Specific protein kinase C isoform exerts chronic inhibition on the slowly activating delayed-rectifier potassium current by affecting channel trafficking

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ABSTRACT
The slowly activating delayed rectifier K\(^+\) current (\(I_{KS}\)) plays a key role in the repolarization of ventricular action potential in the human heart and is formed by the pore-forming \(\alpha\)-subunit encoded by KCNQ1 (Kv7.1) and \(\beta\)-subunit encoded by KCNE1. Evidence suggested that \(I_{KS}\) was regulated through protein kinase C (PKC) pathway, but the mechanism is controversial. This study was designed to identify the specific PKC isoform involved in the long-term regulation of \(I_{KS}\) current. The \(I_{KS}\) current was recorded using whole-cell patch-clamp technique in human embryonic kidney (HEK) 293B cell co-transfected with human KCNQ1/KCNE1 genes. The results revealed that both chronic activation of Ang II and PMA reduced the \(I_{KS}\) current in a long-term regulation (about 24 hours). Further evidence showed that PKCe knockdown by siRNA antagonized the AngII-induced chronic inhibition on the \(I_{KS}\) current, whereas knockdown of cPKC (PKCa and PKCB) alleviated the inhibition effect of PMA on the current. Moreover, the forward transport inhibition of the channel with brefeldin A alleviated the Ang II-induced chronic inhibition on \(I_{KS}\) current, while the channel endocytosis inhibition with dynasore alleviated both Ang II and PMA-induced chronic inhibition on \(I_{KS}\) current. The above results showed that PKCe activation promoted the channel endocytosis and inhibited the channel forward transport to the plasma membrane, while cPKC activation only promoted the channel endocytosis, which both down regulated the channel current.

Introduction
The delayed-rectifier potassium current including \(I_{KS}\) and \(I_{Kr}\) is the major ion channel essential to the cardiac repolarization phase of the action potential in human and determines the action potential duration (APD) in cardiac [1]. \(I_{KS}\) is formed by the pore-forming \(\alpha\)-subunit encoded by KCNQ1 (Kv7.1) and \(\beta\)-subunit encoded by KCNE1. The reduction of \(I_{KS}\) current caused by genetic mutations or drugs is linked to QT interval prolongation in the electrocardiogram (ECG), which can increase the risk of cardiac arrhythmia occurrence and sudden death; meanwhile, the congenital long-QT syndrome 1 (LQT1) patients are mainly related to the mutation of KCNQ1 gene [2]. As one of the repolarization reserves, \(I_{KS}\) current becomes the main current to promote repolarization and prevent the arrhythmia occurrence when other repolarization reserves (such as \(I_{Kr}\)) decrease or adrenergic levels increase in large animals [3]. Therefore, the prolonged APD is likely to cause torsade de pointes and increase the incidence of arrhythmia for the LQT1 patients with a down-regulated \(I_{KS}\) function [4,5]. Therefore, it is of great importance to study the molecular mechanism of \(I_{KS}\) downregulation in pathological conditions (i.e. cardiac hypertrophy and heart failure) for preventing the occurrence of arrhythmia. At present, the regulation of \(I_{KS}\) current under pathological conditions has not been fully explained.

\(I_{KS}\) is regulated by many factors such as nerves and body fluids, among which angiotensin II (AngII) is one of the main body fluids that cause hypertrophic remodeling and electrical remodeling

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of myocardial tissue [6,7]. Our previous studies showed that the QT interval was prolonged in isolated guinea pig hearts by AngII perfusion (200 nM), and further studies indicated that AngII exerted inhibition on the $I_{KS}$ current through protein kinase C (PKC) phosphorylating the channel in an acute regulation manner [8]. Phosphorylation is an effective way to regulate channel function and further affect cardiac electrophysiological characteristics; therefore, it also plays a vital role in the regulation of $I_{KS}$ current [9].

Chronic PKC activation has been implicated in many pathological conditions, such as cancer, lung and kidney diseases and especially heart failure [10–12]. It was reported that Gq-protein-coupled receptors (GqPCR) activation induced the KCNQ1 channel endocytosis by the conventional PKC (cPKC, including α, βI, βII, γ) [13], especially the PKC βII isoform involved in the reduction of KCNQ1 channel expression caused by GqPCR activation [14]. Our previous studies identified that AngII exerted a stronger effect on novel PKC (PKCε) than cPKC (PKCa and PKCb), while PMA exerted a stronger effect on cPKC inversely [8]. Under the pathological conditions, such as cardiac hypertrophy, PKC isoforms expression were significantly up-regulated, which promoted the development of cardiac hypertrophy [15]. Up to now, although we have investigated the acute effect of different PKC isoform on $I_{KS}$ current by different pathways, the chronic effect of specific PKC isoforms on $I_{KS}$ current is not clear. Thus, we will discuss the chronic effect of specific PKC isoforms (cPKC and PKCε) on $I_{KS}$ current. At present, the study was designed (1) to evaluate the chronic effect of specific PKC isoform on $I_{KS}$ current, (2) to define the distinct chronic effect of specific PKC isoform on $I_{KS}$ current using siRNA knockdown technology, and (3) to study the mechanism underlying the regulation $I_{KS}$ current by specific PKC isoform in a chronic regulation manner.

**Material and methods**

**Solutions and chemicals**

Ang II (Enzo Life Sciences) was prepared as a 1 mM stock solution in water. Bis-1 (Sigma) was prepared as a 5 mM stock solution in DMSO. PMA (Sigma) was prepared as a 2 mM stock solution in DMSO. PKCe peptide activator (εV1-2, EAVSLKPT), cPKC peptide activator (cPKC-AP, SVEIWD) and their scramble peptides were synthesized by LifeTein Biotechnology Company (Beijing, China), which were all prepared as 2 mM stock solutions in water. All peptide activators and scramble peptides were conjugated to a delivery peptide, oligo-arginine (R8), to make the peptide cell membrane permeable [16]. All stock solutions were stored at −20°C. The highest final concentration of DMSO in external solution was ≤ 0.1%, a concentration that had no effect on the current recording.

**Cell culture and transfection**

Human embryonic kidney (HEK) 293B cells were cultured in 35 mm petri dishes in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution under 5% CO₂ at 37°C. The cells were stably co-expressing KCNQ1/KCNE1 channel, while the human AT1 receptor cDNA (200 ng) was transiently transfected using a Lipofectamine 2000 Reagent kit (Invitrogen, USA) following the manufacturer’s instructions. Experiments were performed within 24 to 48 hours after the transfection.

**PKC isoenzyme knockdown**

Double-stranded short interfering RNA (siRNA) targeting human PKCa (CGACTGGGAAAAACTGGAG), PKCb (GAAGATGACTCTTCCAT) or PKCe (CTTCAAACCACGCAATAA) (RIBOBIO, China) was transfected (100 nM) into HEK 293B cell stably co-expressing KCNQ1/KCNE1 genes using LipoRNAmax (Invitrogen, UK) according to the manufacturer’s protocol [8]. Transfection media was replaced with culture media 4 hours after transfection, and the experiments were performed 48 hours later.

**Electrophysiology**

The $I_{KS}$ current was measured using a MultiClamp 700B amplifier (Molecular Devices, USA) at room temperature under continuous superfusion with the external solution. When filled with the pipette
solution, the electrodes had a tip resistance between 0.5 and 2.5 MΩ. The electrical signals were sampled at 2.5–10 kHz and filtered at 1 kHz using a low-pass filter and digitized with an A/D converter (Digidata 1322, Molecular Devices, USA). The external solution contained (in mM): NaCl 140, MgCl₂ 1, KCl 5.4, glucose 10, HEPES 10, and CaCl₂ 2 (pH adjusted to 7.4 with NaOH). The pipette solution contained (in mM): KCl 140, HEPES 10, MgCl₂ 1, and EGTA 5 (pH adjusted to 7.2 with KOH). A perforated patch configuration was used in HEK 293B cells to prevent Iₛ current rundown after the cell membrane ruptured, and the patch pipette was back-filled with amphoterin B (600 ng/ml). Following the patch perforation, the whole-cell membrane capacitance was measured from integration of the capacitive transients evoked by voltage steps from −50 mV to −60 mV, which did not activate any time-dependent membrane current. Series resistances were compensated 80% in order to minimize voltage errors. The pClamp software (Clampfit 10.4, Molecular Devices, USA) was used to generate voltage-pulse protocols and acquire data [8].

**Western blotting**

The KCNQ1 membrane protein expression was evaluated by western blot analysis. The membrane protein was extracted with the Pierce Cell Surface Protein Isolation Kit (Thermo scientific). First, the cells were incubated with sulfo-NHS-SS-biotin marker dissolved in PBS for 30 minutes at 4°C. The above ubiquitination reaction was terminated by adding the Quenching Solution, and the supernatant was discarded by centrifugation. The adherent cells were scraped down and washed with TBS, and the lysate was added after the centrifugation. The supernatant was collected centrifugally and mixed with the washed NeutrAvidin Agarose and continued to be incubated at room temperature for 1 hour in a shaking table. The DTT was diluted to a final concentration of 50 mM with sample buffer and incubated for 1 hour. The membrane protein was the filtrate collected by centrifugation and analyzed by western blot. The following primary antibodies were used as follows: anti-Na/K-ATPase (ProteinTech, 1:500) and anti-KCNQ1 (Alomone, 1:700).

**Statistical analysis**

The data were expressed as means±SEM. SPSS 19.0 software was used for the data analysis. Group comparisons were performed with paired or unpaired Student’s t-tests (for single two-group comparisons) and ANOVA with Dunnett’s post hoc tests (for multiple-group comparisons). Differences were considered significant if P < 0.05.

**Results**

**The chronic effect of Ang II on Iₛ current**

As one of the main body fluids, AngII can cause hypertrophic remodeling and electrical remodeling of myocardial tissue. Our previous studies found that AngII produced the acute inhibition on Iₛ current through PKCε phosphorylating the channel directly [8]. But the electrical remodeling of myocardial tissue involved the chronic effect caused by PKC activation was not clearly understood. The HEK 293B cell stably co-expressing KCNQ1/KCNE1 genes transfected with the human AT1 receptor cDNA was used to investigate the chronic effect of Ang II on the Iₛ current. The cells were divided into three groups, including control group, Ang II group, and Ang II plus Bis-1 group, which were treated with normal medium, Ang II (100 nM) and Ang II (100 nM) plus Bis-1 (100 nM) for about 24 hours, respectively. The current was elicited from the holding potential of −80 mV to prepulses from −50 mV to +40 mV for a 5 s duration and was followed by a test pulse to −50 mV to evoke the slowly decaying outward tail currents. The results showed that compared with the control group, AngII markedly reduced both the depolarization and tail currents, which were alleviated in the presence of nonselective PKC inhibitor Bis-1 (Figure 1(a)). Moreover, the tail current density was significantly decreased at the potential range from 0 mV to +40 mV (P < 0.01, Figure 1(b)), with the tail current decreased about 42.19% by the AngII treatment at the potential of +40 mV (from 27.23 ± 1.77 pA/pF to 15.74 ± 1.57 pA/pF, P < 0.01, Figure 1(c)), which
was alleviated by Bis-1 ($P < 0.01$, Figure 1(c)). The tail current amplitude normalized to the maximum tail current amplitude was used to construct the activation curve shown in Figure 1(d). When fit as a Boltzmann function, the half-maximum activation voltage ($V_{1/2}$) and slope factor under control condition were $5.01 \pm 1.32$ mV and $16.75 \pm 1.39$, respectively, which were not significantly different from those after the Ang II treatment ($4.75 \pm 1.47$ mV for $V_{1/2}$, $16.79 \pm 1.57$ for slope factor, $P > 0.05$). We next explored the effect of Ang II on the KCNQ1 channel membrane protein expression which reflected the ion channel number. The membrane protein was extracted and used to detected the KCNQ1 channel membrane protein expression by western blot in all the groups. The results revealed that the KCNQ1 channel membrane protein expression decreased significantly after the Ang II treatment ($P < 0.01$, Figure 1(e)), which suggested that Ang II decreased the $I_{Ks}$ current by reducing the KCNQ1 channel number not by changing the channel gating.

**The chronic effect of PMA on $I_{Ks}$ current**

We next tested the effect of phorbol ester PMA since it is widely used as a PKC activator. We investigated the chronic effect of PMA on $I_{Ks}$ current in HEK 293B cell by activating PKC directly. The cells were divided into two groups, including control group and PMA group, which were treated with normal medium and PMA (100 nM) for about 24 hours, respectively. The results showed PMA markedly reduced both the depolarization and tail currents (Figure 2(a)), and the tail current density of $I_{Ks}$ was significantly decreased at the potential range from 0 mV to +40 mV ($P < 0.01$, Figure 2(b)). Furthermore,
tail current decreased about 32% by the PMA treatment at the potential of +40 mV (from 28.03 ± 1.47 pA/pF to 19.21 ± 0.49 pA/pF, \( P < 0.01 \), Figure 2(c)). The tail current amplitude normalized to the maximum tail current amplitude was used to construct the activation curve shown in Figure 2(d). When fit as a Boltzmann function, the half-maximum activation voltage \( V_{1/2} \) and slope factor under control conditions were 8.61 ± 1.51 mV and 16.84 ± 1.43, respectively, which were not significantly different from those after the PMA treatment (12.59 ± 1.85 mV for \( V_{1/2} \), 16.09 ± 1.50 for slope factor, \( P > 0.05 \)).

We next explored the effect of PMA on the KCNQ1 channel membrane protein expression. The membrane protein was extracted after the PMA treatment for 24 hours and then used to detect the KCNQ1 channel membrane protein expression by western blot. The results revealed that PMA treatment for 24 hours could significantly decrease the KCNQ1 channel membrane protein expression (about 56%, \( P < 0.01 \), Figure 2(e)), which suggested that PMA also decreased the \( I_{K_s} \) current by reducing the KCNQ1 channel number.

**The chronic effect of different PKC isoform on \( I_{K_s} \) current**

We had confirmed that both PMA and Ang II exerted the chronic inhibition effect on \( I_{K_s} \) current by activation of PKC. We next investigated the effect of direct activation of specific PKC isoform (mainly cPKC and PKCe) on the current by different PKC peptide activators. The peptides added with R8-conjugated cell-permeable peptide selectively activate specific PKC isoform [17]. The different PKC isoform peptide activators and their control peptide were synthesized and used as previously reported [8]. The cells were divided into

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**Figure 2.** The chronic effect of PMA on \( I_{K_s} \) current in HEK 293B cell. (a) The representative \( I_{K_s} \) current under the PMA (100 nM) treatment. (b) The current–voltage relationship for the tail currents under the PMA (100 nM) treatment. (c) The summary data for the tail currents under the PMA (100 nM) treatment at +40 mV prepulse. (d) The normalized I–V relationship for \( I_{K_s} \) current. The solid lines represent fits to a Boltzmann function. (e) The representative immunoblot and summary data for the KCNQ1 channel membrane protein under the PMA (100 nM) treatment (**\( P < 0.01 \), compared with the control group).
four groups, including cPKC control peptide group, cPKC peptide activator group, PKCε control peptide group, and PKCε peptide activator group, which were treated with cPKC control peptide (200 nM), cPKC peptide activator (200 nM), PKCε control peptide (200 nM), and PKCε peptide activator (200 nM) for about 24 hours, respectively. For the four groups above, the cPKC control peptide group and cPKC peptide activator group were used to investigate the effect of cPKC activation on the current, while the later two groups were used to investigate the effect of PKCε activation on the current. Compared with the control peptide, the results showed that both the depolarization and tail currents were significantly reduced after the cPKC and PKCε peptide activator treatment (Figure 3(a,e)), and the tail current density was all significantly inhibited at the potential range from +10 mV to +40 mV ($P < 0.01$ or $P < 0.05$, Figure 3(b,f)). Furthermore, the tail current decreased significantly at the potential range of +40 mV (cPKC control peptide: 34.66 ± 2.03 pA/pF, cPKC peptide activator: 17.18 ± 1.08 pA/pF, $P < 0.01$, Figure 3(c); PKCε control peptide: 32.91 ± 1.65 pA/pF, PKCε peptide activator: 19.60 ± 1.31 pA/pF, $P < 0.01$, Figure 3(g)). The tail current amplitude normalized to the maximum tail current amplitude was used to construct the activation curves shown in Figure 3(d and h). When fit as a Boltzmann function, the half-maximum activation voltage ($V_{1/2}$) was 9.98 ± 1.08 mV for cPKC control peptide and 7.96 ± 1.10 mV for PKCε control peptide, while the slope factors were 16.47 ± 0.97 for cPKC control peptide and 14.54 ± 1.03 for PKCε control peptide, which were not significantly different from those in the presence of PKC peptide activators, the $V_{1/2}$ value

Figure 3. The chronic effect cPKC and PKCε activation on $I_{hs}$ current in HEK 293B cell. (a) The representative $I_{hs}$ current under the cPKC activation (200 nM). (b) The current–voltage relationship for the $I_{hs}$ tail currents under the cPKC activation (200 nM). (c) The summary data for the tail currents under the cPKC activation (200 nM) at +40 mV prepulse. (d) The normalized I–V relationship for $I_{hs}$ tail currents. The solid lines represent fits to a Boltzmann function. (e) The representative $I_{hs}$ current under the PKCε activation (200 nM). (f) The current–voltage relationship for the $I_{hs}$ tail currents under the PKCε activation (200 nM). (g) The summary data for the $I_{hs}$ tail currents under the PKCε activation (200 nM) at +40 mV prepulse. (h) The normalized I–V relationship for $I_{hs}$ tail currents. The solid lines represent fits to a Boltzmann function. ($^{* *}$P < 0.01, compared with the cPKC or PKCε control peptide group; $^{*}$P < 0.05, compared with the cPKC or PKCε control peptide group).
was $6.10 \pm 2.23$ mV for cPKC peptide activator and $5.11 \pm 1.42$ mV for PKCε peptide activator, while the slope factors were $23.65 \pm 2.61$ for cPKC peptide activator and $21.50 \pm 1.67$ for PKCε peptide activator ($P > 0.05$). The results confirmed that both chronic cPKC and PKCε activation elicited an inhibitory action on the $I_{K_s}$ current.

The specific PKC isoform involved in the inhibition of Ang II and PMA on $I_{K_s}$ current

In this part, we continued to discuss the specific PKC isoform involved in the chronic inhibition effect of AngII and PMA on the $I_{K_s}$ current. We next explored which PKC isoform mediated the effect of Ang II or PMA by using a gene knockdown approach with custom-designed siRNAs targeting either cPKC (PKCa and PKCβ) or PKCε. To explore the inhibition effect of AngII on the $I_{K_s}$ current, we treated the cells with scramble siRNA (100 nM), AngII (100 nM) plus scramble siRNA (100 nM), AngII plus cPKC siRNA, and AngII plus PKCε siRNA, respectively. As shown, the $I_{K_s}$ current was significantly inhibited by the AngII treatment at the potential range from +10 mV to +40 mV, which was alleviated by the PKCε siRNA transfection, not by cPKC siRNA (scramble siRNA: $33.64 \pm 1.48$ mV, AngII+cPKC siRNA: $16.57 \pm 0.97$ mV, AngII+scPKC siRNA: $18.74 \pm 0.74$ mV, AngII+PKCε siRNA: $34.64 \pm 1.23$ mV, $P < 0.01$ or $P < 0.05$, Figure 4(a–c)). The results revealed that the chronic inhibition effect of AngII on the $I_{K_s}$ current was regulated through PKCε.

We next discussed which PKC isoform involved in the chronic inhibition effect of PMA on $I_{K_s}$ current. To explore the inhibition effect of PMA on the $I_{K_s}$ current, we treated the cells with scramble siRNA (100 nM), PMA (100 nM) plus scramble siRNA (100 nM), PMA plus cPKC siRNA, and PMA plus PKCε siRNA, respectively. As shown, the $I_{K_s}$ current was significantly inhibited under the PMA treatment at the potential range from +10 mV to +40 mV, which was alleviated by cPKC siRNA transfection, not by PKCε siRNA (scramble siRNA: $33.03 \pm 2.53$ mV, PMA+scramble siRNA: $15.45 \pm 1.66$ mV, PMA+cPKC siRNA: $32.86 \pm 1.61$ mV, PMA+PKCε siRNA: $14.38 \pm 1.10$ mV, $P < 0.01$ or $P < 0.05$, Figure 4(d–f)). The results revealed that the chronic inhibition effect of PMA on $I_{K_s}$ current was regulated through cPKC.

The molecular mechanism underlying the chronic inhibition of Ang II and PMA on $I_{K_s}$ current

We further to investigate whether the ion channel transport was involved in the chronic inhibition of Ang II and PMA on $I_{K_s}$ current. We divided the cells into four groups, including control group, brefeldin A group, brefeldin A plus AngII group, and brefeldin A plus PMA group. In the groups above, brefeldin A was used to inhibit the forward transport of ion channel by disrupting the trans-Golgi network [18]. As expected, the $I_{K_s}$ current decreased significantly at the potential range from +10 mV to +40 mV ($P < 0.01$ or $P < 0.05$, Figure 5(a–c)) in the presence of brefeldin A. We next investigated the effect of PMA and AngII on $I_{K_s}$ current in the presence of brefeldin A. The results indicated that both PMA and AngII further decreased the $I_{K_s}$ current based on the effect of brefeldin A ($P < 0.01$ or $P < 0.05$, Figure 5(c)). The results illustrated that both PMA and AngII could accelerate the endocytosis of channel and lead to the current down-regulation.

We next investigated the effect of PMA and AngII on the channel forward transport. We divided the cells into four groups, including control group, dynasore group, dynasore plus AngII group, and dynasore plus PMA group. In the groups above, dynasore was used to inhibit the endocytosis of KCNQ1 channel as an endocytosis inhibitor [19]. The results showed the $I_{K_s}$ current increased at the potential range from +10 mV to +40 mV ($P < 0.01$, Figure 5(d–f)) in the presence of dynasore, which was alleviated by the co-culture of AngII ($P < 0.01$, Figure 5(d–f)), not by PMA ($P > 0.05$, Figure 5(d–f)). The results elucidated that AngII could also inhibit the forward transport of the KCNQ1 channel and lead to the current reduction.

Discussion

The aim of this study was to identify the specific PKC isoform participated in the chronic effect of AngII and PMA on the $I_{K_s}$ current, as well as the
mechanisms involved. In this research, we studied the chronic activation of cPKC and PKCe on the $I_{Ks}$ current. Our data indicated that (1) both cPKC and PKCe chronic activation could inhibit the $I_{Ks}$ current, (2) AngII exerted the inhibition on the $I_{Ks}$ current by PKCe activation, while PMA exerted the inhibition by cPKC activation, (3) AngII inhibited the $I_{Ks}$ current by facilitating the channel endocytosis as well as suppressing the channel forward transport process, and (4) PMA inhibited the $I_{Ks}$ current only by facilitating the channel endocytosis.

There is increasing evidence that phosphorylation is an important way to regulate the channel function. There is no doubt that epinephrine β1 receptor stimulates and increases the $I_{Ks}$ current through cAMP-PKA pathway [17]. Until now, we and others have shown the effect of acute PKC stimulation on the $I_{Ks}$ current, but the long-term regulation of $I_{Ks}$ current by PKC is undefined. GqPCRs such as adrenergic receptor (α1), angiotensin II receptor (AT1), and endothelin receptor (ET1) are known to mediate positive inotropism in human ventricular myocardium [18]. The Gq protein stimulates phosphatidylinositide-specific phospholipase C (PLC) after it is activated. The substrate of PLC is phosphatidylinositol 4, 5-biphosphate (PIP2) so that agonist stimulation of the receptor causes the reduction of PIP2 in the plasma membrane. Hydrolysis of PIP2 generates inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). Stimulation of IP3 receptor by IP3 releases Ca$^{2+}$ from intracellular stores; furthermore, DAG activates PKC. Their stimulation contributes to the increase in contractility at the early stage of heart failure, but chronic stimulation of these receptors has been shown to be associated with myocardial hypertrophy and remodeling. We
and others also reported that KCNQ1/KCNE1 channel phosphorylation was contributed to the acute GqPCR-mediated activation of the channel via PKC [8,19]. In contrast to the inhibition effect on the $I_{Ks}$ current of AT1 receptor activation, our results showed that direct PKC activation by PMA increased the $I_{Ks}$ current acutely [8]. More and more evidences show that activation of different GqPCRs lead to the activation of different PKC isoforms [20]. It was reported that only PKCα, βI, βII, and PKCε isoforms translocated to the cell membrane when the adrenergic α1 receptor excited, while all the PKC isoforms translocated to the cell membrane under the PMA action [21]. The above reports suggest that different PKC agonists lead to activation of different PKC isoforms (whether PKC is activated directly or indirectly), which play biological functions by translocating to the cell membrane. Moreover, due to the different activation and transposition, different PKC isoforms display different regulation on the channel.

It was reported that PKC isoforms expression including PKCα, PKCβ, and PKCε were upregulated significantly under the pathological conditions such as cardiac hypertrophy [22,23]. Chronic stimulation of PKC has been shown to be associated with myocardial hypertrophy and remodeling [15]. Our recent data showed that Ang II and PMA decreased the $I_{Ks}$ current, which was achieved by decreasing the channel number not the channel dynamics. By using specific siRNA knockdown method, we provided further evidence that cPKC (PKCα and

Figure 5. The molecular mechanism underlying the chronic inhibition of Ang II and PMA on $I_{Ks}$ current in HEK 293B cell. (a) The representative $I_{Ks}$ current by the Ang II and PMA treatment under the channel forward transport inhibition. (b) The current–voltage relationship for the $I_{Ks}$ current by the Ang II and PMA treatment under the channel forward transport inhibition. (c) The summary data for the tail currents by the Ang II and PMA treatment under the channel forward transport inhibition at +40 mV prepulse. (d) The representative $I_{Ks}$ current by the Ang II and PMA treatment under the channel endocytosis inhibition. (e) The current–voltage relationship for the $I_{Ks}$ current by the Ang II and PMA treatment under the channel endocytosis inhibition. (f) The summary data for the $I_{Ks}$ tail currents by the Ang II and PMA treatment under the channel endocytosis inhibition at +40 mV prepulse. (**$P < 0.01$, compared with the control group; ***$P < 0.01$, compared with Brefeldin A or Dynasore group; #P < 0.05, compared with Brefeldin A group).
PKCβ underlined the down-regulation of $I_{Ks}$ current by PMA, while PKCc selectively attenuated the inhibitory action of Ang II on the $I_{Ks}$ current.

The ion channel number is dependent on the intracellular trafficking, which includes the forward and reverse transport process. The forward transport is the progress that after the gene transcription, the channel proteins fold and mature on the endoplasmic reticulum and Golgi, which are finally wrapped by secretory vesicles and transported to the cell membrane to perform functions [24]. Reverse transport refers to the endocytosis and degradation progress after the channel proteins invaginated through the cell membrane [25]. For the experimental results above, we speculated that activation of different PKC isoforms may affect the forward transport or reverse transport of the channel, ultimately affecting the channel function. Therefore, we used the endocytosis inhibitor dynasore and forward transport inhibitor brefeldin A to inhibit the reverse and forward transport process, consequently determined the way by which the cPKC and PKCc isoforms inhibited the $I_{Ks}$ current. Our results revealed that PKCc activation exerted a chronic inhibitor on the channel by promoting the channel endocytosis and inhibiting the channel forward transport, while cPKC exerted chronic inhibitor on the channel by promoting channel endocytosis only. Both the forward transport and endocytic degradation processes of the channel involve Rab proteins, which are small GTPases. Rab proteins exist in the cytoplasm as an inactive form binded to GDP (Rab-GDP), but exist as an active form binded to GTP (Rab-GTP) on the cell membrane and transport vesicles. Rab is one of the important factors regulating the channel transport and function [26]. After entering into the early endosome through Rab5, the channel proteins have four ways to go: (1) transport to cell membrane through Rab4 (rapid recycling), (2) degradation by proteasome, (3) degradation by lysosomal through Rab7, and (4) circulation to cell membrane through Rab11 (slow recycling) [27]. Rab4, Rab5, Rab7, and Rab11 are widely expressed in the heart [13], which are all involved in the KCNQ1/KCNE1 channel transport in the cell [25,28]. We hypothesize whether different PKC isoforms affect the channel transport through different Rab proteins, which we will research in our future work.

**Conclusion**

Collectively, we show that Ang II-stimulated PKCc activation promoted the endocytosis of KCNQ1/KCNE1 channel and inhibited the channel forward transport to the plasma membrane, while PMA-stimulated cPKC activation only promoted the internalization of KCNQ1/KCNE1 channel. To our knowledge, this is the first time to show that different PKC isoform inhibit the $I_{Ks}$ current by affecting different progress of the ion channel intracellular trafficking. This study is of great significance to reveal the molecular mechanism of cardiac hypertrophy electrical remodeling and to find new targets for arrhythmia.

**Limitations**

Our data had identified that specific PKC isoform suppressed $I_{Ks}$ in a long-term manner by affecting different channel trafficking process, which may involve direct modulation of the channel, or potentially other signaling proteins.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This study was supported by the National Natural Science Foundation of China (No 21674080)

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