Original Paper

Modulation of HERG K⁺ Channels by Chronic Exposure to Activators and Inhibitors of PKA and PKC: Actions Independent of PKA and PKC Phosphorylation

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Key Words

HERG • Protein kinase A • Protein kinase C • Reactive oxygen species (ROS) • Vitamin E • MnTBAP

Abstract

Background: Human ether-a-go-go-related gene (HERG) channel is the major molecular component of the native rapid delayed rectifier K⁺ current (I_{Kr}) that is a crucial determinant of cardiac repolarization. Impairment of I_{Kr}/HERG function is commonly believed to be a mechanism causing long QT syndromes (LQTS), a lethal ventricular tachyarrhythmia. The cAMP-dependent protein kinase A (PKA) and PKC activities are markedly increased in some pathological conditions of the heart such as heart failure. This study was designed to investigate the effects of acute and chronic exposure to PKA or PKC activators and inhibitors on HERG channel activities and to provide insight into the mechanisms for the modulations. **Methods:** Channel activity was measured in HEK293 cells stably expressing HERG using whole-cell patch-clamp techniques. Intracellular reactive oxygen species (ROS) were measured by CM-H2DFDA. Mitochondrial membrane potential (ΔΨ_m) was measured using JC-1 dye. HERG channel phosphorylation was assayed by [³²P]orthophosphate methods. **Results:** Acute exposure of cells to PKA or PKC activators by bath superfusion minimally affected I_{HERG} and so did the PKA or PKC inhibitor. By comparison, prolonged exposure (chronic incubation) of cells to PKA or PKC activators significantly impaired HERG K⁺ channel function as reflected by reduced I_{HERG} density and positive shift of the steady-state activation curve. Antioxidants vitamin E and MnTBAP both abolished the depressive effects of PKA or PKC activators on HERG function. Further, both PKA and PKC activators stimulated production of intracellular reactive oxygen species (ROS), an effect efficiently prevented by antioxidants or by PKA and PKC inhibitors.

Conclusions: HERG function is insensitive to PKA or PKC phosphorylation modulation per se, but can be impaired by the activators of PKA or PKC with long exposure likely via generation of ROS. In view of the critical role of HERG K⁺ channel in regulating cardiac repolarization and the sustained activation of both PKA and PKC in many pathological conditions of the heart such as heart failure, it is conceivable that HERG impairment by ROS accumulation induced by PKA and PKC contributes to the impaired cardiac repolarization.

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Introduction

The physiological function of the K⁺ currents carried by the channel proteins encoded by human *ether-a-go-go*-related gene (*HERG*) is versatile. It has been shown to be involved in regulating electrical activities of neurons [1] and cardiac myocytes [2], hormone secretion [3-5], tumor cell proliferation and apoptosis [6-8]. In the heart, HERG is the major molecular composition of the native current rapid delayed rectifier K⁺ current (I_{Kr}) that is a crucial determinant of cardiac repolarization, particularly the plateau phase of action potentials [1, 9, 10]. Impairment of I_{Kr} /HERG function can cause excessive prolongation of action potential duration (APD), which is commonly believed to be a mechanism for long QT syndromes (LQTS), a lethal ventricular tachyarrhythmia. Indeed, HERG is known as one of genetic sites for mutations causing familial LQTS and as a major molecular target for drug actions leading to drug-induced LQTS. One unique characteristic of HERG K⁺ channels is its high susceptibility to blockade by a vast variety of chemicals of different structures [11]. In addition, HERG is also known to be sensitive to modulation by a variety of protein kinases [12-20] and metabolites [21-26]. Modulation of HERG channels by these endogenous molecules may also cause QT prolongation, potentially leading to pathological LQTS, another form of acquired LQTS. Indeed, Tsuji et al. [27] showed I_{Kr} , measured as E-4031-sensitive tail current, to be ~36% smaller in rabbits with ventricular tachypacing-induced congestive heart failure than in healthy rabbits. Lodge and Normandin [28] demonstrated earlier that I_{Kr} , measured as dofetilide-sensitive tail current, reduced by ~45% in the BIO TO-2 strain of cardiomyopathic hamster of 10-month-old, derived from the BIO 53.58 animals and providing a model of dilated low output heart failure, compared with the 10-month-old control (BIO F1B) hamsters. Simulations of cellular electrophysiology predict I_{Kr} inhibition to cause EADs in failing, but not nonfailing, myocytes [29]. These studies indicate the potential impact of intracellular environmental changes on HERG function thereby heart function. More precise understanding of HERG modulation by cellular signaling molecules are therefore of paramount importance for our understanding of HERG function thereby arrhythmogenicity under various pathophysiological conditions.

It has been documented that the cAMP-dependent protein kinase A (PKA) signaling pathway produced decrease in HERG current amplitude (I_{HERG}) and the reduction of I_{HERG} was attributable to the positive shift of HERG activation caused by PKA phosphorylation [13-16]. The data from previous studies concerning protein kinase C (PKC) modulation of HERG K⁺ channel have been controversial. Some investigators demonstrated a role of PKC in regulating I_{HERG} [17-18], while some failed to observe any effects [20, 22, 25, 30]. It remains unresolved as to what cause the disparities. An important note to these previous studies is that the data were acquired with acute or short-term (<20 min) superfusion of activators or inhibitors of PKA or PKC. It is unclear how sustained exposure of cells to activators or inhibitors of PKA or PKC affect HERG function. This study was designed to shed light on this issue by comparing the effects of acute and chronic exposure to PKA or PKC activators and inhibitors on HERG channel activities and getting insight into the mechanisms for the modulations.

Materials and Methods

Cell Culture

HEK293 cells stably expressing HERG [31] were used in our study. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 200 μ M G418, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were subcultured to ~85% confluency and harvested by trypsinization and stored in the Tyrode solution containing 0.5% bovine serum albumin at 4°C. Electrophysiological recordings were made within 10 hours of harvest.

Whole-Cell Patch-Clamp Recording

We followed the methods and protocols for patch clamp recordings of HERG K⁺ currents (I_{HERG}) as described in detail elsewhere [12, 23]. I_{HERG} was recorded in the whole-cell voltage clamp mode with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes were prepared to have tip resistances of 1–3 megohms when filled with the internal solution containing (mM) 130 KCl, 1 MgCl₂, 5 Mg-ATP, 10 EGTA, and 10 HEPES (pH 7.3). The extracellular (Tyrode) solution contained (mM) 136 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). Experiments were performed at 36 ± 1 °C. Junction potentials, if any, were zeroed before formation of the membrane-pipette seal. Leak currents, if any, were subtracted and series resistance and capacitance were efficiently compensated.

Drugs and Treatment

PKA activator or cAMP elevating agent forskolin (FSK), PKA inhibitor H89, PKC-stimulating phorbol ester 12,13-didecanoate (PDD) and phorbol 12-myristate 13-acetate (PMA), PKC inhibitor bisindolylmaleimide (Bis), and antioxidants vitamin E (VitE) and manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP, a cell permeable superoxide dismutase mimetic & peroxynitrite scavenger) were all purchased from Sigma. FSK, H89, PDD, PMA, and Bis were prepared as a 1000 $\times$ final concentrations stock solutions in DMSO and diluted into patch-clamp recording Tyrode solution at the time of experiments. VitE was dissolved in ethanol and diluted by 1000 times to reach the final concentration. MnTBAP (100 mM) was dissolved in NaHCO₃ (1 M). All the solvents other than the Tyrode solution, which were used to dissolve the drugs, had an experimental concentration of 0.1%, and for the experiments involving these solvents the control recordings were made in the presence of 0.1% of such solvents.

For acute exposure, drugs were added to the patch-clamp bathing solution 10 min after baseline recording of I_{HERG} and the current amplitudes before and after drugs were compared. For prolonged exposure, HERG-expressing HEK293 cells were incubated in normal culture medium containing agents at the desired final concentrations for 10 hrs prior to patch-clamp recordings and group comparison between treated and untreated cells was made. Step I_{HERG} was defined as the current amplitude at the end of the 2.5-sec depolarizing voltage steps and tail I_{HERG} was measured as the peak value of the decaying tail currents upon repolarization to -50 mV. To have more rational comparisons between groups, I_{HERG} density was calculated by dividing the current amplitude by the capacitance of the same cell. For experiments involving protein kinase inhibitors, cells were pre-incubated with the drugs for 2 hrs before exposure to ceramide or other agents.

Intracellular Reactive Oxygen Species (ROS) Measurement

The procedures for measuring ROS were essentially the same as described in detail elsewhere [21, 24]. Briefly, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DFDA; Molecular Probes-Invitrogen, Eugene, USA) was used to detect oxidative status in living cells. In our pilot experiments, we measured the time-course of ROS production at 10 min, 60 min, 5 hrs, 10 hrs and 24 hrs in HEK293 cells treated with PKA or PKC activators and found that significant generation of ROS occurred only from 10 hrs after treatment. At this time point, no cell death was observed; however, 24 hrs after exposure, cell death began. We therefore choose to measure ROS production and to study the effects of the drugs at 10 hrs after exposure. CM-H2DCFDA was prepared in DMSO immediately prior to loading. Glass coverslips were coated with laminin and placed in the wells of a 12-well culture plate before the cells were seeded into the well in a density of 5×10^4 /well. For experiments involving VitE or MnTBAP, the cells were pretreated with VitE (100 μ M) or MnTBAP (5 μ M) for 2 hrs then incubated in the culture medium containing varying agents in the continual presence of VitE or MnTBAP. After wash with pre-warmed (37°C) PBS, cells were incubated

with the fluorescence dye CM-H2DCFDA (10 μ M) for 30 min. Next, the cells were examined under a laser scanning confocal microscope (Nikon A1R-A1), with an excitation wavelength of 480 nm and an emission wavelength at 505–530 nm. The percentage of stained cells and the fluorescence intensity of staining were determined by densitometric scanning with LSM software (Zeiss).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using JC-1 dye (Molecular Probes) to assess mitochondrial integrity, as described elsewhere [32]. JC-1 dye was added to cell culture to reach a final concentration of 10 μ g/ml and incubated for 20 min at 37°C. The cells were then washed three times with PBS and seeded to culture dishes containing cover slips. Next, the coverslips were mounted on the slides. The slides were immediately examined under a confocal microscope (Zeiss). JC-1 dye aggregates at high $\Delta\Psi_m$ and can be excited at 488 nm. The emission shifts from green (525 nm) to red (590 nm) when J aggregates form.

HERG Phosphorylation Assay

HERG channel phosphorylation was assayed according to the methods described by Sroubek & McDonald [33]. Briefly, HERG-expressing HEK293 cells were first phosphate-starved in FBS- and phosphate-free DMEM for 3 h at 37 °C. The cells were then loaded with [^{32} P]orthophosphate (50–80 mCi/well in 750 μ l of total medium) for 1 h and immediately treated with 20 μ M forskolin (or DMSO for control) or 0.1 μ M PDD for 10 min. Lysis with ice-cold NDET buffer supplemented with phosSTOP phosphatase inhibitors (Roche Applied Science). Post-nuclear supernatant was then incubated with 20 μ l of mouse anti-Myc 9e10 (Santa Cruz Biotechnology, Inc.) antibody for 2 h and incubated overnight with Dynabeads® Protein G (Invitrogen), shaking at 4 °C. Following three washes with NDET buffer, the protein sample was eluted, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Autoradiography was detected using UltraCruz™ Autoradiography Film (Santa Cruz Biotech). Duplicate measurements for each sample were averaged.

Data Analysis

Group data are expressed as mean $\pm$ S.E. Comparisons among groups were made by analysis of variance (*F*-test), and Bonferroni-adjusted *t*-tests were used for multiple group comparisons and paired or unpaired *t*-test was used, as appropriate, for two-group comparisons. A two-tailed *p* < 0.05 was taken to indicate a statistically significant difference. Nonlinear least square curve fitting was performed with CLAMPFIT in pCLAMP 9.0 or Excel 2001.

Results

Effects of Protein Kinase A Activator and Inhibitor on I_{HERG}

Depolarizing pulses from –60 mV to +40 mV elicited outward currents with characteristic inward rectification that manifested at stronger depolarization. Slowly decaying tail currents were recorded upon membrane repolarization back to –50 mV.

PKA phosphorylation of HERG channels has been shown to cause reduction of current amplitude due to the positive shift of HERG activation voltages [13–16]. However, with acute exposure (bath application for 20 min) of cells to the PKA activator FSK at 20 μ M did not produce any appreciable effects on I_{HERG} (Fig. 1A). The PKA inhibitor H89 (1 μ M) shifted the current-voltage (I-V) curve to negative potentials causing apparent increase in the current amplitude (Fig. 1B).

In contrast to acute exposure, incubation of cells with FSK (20 μ M) for 10 hrs considerably decreased both step and tail I_{HERG} (Fig. 2A & 2B) and the effects were only partially reversed by H89. FSK also caused a positive shift of the activation curve with the values of the voltage for half activation $V_{1/2}$ being changed from -28.0 ± 0.9 mV under control conditions to -23.7 ± 0.7 mV in the presence of FSK (*p* < 0.01, *n* = 20 and 15, respectively). The slope factor *k* was not significantly affected by FSK. Pretreatment of cells with H89 to inhibit PKA completely corrected the positive shifts of I-V relationship and activation curve ($V_{1/2}$ = -29.6 ± 1.0 mV

Fig. 1. Lack of effects of acute exposure (bath superfusion) of PKA activator (forskolin, FSK; 20 μ M) on HERG current (I_{HERG}) in HERG-expressing HEK293 cells. For acute exposure, FSK was applied to the Tyrode solution for patch-clamp recordings and currents recorded up to 20 min superfusion with FSK were taken to measure the drug effect as compared with the before-drug values. I_{HERG} was elicited by 2.5-s depolarizing pulses to voltages ranging from -60 to +40 mV to record the step current, followed by 2.5-s repolarization step to -50 mV to record the tail current, as shown with the voltage protocol shown in the inset. **A**, Current-voltage (I-V) relationship before and after FSK treatment; **B**, Current-voltage (I-V) relationship before and after treatment with PKA inhibitor H89 (1 μ M). Note the negative shift of the I-V curve.

for H89 + FSK, $p < 0.05$ vs. FSK alone), but only slightly relieved the I_{HERG} reduction induced by FSK (the peak I_{HERG} was 26.8 ± 3.0 pA/pF with FSK alone and 34.6 ± 3.9 pA/pF for H89 + FSK, $p > 0.05$). As illustrated in Fig. 2C, on the other hand, incubation of cells with H89 (1 μ M) alone for 10 hrs produced a remarkable shift of the HERG I-V relationship and activation curve towards more negative potentials. The maximum step I_{HERG} was unaltered despite that because of the positive shift, I_{HERG} was increased at potentials negative to -10 mV and decreased at potentials positive to -10 mV. The maximum tail I_{HERG} , however, was reduced by approximately 11%. The activation $V_{1/2}$ was changed from -24.5 ± 2.1 mV ($n = 13$) in control to -32.5 ± 1.1 mV in the presence of H89 ($p < 0.05$, $n = 12$), but the slope factor was not significantly altered ($k = 5.7 \pm 0.1$ mV for control and $k = 5.9 \pm 0.3$ mV for H89, $p > 0.05$).

Effects of Protein Kinase C Activators and Inhibitors on I_{HERG}

Similar to the PKA activator FSK, and also consistent with previous findings by Schledermann et al. [20], when acutely applied the PKC activators PMA (1 μ M; Fig. 3A) and PDD (0.1 μ M; Fig. 3B) both failed to affect I_{HERG} (Fig. 3A). By comparison, the PKC inhibitor Bis (0.1 μ M) diminished I_{HERG} amplitude by roughly 20~25% (Fig. 3C).

Also similar to the effects of the PKA activator with prolonged incubation, PDD treatment for 10 hrs considerably decreased I_{HERG} amplitude and positively shifted the activation curve (Fig. 4A). Co-application of Bis only partially recovered the depressed current density and failed to alter the positive shift of activation curve induced by PDD. Intriguingly, cells treated with Bis (0.1 μ M) alone for 10 hrs showed moderate but statistically significant ($p < 0.05$, $n = 22$) suppression of I_{HERG} density at all test potentials studied compared with the value from control untreated cells (Fig. 4B). The I_{HERG} activation property was not different between Bis and control. Also notably, unlike PDD, another PKC activator PMA did not affect HERG channel and its steady-state activation properties (Fig. 4C).

Fig. 2. Effects of prolonged exposure to PKA activator FSK (20 μ M) and inhibitor H89 (1 μ M) on I_{HERG} in HERG-expressing HEK293 cells. For prolonged exposure, HEK293 cells were preincubated with FSK in the normal culture medium for 10 hrs before patch-clamp studies and the current amplitude was normalized to cell capacitance to minimize the inter-cell variations of cell size for group comparison with untreated cells. **A**, Typical examples of I_{HERG} traces recorded before drug, in the presence of FSK, and in the presence of FSK+H89; **B**, left: I-V relationships and right: steady-state activation curves, showing the effects of FSK or FSK+H89 on I_{HERG} . The activation curves were constructed by plotting the conductance G as a function of depolarizing potentials. G was calculated by normalizing the tail currents at -50 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at +40 mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{\text{max}} = 1/\{1 + \exp[(V_{1/2} - V)/k]\}$, where G_{max} represents the maximal conductance at +40 mV, $V_{1/2}$ is a half-maximal activation voltage, and k is a slope factor. $P < 0.05$ vs control for test potentials from -40 mV to +30 mV. **C**, Effects of H89 alone on I_{HERG} . H89 apparently increased I_{HERG} because of the negative shift of I-V curve and activation curve. $P < 0.05$ vs Ctl (control) for test potentials from -50 mV to -20 mV.

Role of Reactive Oxygen Species (ROS) in I_{HERG} Modulation by PKA or PKC Activators and Inhibitors

Our observations that PKA and PKC inhibitors were not able to completely reverse the suppressive effects of PKA and PKC activators on I_{HERG} suggest that the kinases activators likely act via mechanisms in addition to on PKA and PKC activities. Indeed, HERG K^+ channel function has been shown to be prone to oxidative damage [12, 21, 24]. Thus, one possible mechanism is related to increased generation of intracellular reactive oxygen species (ROS). We tested this notion by preincubating the cells with vitamin E (VitE, 100 μ M) for 2 hrs and then incubating the cells with FSK in the continual presence of VitE for 10 hrs. As shown in Fig. 5A, VitE substantially weakened the depressing effects of FSK on I_{HERG} and prevented the positive shift of the activation curve. For example, the I_{HERG} density at 0 mV was 38.2 ± 2.8 pA/pF ($n=20$) under control conditions, and the value was reduced to 22.2 ± 2.1 pA/pF ($n=17$, $p < 0.05$) in the presence of FSK alone. In the presence of VitE, the I_{HERG} density was nearly the same as the control value: 38.0 ± 3.0 pA/pF ($n=16$). Another antioxidant MnTBAP produced qualitatively the same effects as VitE (Fig. 5B): complete reversal of FSK-induced depression of I_{HERG} density and the positive shift of HERG activation.

Similarly, the depressing effect of PDD was considerably prevented by pretreatment with VitE. For example, the I_{HERG} density at 0 mV was 60.4 ± 7.3 pA/pF ($n=20$) for control, 34.1 ± 2.7 pA/pF for PDD alone ($n=18$, $p < 0.05$ vs. control) and 51.8 ± 4.9 pA/pF for VitE+PDD ($n=19$, $p > 0.05$ vs. control; $p < 0.05$ vs. PDD alone). Moreover, VitE partially corrected the

Fig. 3. Lack of effects of acute exposure of PKC activators and inhibitor on I_{HERG} in HERG-expressing HEK293 cells. *A*, I-V relationship before and after PKC activator PMA (1 μM) treatment; *B*, I-V relationship before and after PKC activator PDD (0.1 μM) treatment; *C*, I-V relationship before and after treatment with PKC inhibitor Bis (1 μM). $P < 0.05$ vs before Bis for test potentials from -20 mV to +10 mV.

Fig. 4. Effects of prolonged exposure to PKC activator PDD (1 μM) and inhibitor Bis (1 μM) on I_{HERG} in HERG-expressing HEK293 cells. *A*, left: I-V relationships and right: steady-state activation curves, showing the effects of PDD or PDD+Bis on I_{HERG} . $P < 0.05$ vs control for test potentials from -50 mV to +10 mV. *B*, Effects of H89 alone on I_{HERG} . H89 apparently increased I_{HERG} because of the negative shift of I-V curve and activation curve. $P < 0.05$ vs control for test potentials from -50 mV to -20 mV.

positive shift of I_{HERG} activation caused by PDD (Fig. 5C). Preincubation with MnTBAP nearly abolished the depressing effects of PDD on I_{HERG} but failed to correct PDD-induced positive shift of HERG activation (Fig. 5D).

To confirm that ROS production was indeed increased by PKA or PKC activators after chronic incubation, we proceeded to measure the intracellular ROS levels using CM-H2DCFDA fluorescence dye. The cells with staining and with fluorescence intensity ≥ 5 times the background were defined as positive staining, and the number of cells with positive staining was pooled from 5 fields for each batch of cells (a total of 4 independent batches of cells for each group were studied). The intensity of staining by the fluorescent probe for ROS was analyzed by densitometric scanning using the LSM program, and cells with positive staining were taken for analysis, and the data were normalized to the control values the PKA activator. Under control conditions, cells with positive staining were sparse (Fig. 6A). Yet in cells treated with FSK, the number of cells with positive staining (Fig. 6B), as well as the intensity of staining (Fig. 6C), was consistently higher. The ROS level was significantly lower, as indicated by the smaller number of cells with positive staining and the weaker intensity of staining in individual cells, in the cells pretreated with VitE prior to FSK treatment. Notably, the PKA inhibitor H89 was able to eliminate the ROS production by FSK, just as VitE did (Fig. 6A). Consistently, MnTBAP also abolished the FSK-induced ROS generation; the number of stained cells (Fig. 6D) and the intensity of staining (Fig. 6E) in the presence of MnTBAP were nearly the same as those under control conditions.

Fig. 5. Role of reactive oxygen species (ROS) on I_{HERG} modulation by PKA activator forskolin (FSK) or PKC activator PDD. **A**, Effects of vitamin E (VitE) on FSK-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves (same below). The cells were preincubated with VitE (100 μ M) for 2 hrs and then incubated with FSK (20 μ M) in the presence of VitE for 10 hrs before patch-clamp recordings. $P < 0.05$ FSK vs Ctl for test potentials from -40 mV to +40 mV and $P < 0.05$ FSK+VitE vs FSK alone for test potentials from -40 mV to +40 mV; $n = 18$ cells; **B**, Effects of MnTBAP on FSK-induced I_{HERG} depression. The cells were preincubated with MnTBAP (5 μ M) for 2 hrs and then incubated with FSK (20 μ M) in the presence of MnTBAP for 10 hrs before patch-clamp recordings. $P < 0.05$ FSK vs Ctl for test potentials from -30 mV to +30 mV and $P < 0.05$ FSK+MnTBAP vs FSK alone for test potentials from -30 mV to +40 mV; $n = 18$ cells; **C**, Effects of VitE on PDD-induced I_{HERG} depression. The cells were preincubated with VitE (100 μ M) for 2 hrs and then incubated with PDD (0.1 μ M) in the presence of VitE for 10 hrs before patch-clamp recordings. $P < 0.05$ PDD vs Ctl for test potentials from -40 mV to +40 mV and $P < 0.05$ PDD+VitE vs PDD alone for test potentials from -20 mV to +30 mV; $n = 16$ cells; **D**, Effects of MnTBAP on PDD-induced I_{HERG} depression. The cells were preincubated with MnTBAP (5 μ M) for 2 hrs and then incubated with PDD (0.1 μ M) in the presence of MnTBAP for 10 hrs before patch-clamp recordings. $P < 0.05$ PDD vs Ctl for test potentials from -40 mV to +40 mV and $P < 0.05$ PDD+MnTBAP vs PDD alone for test potentials from -30 mV to +40 mV; $n = 16$ cells.

Similarly, the PKC activator PDD also boosted up the generation of ROS in the cells and either VitE (Fig. 7A, B & C) or MnTBAP (Fig. 7A, D & E) abolished this effect. Moreover, the PKC inhibitor Bis prevented the ROS-production induced by PDD.

Comparison of ROS Production and HERG Phosphorylation

Next, we compared the time course of ROS production and HERG phosphorylation at 10 min, 60 min, 5 hrs, 10 hrs and 24 hrs in the presence of PKA or PKC activator. As shown in Fig. 8A, cellular production of ROS in the presence of FSK (20 μ M) or PDD (0.1 μ M) developed with time. Specifically, no significant cellular production of ROS was detected until 10 hrs after FSK (20 μ M) or PDD (0.1 μ M); thereafter, ROS production continued to increase.

Fig. 6. Increased intracellular level of reactive oxygen species (ROS) by PKA activator forskolin (FSK; 20 μ M), measured by CM-H2DCFDA fluorescence dye. **A**, Laser scanning confocal microscopic images of CM-H2DCFDA staining reflecting the intracellular ROS levels in the presence of FSK, VitE, or H89. **B & D**, Percentage of positively stained cells, obtained from at least 5 fields of 4 experiments by counting the cells with staining intensity ≥ 5 times the background; **C & E**, Averaged intensity of CMH-2DCFDA fluorescence measured from the positively stained cells. * $P < 0.05$ FSK vs Ctl; + $P < 0.05$ FSK+VitE/MnTBAP or FSK+H89 vs FSK alone.

On the other hand, phosphorylation of both mature and immature forms of HERG channel proteins occurred right after FSK or PDD treatment at 10 min, and dissipated rapidly with time (Fig. 8B). By 60 min, HERG phosphorylation was virtually abolished.

To see if ROS production caused cellular damage, we monitored the alterations of mitochondrial integrity by detecting mitochondrial transmembrane potential $\Delta\Psi_m$. $\Delta\Psi_m$ is known to be a critical factor determining the integrity of mitochondria, and loss of $\Delta\Psi_m$ can lead to apoptosis. $\Delta\Psi_m$ was detected using JC-1 dye. Untreated control cells that were stained with the fluorescent dye JC-1 exhibited numerous, brightly stained mitochondria that emitted red orange fluorescence (Fig. 8C), representing J aggregates that accumulate at normally hyperpolarized membrane potential. The earliest dissipation of $\Delta\Psi_m$ representing depolarization of mitochondrial membrane potential, as indicated by green fluorescence of monomers, was detected 24 hrs after FSK or PDD treatment. Only minor changes of $\Delta\Psi_m$ were seen at 10 hrs after drug treatment, indicating minimal cellular damage at this time point.

Discussion

In the present study, we have investigated the effects of PKA and PKC activators on HERG current generated in HERG-expressing HEK293 cells and the cellular mechanisms underlying the effects. The main findings of the study include: (1) acute exposure of cells to PKA or PKC activators by bath superfusion minimally affected I_{HERG} , and so did the PKA or PKC inhibitor; (2) prolonged exposure (chronic incubation) of cells to PKA or PKC activators significantly impaired HERG K^+ channel function as reflected by reduced I_{HERG} density and positive shift of the steady-state activation curve; (3) antioxidants vitamin E and MnTBAP both were able to cancel the depressive effects of PKA or PKC activators on HERG function; and (4) both PKA and

Fig. 7. Increased intracellular level of reactive oxygen species (ROS) by PKC activator PDD (0.1 μ M), measured by CM-H2DCFDA fluorescence dye. **A**, Laser scanning confocal microscopic images of CM-H2DCFDA staining reflecting the intracellular ROS levels in the presence of PDD, VitE, or Bis. **B & D**, Percentage of positively stained cells, obtained from at least 5 fields of 4 experiments by counting the cells with staining intensity ≥ 5 times the background; **C & E**, Averaged intensity of CMH-2DCFDA fluorescence measured from the positively stained cells. * $P < 0.05$ PDD vs Ctl; * $P < 0.05$ PDD+VitE/MnTBAP or PDD+Bis vs FSK alone.

PKC activators stimulated production of intracellular reactive oxygen species (ROS), which was efficiently prevented by antioxidants or by PKA and PKC inhibitors. These data suggest that HERG function is insensitive to PKA or PKC phosphorylation modulation per se, but can be impaired by the activators of PKA or PKC with prolonged exposure likely via generation of ROS. In view of the critical role of HERG K^+ channel in regulating cardiac repolarization and the fact that both PKA and PKC are sustainably activated in many pathological conditions of the heart such as heart failure, it is conceivable that HERG impairment by ROS accumulation induced by PKA and PKC contributes to the electrical disturbance in failing heart.

Our observation that acute application of PKA or PKC activator failed to produce any appreciable effects on I_{HERG} properties suggests that PKA or PKC phosphorylation does not play any significant role in modulating HERG function. This is in contrast to the early report by Kiehn et al. [13], the authors suggested that PKA phosphorylation rendered a remarkable positive shift of HERG activation and I_{HERG} reduction. While the interpretation of this study is confounded by the use of PMA as a PKA activator (which is commonly used as a PKC activator), their subsequent study using mutagenesis seemed to confirm the role of PKA phosphorylation in HERG modulation [14]. Similar results were later reported by other groups [15, 16]. It is noted that the reduction of I_{HERG} in these studies was solely due to the positive shift of HERG activation caused by PKA phosphorylation, but not due to actual depression of the current density. Consistent with this view, FSK did not affect I_{HERG} density upon superfusion for acute action. Moreover, our data from the PKA inhibitor H89 experiments suggests that basal PKA activities produce tonic regulation of HERG activation, maintaining the I_{HERG} activation ($V_{1/2}$) at relatively positive potentials, but have no effects on I_{HERG} amplitude because H89 alone only shifts I_{HERG} activation to hyperpolarizing voltages (or

Fig. 8. Time courses of changes of ROS production (A), HERG channel phosphorylation (B), and mitochondrial membrane potential ($\Delta\Psi_m$) (C) in the presence of FSK (20 μ M) or PDD (0.1 μ M). HEK293 cells expressing HERG were loaded with ^{32} P radioisotope and then treated with DMSO or 20 μ M FSK or 0.1 μ M PDD for 10 min. Immunoprecipitated HERG was then analyzed using autoradiography (upper panels). The data obtained at varying time points were normalized to 0 min (prior to FSK treatment) and were plotted as a function of time (lower panels). Note that phosphorylation of HERG channel of both 150 kDa and 135 kDa occurred at 10 min following FSK or PDD treatment and rapidly declined thereafter. $\Delta\Psi_m$ was determined by JC-1 staining. Similar data were obtained from another 3 experiments. * P <0.05 10 min vs 0 min; * P <0.05 1 hr or longer vs 10 min.

corrects the positive shift caused by basal PKA) but does not alter the peak current amplitude despite that due to the activation shift H89 increases I_{HERG} at negative potentials (Fig. 1). On the other hand, the chronic forskolin (FSK) data would suggest that elevated cAMP level or/and enhanced PKA activity above basal levels further set I_{HERG} activation to even more positive potentials and meanwhile decreased I_{HERG} amplitude/density, as indicated by the fact that FSK caused a rightward shift of I_{HERG} activation and a marked decrease in I_{HERG} amplitude as well (Fig. 2A). The degree of I_{HERG} reduction cannot be explained by the amount of rightward shift of HERG activation; FSK-induced I_{HERG} reduction took place at all voltages tested ranging from -60 to +40 mV. This notion was supported by the partial recovery, produced by H89,

of FSK-induced depression of I_{HERG} . Effects of H89 on FSK-induced depression of I_{HERG} also indicate that FSK acts on HERG channels at least partly via PKA activation. However, our data could not explain why H89 failed to reverse FSK-induced I_{HERG} depression despite that it did prevent the positive shift of I_{HERG} activation. Furthermore, VitE or MnTBAP entirely restored FSK-induced I_{HERG} reduction. Collectively, it appears that PKA phosphorylation only regulates the voltage-dependence of I_{HERG} activation but not the I_{HERG} amplitude, in line with previous findings [13-16], and the FSK-induced I_{HERG} depression is likely due to ROS formation and accumulation directly stimulated by FSK via activation of PKA.

The data from previous studies concerning PKC modulation of HERG K^+ channel have been controversial. Some investigators demonstrated a role of PKC in regulating I_{HERG} [17, 18], while some failed to observe any effects [20, 22, 30]. The results from our present study are not straightforward either in terms of the role of PKC in HERG modulation. First, our results that PKC activator PDD decreased I_{HERG} density and shifted HERG activation to more positive potentials, effects largely reversed by PKC inhibitor bisindolylmaleimide (Bis) would favor that PKC phosphorylation is involved in I_{HERG} modulation. However, data arguing against the role of PKC phosphorylation were also obtained. First, the effects of PDD or PMA could only been seen with prolonged exposure (10 hrs) and acute exposure by superfusion for 20 min failed to cause any alterations of I_{HERG} . In addition, Bis markedly inhibited I_{HERG} in the absence of PKC activator. Were the inhibitory effect of PKC inhibitors on I_{HERG} interpreted as an inhibition of basal PKC activity, then it would imply that PKC phosphorylation enhances HERG function; this is obviously in variance with the PDD data. One plausible explanation for this apparent contradiction is that the PKC inhibitors can directly block HERG channels independently of PKC activity on one hand and indirectly enhance HERG function via inhibiting PKC activation on the other hand. The question yet to be answered is whether PKC phosphorylation of HERG channel proteins is required for I_{HERG} depression by PKC activators. A recent study on HERG modulation by PKC with multiple mutations of PKC phosphorylation sites in HERG sequence elegantly ruled out the requirement of PKC phosphorylation of HERG channel proteins for modulation by PKC activators [18]. They observed that PKC activation caused a remarkable reduction of I_{HERG} amplitude and a positive shift of HERG activation in *Xenopus* oocytes, and these effects were attenuated by PKC inhibitors and reproduced in mutated HERG with the PKC phosphorylation sites omitted. Based on these observations, the authors proposed that additional intermediate signal transduction factors/proteins mediated the effects. However, it was not discussed by the authors what could be the intermediate factor(s). Here we demonstrate that ROS is likely the intermediate factor or at least is one of the intermediate factors, which will be discussed in more detail below.

Here we demonstrated that PKA and PKC activators suppressed HERG K^+ channel function in two ways: markedly diminishing I_{HERG} density and slightly shifting the voltage-dependent HERG activation towards more positive potentials. The inhibitory effect of PKA or PKC is prevented by antioxidants vitamin E (VitE) and MnTBAP, but the positive shift of HERG activation caused by PKA or PKC is unaffected by the antioxidants. The effects of these agents closely resemble those described for a sphingolipid metabolite ceramide by Bai et al. [34]: remarkable depression of I_{HERG} density along with moderate positive shift of HERG activation. An intriguing finding of this study is that both FSK, a cAMP elevating agent/PKA activator, and PDD, a PKC activator, pronouncedly enhanced the intracellular ROS level, and the increases were effectively prevented by VitE and abolished by MnTBAP. In line with these results, VitE or MnTBAP also considerably attenuated the I_{HERG} depression caused by FSK or PDD. More intriguingly, Bai et al. [34] demonstrated that ceramide stimulates intracellular production of ROS and the inhibitory effects of ceramide on I_{HERG} are abolished by antioxidants VitE and MnTBAP. It appears that PKA/PKC activators and ceramide produce the same pattern of HERG channel impairment, likely by the same subcellular and biochemical mechanisms: production and accumulation of ROS. Furthermore, our data also suggest that the generation of ROS induced by PKA or PKC activators was mainly of $O_2^{\cdot -}$ because MnTBAP, nearly abolished the ROS increases. This is again in line with the action of ceramide [34].

PKA stimulation of ROS has been documented previously. For instance, Yamagishi et al. [35] found that PKA activation is a key step for the leptin-induced ROS generation in bovine aortic endothelial cells, which was competently prevented by H89 and by Rp-cAMPS an antagonist of cAMP. However, evidence for PKA inhibition of ROS, in particular, superoxide anion ($O_2^{\cdot-}$) also exists; Raha et al. [36] demonstrated that PKA and cAMP derivatives caused a decrease in NADH-driven $O_2^{\cdot-}$ formation in rat heart sub-mitochondrial particles and mitochondria incubated with ATP. It is possible that whether PKA stimulates or inhibits ROS generation is cell type-dependent. It is evidently from our experiments that in HEK293 cells PKA activation stimulates ROS generation and the latter mediates the deleterious effects of PKA on HERG function. The ability of PKC to participate in the generation of ROS has been better established. Inoguchi et al. [37] showed that PMA, a PKC activator, stimulated ROS production through NAD(P)H activation in both cultured aortic smooth muscle cells and endothelial cells. Furthermore, the increased ROS production induced by high glucose level in cultured vascular cells was significantly inhibited by PKC-specific inhibitors, suggesting the role of PKC. Similarly, in rat preovulatory follicles PKC mediates ROS production through NADPH/NADH oxidase and superoxide anion ($O_2^{\cdot-}$) is the major species because incubation of follicular cells with SOD blocked the PKC activator TPA (12-O-tetradecanoylphorbol-13-acetate)-stimulated ROS production, whereas catalase was without effect [38]. Wang et al. [39] have also found that PKC activation by PMA stimulated $O_2^{\cdot-}$ generation in rat neutrophils. Datta et al. [40] showed that PKC- β II is required for TPA-induced ROS production in myeloid leukemia cells. Shizukuda et al. [41] demonstrated that in adult ventricular myocytes, the membrane translocation of PKC- δ mediated the increased ROS production induced by hyperglycemia. Our present study verifies the ability of PKC activity to enhance intracellular ROS production and suggests it is this ROS generation that mediates I_{HERG} depression by PDD because VitE or MnTBAP rescues the impaired HERG function.

Collectively, PKA or PKC activation decreases I_{HERG} density and shifts HERB activation to positive potentials by directly stimulating ROS production but not by phosphorylation. In other words, ROS or likely $O_2^{\cdot-}$ is a mediator for the inhibitory effects of PKA and PKC activators, because the effects were corrected by antioxidants VitE or MnTBAP. One important limitation of the present study is the lack of mechanistic link between PKA or PKC activators and ROS production in our cells. We do not know what is the source of ROS, mitochondrial or cytosolic, neither do we answer how PKA or PKC increases intracellular ROS. Obviously, to delineate the mechanisms, rigorous studies are required. The second weakness of the work is that our study was carried out in HEK293 cells expressed only the pore-forming subunit of I_{Kr} , HERG, which may not be adequate to address the questions because it has been proposed that I_{Kr} is formed by co-assembly of HERG with mink [42] or with MirP1 [43]. These auxiliary subunits might well affect the effects of ceramide on I_{HERG} , although a recent study indicates that HERG itself is sufficient to account for the biophysical and pharmacological properties of the native I_{Kr} [44].

Abbreviations

Bis (bisindolylmaleimide); C2 (membrane permeable ceramide); C2 (N-acetyl-D-erythro-sphingosine); CHF (congestive heart failure); CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate); Dihydro-C2 (dihydro-, N-acetyl-D-erythro-sphingosine); EAD (early afterdepolarization); FSK (forskolin); Gen (genestein); HA (herbimycin A); HERG (human *ether-à-go-go* related gene); I_{Kr} (rapid delayed rectifier K^+ current); MnTBAP (Mn(III) tetrakis(4-benzoic acid) porphyrin chloride); $O_2^{\cdot-}$ (superoxide anion); OA (okadiac acid); PDD (phorbol ester 12,13-didecanoate); PKA (protein kinase A); PKC (protein kinase C); PKC ξ (atypical protein kinase C); PMA (phorbol 12-myristate 13-acetate); PTK (protein tyrosine kinase); ROS (reactive oxygen species); SMase (sphingomyelinase); SOD (superoxide dismutase); VitE (vitamin E).

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