

## PKC isozyme selective regulation of cloned human cardiac delayed slow rectifier K current

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### Abstract

Delayed rectifying K<sup>+</sup> channel,  $I_{Ks}$ , plays a vital role in normal and arrhythmogenic heart.  $I_{Ks}$  is modulated by PKC but the identity of which PKC isozymes is involved in this modulation is not known. To dissect the role of individual PKC isozymes in the regulation of  $I_{Ks}$ , human cardiac  $I_{Ks}$  channel (minK + KvLQT1) was expressed in *Xenopus* oocytes. Peptide PKC isozyme-specific activator and inhibitors, in addition to the general PKC activator, PMA, were used. Whole-cell  $I_{Ks}$  was recorded using two-electrode voltage clamp technique. PMA and  $\epsilon$ PKC specific activator peptide, but not the inactive analog, 4 $\alpha$ PDD, significantly increased  $I_{Ks}$ . Peptide specific inhibitors for  $\beta_{II}$ PKC, and a general PKC inhibitor, calphostin C antagonized PMA-induced activation of  $I_{Ks}$ . However, control peptide, pentylsine, and specific inhibitor peptide for  $\alpha$ PKC,  $\beta_I$ PKC,  $\delta$ PKC, or  $\eta$ PKC did not alter PMA effect on  $I_{Ks}$ . The present study demonstrates that  $\beta_{II}$ PKC,  $\epsilon$ PKC but not  $\beta_I$ PKC,  $\alpha$ PKC,  $\delta$ PKC, and  $\eta$ PKC, are involved in PMA-induced activation of the cloned human  $I_{Ks}$  expressed in *Xenopus* oocyte. Furthermore, this is the first report to dissect the fine functional role of  $\beta_{II}$ PKC and  $\beta_I$ PKC in the regulation of  $I_{Ks}$ . Identification of the particular isozyme(s) that mediates the regulation of  $I_{Ks}$  channels is of importance for the understanding of the mechanism of ion channel regulation and the development of new therapeutic agents.

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Delayed rectifying K<sup>+</sup> channels are of importance in determining the shape and repolarization of cardiac action potentials. One component of the cardiac delayed rectifier is called the slow K<sup>+</sup> current ( $I_{Ks}$ ). It is composed of two molecular entities: KvLQT1 and minK [1,2].  $I_{Ks}$  plays an important role in cardiac repolarization and its alteration is responsible for the most common forms of inherited long-QT syndrome in human [1–3].  $I_{Ks}$  is modulated in the heart by  $\beta$ -adrenergic receptors through a cAMP pathway [4] and by  $\alpha$ -adrenergic receptors through the PKC pathway [5–7]. minK has been cloned from many species, including mouse [8], rat [9], rabbit [10], and human [11].  $I_{Ks}$  channel is the target of several anti-arrhythmic drugs [12].

Two subfamilies of PKC isozymes, which can be stimulated by the tumor-promoting drug, 4- $\beta$  phorbol ester 12-myristate-13-acetate, PMA, are conventional cPKC isozymes,  $\alpha$ ,  $\beta_I$ ,  $\beta_{II}$ , and  $\gamma$ PKC that contain the Ca<sup>2+</sup> binding domain (C2-containing), and the novel nPKC ( $\delta$ ,  $\theta$ ,  $\epsilon$ , and  $\eta$ PKC) or C2-less isozymes [13]. The characterization of the role of individual PKC isozymes in the regulation of ion channels has been largely limited by the lack of isozyme selective modulators (antagonists and agonists). Identification of the particular isozyme(s) that mediates the regulation of K<sup>+</sup> channels is essential for better understanding of the regulatory mechanism of  $I_{Ks}$  in physiological and pathological settings. Recently, we have demonstrated that C2-containing isozymes play an important role in mediating PMA-induced inhibition of L-type Ca<sup>2+</sup> using peptide inhibitors specific for individual isozymes [14,15]. PKC activation has been

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associated with the translocation of PKC isozymes from one intracellular compartment to another [16,17]. This translocation is mediated, at least, in part, by the binding of activated PKC isozymes to the selective anchoring proteins (RACKs, receptors for activated C-kinase) that anchor them to different subcellular sites and consequently activate PKC [18]. Peptides that mimic either the PKC binding site on RACKs or the RACK binding site on PKC are translocation inhibitors of PKC that inhibit the function of the enzyme [19]. Based on this rationale design, peptide inhibitors of particular PKC isozymes have been developed to inhibit the interaction of individual PKC isozymes with their respective RACKs, thus altering their translocation and function [19,20]. Using these peptides, we examined the potential role of individual PKC isozymes in the regulation of human cardiac  $I_{Ks}$  expressed in *Xenopus* oocytes. We report here that the expressed  $I_{Ks}$  through both minK and KvLQT1 in *Xenopus* oocyte is selectively activated by  $\beta_{II}$ PKC and  $\epsilon$ PKC.

## Materials and methods

**Preparation of *Xenopus* oocyte and cRNA injection.** Mature female *Xenopus* frogs, purchased from *Xenopus* I (Ann Arbor, Michigan), were anesthetized with 1.5 mg/ml tricaine. Surgically removed ovarian lobes were dissected and treated for 1.5 h with 1.5 mg/ml collagenase type IA dissolved in Ca-free ND96 medium (mmol/L: NaCl 96, KCl 2, MgCl<sub>2</sub> 2, and HEPES 5, pH 7.4). Stages IV and V oocytes were selected. Plasmids encoding human cardiac K channel  $I_{Ks}$  subunit, pCDNA3.1(+)-minK and pCDNA3.1(+)-KvLQT, were generously given by Dr. Robert S. Kass from Columbia University. Plasmids were first linearized with restriction enzymes and in vitro transcription was carried out using the mMESSAGE mMACHINE (Ambion, TX). Each oocyte was injected with 50 nl hH1 cRNA. The injected oocytes were stored at 18°C in Leibovitz's L-15 medium (Gibco-BRL, MD) supplemented with 50 U/ml penicillin/streptomycin. Currents were recorded from the 3th to the 4th day.

**Solutions and drugs.** The composition of external solution for  $I_{Ks}$  recording is ND96 [6,21]. V1 or C2 region derived peptides (100 nM) were injected individually or in combination as indicated in a total volume of 50 nl (1/20 of oocyte volume). Proper diffusion of the peptides into the cytoplasm is reached within 10–15 min as previously reported [22]. Ten to 15 min following injection of the antagonist peptide, oocytes were superfused with PMA. For  $\epsilon$ V1-7 ( $\epsilon$ PKC agonist peptide), the time course of  $I_{Ks}$  was recorded immediately following injection. The peptides  $\epsilon$ V1-7 (HDAPIGYD,  $\epsilon$ PKC agonist),  $\alpha$ C2-4 (SLNPQWNET;  $\alpha$ PKC antagonist),  $\beta_1$ V5-3 (KLFIMN,  $\beta_1$ PKC antagonist),  $\beta_{II}$ V5-3 (QEVIRN,  $\beta_{II}$ PKC antagonist),  $\eta$ V1-2 (EAVGLQPT;  $\eta$ -PKC antagonist), and pentylsine (K-K-K-K-K, control peptide) were synthesized from Genemed Synthesis, South San Francisco, CA. All peptides used were over 90% pure. All chemicals were purchased from Sigma Chemicals or otherwise indicated.

**Oocyte  $I_{Ks}$  current recordings.** The expressed  $I_{Ks}$  was recorded with two-electrode voltage clamp technique using GeneCLAMP 500 amplifier (Axon Instrument, Foster City, CA). The volume of the recording chamber was 0.3 ml and the rate of perfusion was 0.3 ml/min. Oocytes were impaled with electrodes filled with 3 mol/L KCl in ND96 external solution. For  $I_{Ks}$  current–voltage (I–V) relations, oocytes were depolarized from a holding potential of  $-60$  mV to tests ranging from

$-50$  to  $+70$  mV with increments of 10 mV [21]. The time course for  $I_{Ks}$  was recorded by a depolarization pulse to  $+40$  mV from a holding potential of  $-60$  mV.

**Data analysis.** Data acquired were stored and then analyzed offline with Pclamp 6 software (Axon Instrument). All values are measured as the difference between zero and the peak current. All measurements of  $I_{Ks}$  changes were performed at 20 min to avoid potential time-dependent internalization of plasma membrane in oocytes reported after 20 min of exposure to phorbol esters [23]. Microcal Origin v5.0 (Microcal Software) program was used to generate figures and perform statistical analysis. Data are presented as means  $\pm$  SEM. Percent activation was calculated as the difference of the current amplitude by the intervention(s) over the control value. Student's paired *t* test was used to compare the data before and after interventions. Unpaired *t* test or ANOVA was used to compare the data between groups. A value of  $p < 0.05$  was considered statistically significant.

## Results

### PMA activated $I_{Ks}$

To characterize the modulation of  $I_{Ks}$  by PKC under our experimental conditions, we first used a general

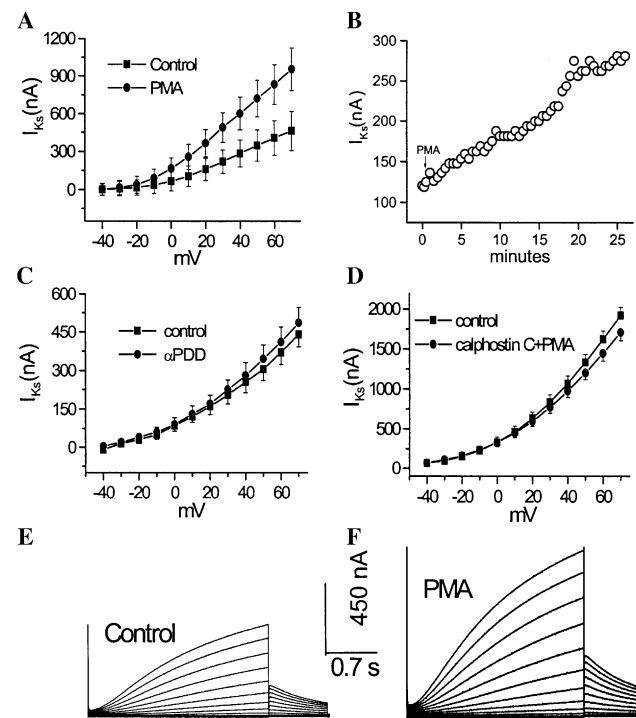


Fig. 1. Activation of  $I_{Ks}$  by PMA. Panel A shows the current–voltage relations of  $I_{Ks}$  during control and following 20 min superfusion of PMA (20 nM) in five oocytes. Panel B shows the time course of  $I_{Ks}$  activation by PMA (20 nM) at  $+40$  mV. Panel C shows the current–voltage relations of  $I_{Ks}$  during control and 20 min after  $4\alpha$ PDD (20 nM) superfusion of six oocytes. Panel D shows the current–voltage relations of  $I_{Ks}$  during control and 20 min after PMA superfusion of five oocytes pretreated with calphostin C, a general PKC inhibitor. Panels E and F are the selected whole current traces from control group and PMA superfused oocytes, respectively. Time 0 corresponds to the time of oocyte impalement and the arrow refers to the onset of drug superfusion.

PKC activator, PMA, and a general PKC inhibitor, calphostin C. Figs. 1A and B illustrate the effect of PMA on  $I_{Ks}$ . Perfusion of oocytes with PMA (20 nM) resulted in a slow and time-dependent activation of peak  $I_{Ks}$ . Panel A shows the I–V relations during control and superfusion with PMA at 20 min. PMA activated  $I_{Ks}$  by  $116 \pm 36.9\%$  at +40 mV ( $n = 5$ ,  $p < 0.05$ , compared with control). Panel B is the time course recorded from one oocyte at a pulse of +40 mV. The specificity of PMA effect on  $I_{Ks}$  was confirmed by comparing its effect to that of another phorbol ester,  $\alpha$ PDD, which does not activate PKC. The results are shown in panel C. Perfusion of oocytes with  $\alpha$ PDD (20 nM) did not significantly affect  $I_{Ks}$  ( $10.8 \pm 6.4\%$ ,  $n = 6$ ,  $p = \text{NS}$ ) at 20 min. Panel D illustrates the effects of PMA (20 nM) in the presence of calphostin C, a general PKC inhibitor. Oocytes were treated with calphostin C (10  $\mu\text{M}$ ) for 10–15 min before the onset of PMA application. Superfusion of calphostin C treated oocytes with PMA (20 nM) completely blocked PMA activation of  $I_{Ks}$  ( $-9.7 \pm 4.5\%$ ,  $n = 5$ ,  $p = \text{NS}$ , compared with control). Panels E and F show  $I_{Ks}$  current traces during control (panel E) and after 20 min of perfusion with PMA (panel F), respectively. These experiments indicate that PMA activation of  $I_{Ks}$  is mediated through PKC. We used the currently available peptide specific activator ( $\epsilon$ V1-7) and inhibitors of PKC isozymes ( $\beta_{II}$ PKC,  $\beta_I$ PKC,  $\alpha$ PKC,  $\delta$ PKC, and  $\eta$ PKC) to dissect which PKC isozyme is involved in this up-regulation of  $I_{Ks}$ . For clarity purposes, we first present the  $\beta$ PKC data.

*PMA-induced activation of  $I_{Ks}$  was antagonized by  $\beta_{II}$ V5-3, but not altered by  $\beta_I$ V5-3*

We investigate the possible involvement of  $\beta$ PKC in PMA-induced activation of  $I_{Ks}$  and furthermore to identify the functional difference between the two isoforms of the  $\beta$ PKC. V-region derived  $\beta_I$ V5-3 and  $\beta_{II}$ V5-3 inhibitory peptides, which are specific for  $\beta_I$ PKC and  $\beta_{II}$ PKC, respectively [24], were used. Fig. 2 shows the effects of these two  $\beta$ PKC isozyme specific peptides on PMA-induced activation of  $I_{Ks}$ . After injection of peptide, 10–15 min were allowed for appropriate diffusion before perfusion of PMA and current recording. Fig. 2A shows the I–V relations of  $I_{Ks}$  during control and after 20 min following the perfusion of  $\beta_{II}$ V5-3 injected oocytes with PMA (20 nM).  $\beta_{II}$ V5-3 completely antagonized PMA-induced  $I_{Ks}$  activation ( $3.0 \pm 13.5\%$ ,  $n = 6$ ,  $p < 0.05$ , compared with PMA alone). Panel C is the time course recorded from one oocyte at pulse +40 mV.

Interestingly, injection of  $\beta_I$ V5-3 (100 nM) did not significantly modulate PMA-induced  $I_{Ks}$  activation ( $104 \pm 4.1\%$ ,  $n = 4$ ,  $p > 0.05$ , compared with PMA alone) (panel B). Figs. 2D and E show current traces during control and  $\beta_{II}$ V5-3 plus PMA, respectively. Taken together, these results demonstrate the involvement of  $\beta_{II}$ PKC, but not  $\beta_I$ PKC, in PMA-induced  $I_{Ks}$  activation. *This is the first study* establishing fine functional separation of two  $\beta$ PKC isozymes,  $\beta_I$ PKC and  $\beta_{II}$ PKC, in the regulation of an ion channel.

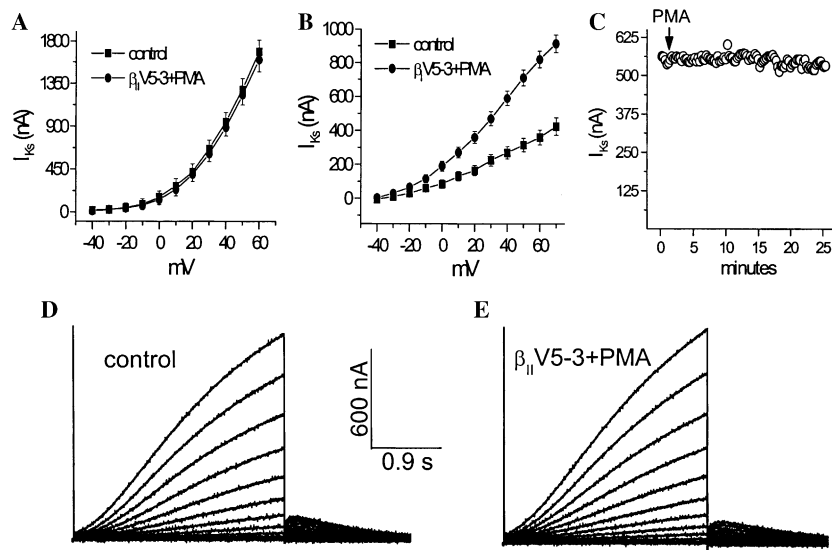


Fig. 2. Effect of PMA on  $I_{Ks}$  in the presence of the peptide inhibitor of  $\beta_{II}$ PKC ( $\beta_{II}$ V5-3) and the  $\beta_I$ PKC ( $\beta_I$ V5-3). Panel A shows the current–voltage relations of  $I_{Ks}$  during control and 20 min following PMA (20 nM) superfusion of the  $\beta_{II}$ V5-3 injected oocytes ( $n = 6$ ). Panel C shows the time course of  $I_{Ks}$  recorded at +40 mV in one such oocyte. Panel B shows the current–voltage relations of  $I_{Ks}$  during control and 20 min after superfusion of PMA in four oocytes injected with  $\beta_I$ V5-3 peptide. Panels D and E illustrate selected traces from control group and PMA superfusion of  $\beta_{II}$ V5-3 injected oocytes, respectively. Time 0 corresponds to the time of oocyte impalement and the arrow refers to the onset of drug superfusion.

### Peptide activator of $\epsilon$ PKC, $\epsilon$ V17 activated $I_{Ks}$

To selectively activate  $\epsilon$ PKC, we used a novel peptide,  $\epsilon$ V1-7, which is derived from the regulatory V1-region of  $\epsilon$ PKC and was previously shown to selectively activate the translocation of  $\epsilon$ PKC [17]. This is the first available agonist peptide activator of one single PKC isozyme. Fig. 3 shows that  $\epsilon$ PKC agonist,  $\epsilon$ V1-7, increased  $I_{Ks}$  by  $100.8\% \pm 12.9$  ( $n = 6$ ,  $p < 0.05$ ) at 20 min following injection of the  $\epsilon$ PKC agonist peptide ( $\epsilon$ V1-7, 100 nM).

### PMA-induced activation of $I_{Ks}$ was not affected by peptide specific antagonists for $\alpha$ , $\delta$ , and $\eta$ PKC isozymes and by a control peptide

To investigate whether other PKC isozymes may also be involved in the modulation of  $I_{Ks}$ , we tested other peptide specific antagonists of PKC isozymes  $\alpha$ ,  $\delta$ , and  $\eta$ PKC (see Materials and methods for detail). Fig. 4A shows the I–V relations of  $I_{Ks}$  during control and 20 min following PMA superfusion of oocytes injected with  $\alpha$ C2-4 (100 nM).  $I_{Ks}$  was increased by  $105.3 \pm 9.0\%$  ( $n = 4$ ,  $p = NS$ , compared with PMA alone). Figs. 4B and C show the I–V relations of  $I_{Ks}$  during control and 20 min following PMA superfusion of oocytes injected with  $\delta$ V1-1 (100 nM) and  $\eta$ V1-2, respectively, and  $I_{Ks}$  was increased by  $113.5 \pm 24\%$  ( $n = 5$ ,  $p = NS$ , compared with PMA alone) and  $109 \pm 18\%$  ( $n = 5$ ,  $p = NS$ , compared with PMA alone), respectively. These results demonstrate that  $\alpha$ ,  $\delta$ , and  $\eta$ PKC isozymes are not involved in PMA-induced  $I_{Ks}$  activation. Negative control experiments were performed using pentylsine (100 nM), a control peptide [15,25]. PMA (20 nM) exerted its activation of  $I_{Ks}$  in the presence of pentylsine ( $122 \pm 28\%$  ( $n = 5$ ,  $p > 0.05$ )). Therefore, the effect of PMA on  $I_{Ks}$  was not altered by the control peptide, pentylsine, indicating that non-specific peptide activity is not responsible for the effects of these PKC isozyme specific

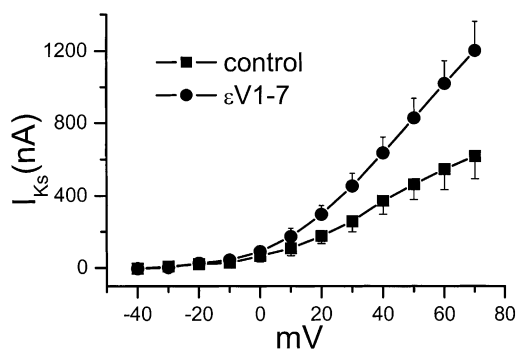


Fig. 3. Effect of peptide activator of  $\epsilon$ PKC,  $\epsilon$ V17 on  $I_{Ks}$ . Figure shows the current–voltage relations of  $I_{Ks}$  during control and after injection of  $\epsilon$ V17 (100 nM) peptide in six oocytes.

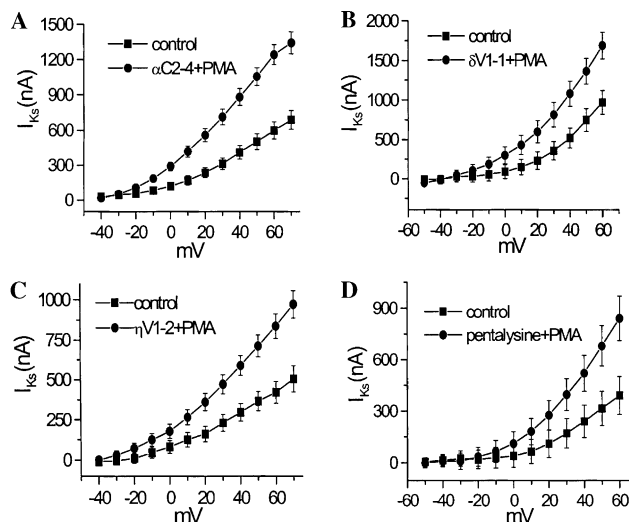


Fig. 4. Effect of PMA on  $I_{Ks}$  in the presence of peptide inhibitors for  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\eta$ PKC isozymes, and pentylsine, a control peptide. Panels A–D show the current–voltage relations of  $I_{Ks}$  during control and 20 min after superfusion of PMA (20 nM) in the presence of  $\alpha$ C2-4 (100 nM,  $n = 4$ ),  $\delta$ V1-1 (100 nM,  $n = 5$ ),  $\eta$ V1-2 (100 nM,  $n = 5$ ), and a control peptide, pentylsine (100 nM,  $n = 5$ ), respectively.

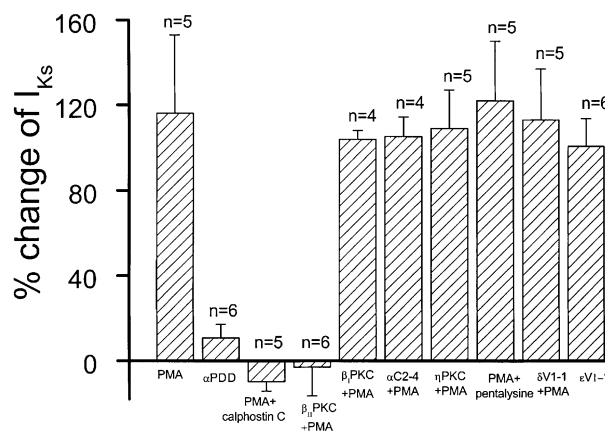


Fig. 5. Summary of the percentage change of  $I_{Ks}$  by PMA, calphostin C, PKC isozyme specific peptide activator, inhibitors, and control peptide.

peptides. The summary of all the above results is shown in Fig. 5.

### Discussion

Our present study demonstrates that  $\beta_{II}$ PKC,  $\epsilon$ PKC but not  $\beta_I$ PKC,  $\alpha$ PKC,  $\delta$ PKC, and  $\eta$ PKC, are involved in PMA-induced activation of the cloned human  $I_{Ks}$  expressed in *Xenopus* oocyte. This is the first report that dissected the fine functional role of  $\beta_{II}$ PKC and  $\beta_I$ PKC in the regulation of  $I_{Ks}$ . Identification of the particular isozyme(s) that mediates the regulation of  $I_{Ks}$  channels is of importance not only for the understanding of the mechanism of ion channel regulation, but also for the

development of new therapeutic strategies for cardiac arrhythmias.

The role of individual PKC isozymes in the regulation of ion channels has been largely limited by the lack of isozyme specific activators and inhibitors. Several previous studies implicated the role of PKC in the regulation of  $I_{Ks}$  channels. The role and the identity of the PKC isozyme(s) responsible for this regulation remain largely unexplored.  $I_{Ks}$  current, expressed in *Xenopus* oocytes from several species, was activated by PKA activation [26]. However, mouse and rat  $I_{Ks}$  currents were decreased by PKC activation [8], whereas guinea pig  $I_{Ks}$  current was activated by PKC [27]. In the mouse and rat minK there is a putative PKC phosphorylation site at Ser<sup>102</sup>. Position 102 in the minK protein has been shown critical in determining the effect of PKC. Human minK also has a serine residue at position 102.

Our present findings demonstrating that human cardiac  $I_{Ks}$  is consistently activated by PMA, a general PKC activator, are consistent with previous studies using heterologous expression system [28]. In addition,  $I_{Ks}$  was also activated by the peptide activator of  $\epsilon$ PKC,  $\epsilon$ V1-7. The general PKC antagonist, calphostin C, and  $\beta_{II}$ PKC specific peptide antagonist, were able to abolish PMA effects. This clearly implicates  $\beta_{II}$ PKC in PMA- and  $\epsilon$ PKC-induced activation of  $I_{Ks}$  but not other PKC isoforms such as  $\beta_I$ ,  $\alpha$ ,  $\delta$ , and  $\eta$ PKC. However, we do not exclude the possibility that other isoforms, which were not tested here, may be involved in the regulation of  $I_{Ks}$  channels.

Phosphorylation of ion channel proteins is one of the key mechanisms in signal transduction pathways that alter channel properties and influence excitability and thus the physiological function of excitable cells [29]. The molecular mechanisms by which PKC regulates cardiac  $I_{Ks}$  channels are not completely defined.

In the last few years, research in the general area of signal transduction has advanced significantly. As a result, PKC emerged as a key component along signal transduction pathways. PKC has been involved in the modulation of ion channels [15,30–34], inotropic and chronotropic effects [30,35–38], gene expression [39,40], secretion of cardiac factors [41,42], hypertrophy [43,44], ischemia, and infarction [45]. There is growing clinical and experimental evidence implicating PKC activation in many pathological conditions, such as cardiac hypertrophy, heart failure, and ischemic preconditioning [46–50]. It becomes therefore critical to characterize and gain insight into how PKC and most importantly its multiple isozymes regulate cardiac ion channels, both in physiological and pathological settings. In cardiac myocytes, the resting membrane potential and the rate of repolarization during an action potential are largely controlled by K currents.  $I_{Ks}$  has important role in diastolic repolarization of cardiac myocytes and determines the cardiac action potential duration.  $I_{Ks}$  channels

thus constitute the key elements in the genesis of arrhythmias. Defect of  $I_{Ks}$  causes long QT syndrome, which is an inherited and sometimes spontaneously occurring disease characterized by an abnormal long action potential duration that predisposes the individual to lethal arrhythmia [51].

Given the important physiological and pathophysiological role of  $I_{Ks}$  in the cardiac rhythm, the ability to up- or down-regulate  $I_{Ks}$  could be a means of controlling the duration of action potential in human heart, and subsequently have preventive and therapeutic implication for arrhythmias. The ability to dissect the individual role of PKC isozymes in the regulation of  $I_{Ks}$  channels may provide functional information that will help in the design of isozyme-targeted therapeutics.

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