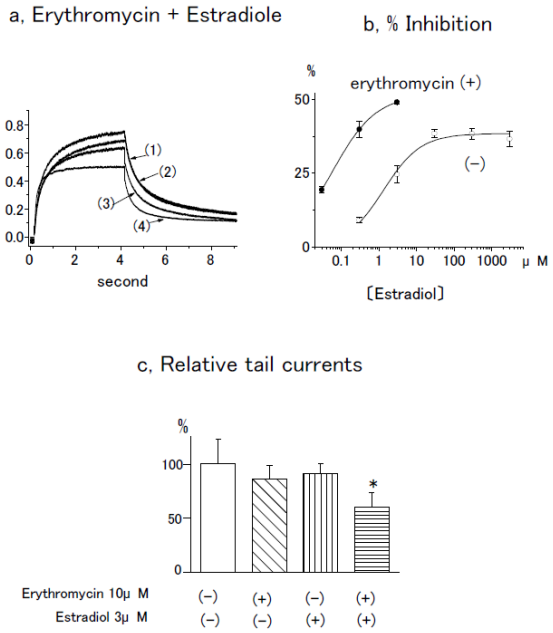



Figure 5,
*a*, Superimposed currents depolarized to +10 mV test potential form -80 mV holding potentials in control (1), at 6 minutes after application of 10 μM erythromycin (2), 3 μM β-estradiol (3), and both 3 μM β-estradiol and 10 μM erythromycin (4). The ordinate indicates relative currents normalized by control ones.
*b*, Dose-response curves of β-estradiol in the absence (-) and presence of 10 μM erythromycin (+). Data were obtained by measuring normalized tail currents at the voltage of +20 mV. The apparent $IC_{50}$ is 1.31 μM in the absence of erythromycin and 59 nM in the presence of 10 μM erythromycin, respectively. The maximal inhibition of estradiol is increased to 48 % from 38 % by the co-application of erythromycin.
*c*, Summary of blocking effects of 10 μM erythromycin or 3 μM β-estradiol on hERG currents measured by tail currents at +10 mV test potential. Data were normalized by control tail currents and indicated mea ± S.E. (n= 7 cells). Significant blocks were observed while simultaneous application of both drugs. * P value was < 0.02 (n= 7). All data were normalized.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　１件）
Fumiaki Ando, Akinori Kuruma Seiko Kawano Synergic effects of β-estradiol and erythromycin on hERG currents. (2011) Journal of Membrane Biology May;241(1):31-8.

〔学会発表〕（計　１件）
Ying-Chun Yu、Yoshiro Sohma and Seiko Kawano et al
“Blocking kinetics of cftr channel by aromatic carboxylate positional isomers characterised using a novel amplitude distribution analysis method.” in 54th Biophysical Society (USA), at San Francisco

〔図書〕（計　０件）

〔産業財産権〕

○出願状況（計０　件）

○取得状況（計０◇件）

６．研究組織

(1)研究代表者
川野誠子（Siko Kawano）
駒沢女子大学・人間健康学部・教授
研究者番号：00177718

(2)研究分担者
なし　　（　　）