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RESEARCH PAPER

Stimulatory action of protein kinase Cε isoform on the slow component of delayed rectifier K^+ current in guinea-pig atrial myocytes

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Background and purpose: Protein kinase C (PKC) comprises at least twelve isoforms and has an isoform-specific action on cardiac electrical activity. The slow component of delayed rectifier K^+ current (I_{Ks}) is one of the major repolarizing currents in the hearts of many species and is also potentiated by PKC activation. Little is known, however, about PKC isoform(s) functionally involved in the potentiation of I_{Ks} in native cardiac myocytes.
Experimental approach: I_{Ks} was recorded from guinea-pig atrial myocytes, using the whole-cell configuration of patch-clamp method.
Key results: Bath application of phenylephrine enhanced I_{Ks} concentration-dependently with EC_{50} of 5.4 μM and the maximal response (97.1 ± 11.9% increase, $n = 16$) was obtained at 30 μM. Prazosin (1 μM) almost totally abolished the potentiation of I_{Ks} by phenylephrine, supporting the involvement of α_1-adrenoceptors. The stimulatory action of phenylephrine was significantly, if not entirely, inhibited by the general PKC inhibitor bisindolylmaleimide I but was little affected by Gö-6976, Gö-6983 and rottlerin. Furthermore, this stimulatory effect was significantly reduced by dialyzing atrial myocytes with PKCε-selective inhibitory peptide εV1-2 but was not significantly affected by conventional PKC isoform-selective inhibitory peptide βC2-4. Phorbol 12-myristate 13-acetate (PMA) at 100 nM substantially increased I_{Ks} by 64.2 ± 1.3% ($n = 6$), which was also significantly attenuated by an internal dialysis with εV1-2 but not with βC2-4.
Conclusions and implications: The present study provides experimental evidence to suggest that, in native guinea-pig cardiac myocytes, activation of PKC contributes to α_1-adrenoceptor-mediated potentiation of I_{Ks} and that ε is the isoform predominantly involved in this PKC action.
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Keywords: I_{Ks}; PKC; PKCε; phenylephrine; α_1-adrenoceptor

Abbreviations: BIS-I, bisindolylmaleimide I; DAG, diacylglycero1; $I_{Ca,L}$, L-type Ca^{2+} inward current; I_K, delayed rectifier K^+ current; I_{Kr}, rapid component of delayed rectifier K^+ current; I_{Ks}, slow component of delayed rectifier K^+ current; PDBu, phorbol 12,13-dibutyrate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; RACKs, receptors for activated C kinases; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate

Introduction

Protein kinase C (PKC) mediates the regulation of cardiac muscle function by a variety of neurotransmitters, hormones and extracellular signalling molecules. Currently, at least 12 isoforms of PKC have been identified in various tissues and are classified into three groups based on their structure and activation mechanisms (Mellor and Parker, 1998); namely, conventional PKC (α, βI, βII and γ) that have a C2-domain and are activated by Ca^{2+}, diacylglycero1 (DAG) and phosphatidylserine (PS); novel PKC (δ, ε, μ, θ and η) that do not have a C2-domain and are activated by DAG and PS; and atypical PKC (ζ and λ) that are regulated by PS. Expression of PKC isoforms in the heart displays species- and/or developmental stage-dependent differences (Pucéat *et al*., 1994; Rybin and Steinberg, 1994). It has been demonstrated that guinea-pig heart coexpresses conventional (α, βII and γ), novel (ε) and atypical (ζ) PKC isoforms (Takeishi *et al*., 1999). There is accumulating evidence to indicate that PKC isoforms in the heart are differentially regulated by various

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stimuli under physiological and pathological conditions (Takeishi *et al*., 1999; Hool, 2000; Ruf *et al*., 2002) and have different functional roles in the modulation of electrical activity (Johnson and Mochly-Rosen, 1995) and the development of cardiac hypertrophy and heart failure (Bowling *et al*., 1999; Takeishi *et al*., 2000). In this regard, it is important to note that PKCε has been shown to contribute to cardiac protection associated with ischemic preconditioning (Gray *et al*., 1997; Qiu *et al*., 1998; Liu *et al*., 1999).

The slow component of the delayed rectifier K^+ current (I_{Ks}) is important for the repolarization of atrial (Sanguinetti and Jurkiewicz, 1991; Wang *et al*., 1993; Gintant, 1996; Bosch *et al*., 2003; Ding *et al*., 2004) and ventricular action potentials (Sanguinetti and Jurkiewicz, 1990; Li *et al*., 1996; Bosch *et al*., 1998) in the heart of several mammalian species including humans. I_{Ks} also represents a relevant target for the action of the sympathetic neurotransmitters, adrenaline and noradrenaline, and thereby mediates sympathetic control of cardiac excitability and contractility (Sanguinetti *et al*., 1991). Stimulation of β-adrenoceptors has been shown to markedly enhance I_{Ks} via activation of the cyclic AMP-dependent protein kinase (PKA) in some types of myocardial cells, including atrial and ventricular myocytes (Walsh and Kass, 1988; Yazawa and Kameyama, 1990; Matsuura *et al*., 1996). On the other hand, it has been demonstrated in guinea-pig ventricular myocytes that α_1-adrenoceptor stimulation produced a modest (approximately 20–30%) increase in I_{Ks} via activation of PKC (Tohse *et al*., 1992). The elevated sympathetic tone is accompanied by an enhancement of outward K^+ current through I_{Ks}, which is assumed to counteract the depolarizing effect of the simultaneously potentiated L-type Ca^{2+} inward current ($I_{Ca,L}$) and thereby prevents the excess prolongation of action potential duration, potentially leading to the occurrence of arrhythmias as well as Ca^{2+} overload in cardiac muscle. Thus, the stimulatory action of both α_1- and β-adrenoceptors on I_{Ks} appears to play an important physiological role in protecting the heart from the arrhythmogenic and cardiotoxic effects of excess sympathetic activity.

The present study was undertaken to determine which PKC isoform(s) is functionally involved in mediating the stimulatory action of α_1-adrenoceptor activation, as well as PMA, on I_{Ks}, with the use of the PKC isoform-selective inhibitory peptides and pharmacological inhibitors. Our results provide experimental evidence supporting a functional role of ε isoform of PKC (PKCε) in mediating α_1-adrenergic potentiation of I_{Ks} in native atrial myocytes of guinea-pig hearts.

Materials and methods

Isolation of atrial myocytes

All animal procedures were performed in accordance with the guidelines established by the institution's Animal Care and Use Committee. Single atrial myocytes were enzymatically isolated from adult Hartley guinea-pig hearts using a retrograde Langendorff perfusion method as described previously (Powell *et al*., 1980; Ding *et al*., 2004).

Solutions and chemicals

Normal Tyrode solution contained (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose and 5.0 HEPES (pH adjusted to 7.4 with NaOH). The extracellular bath solution used for measuring I_{Ks} was normal Tyrode solution supplemented with 0.4 μM nisoldipine (a generous gift from Bayer AG, Wuppertal-Elberfeld, Germany) and 5 μM E-4031 (Wako, Osaka, Japan). Agents added to the extracellular solution included phenylephrine hydrochloride (Sigma, St Louis, MO, USA), prazosin hydrochloride (Sigma), phorbol 12-myristate 13-acetate (PMA, Sigma), bisindolylmaleimide I (BIS-I, Sigma), Gö-6976 (Calbiochem, San Diego, CA, USA), Gö-6983 (Biomol, Plymouth Meeting, PA, USA), rottlerin (Calbiochem) and thymeleatoxin (Calbiochem). The control pipette solution contained (in mM) 70 potassium aspartate, 50 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 3 Na_2-ATP (Sigma), 0.1 Li_2-GTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 EGTA, 0.5 $CaCl_2$ and 5 HEPES (pH adjusted to 7.2 with KOH). The concentration of free Ca^{2+} and Mg^{2+} in the pipette solution was calculated to be approximately 1.7×10^{-8} (pCa = 7.8) and 3.7×10^{-5} M (pMg = 4.4), respectively (Fabiato and Fabiato, 1979; Tsien and Rink, 1980). It should be noted that conventional PKC isoforms can be functionally activated by the presence of intracellular free Ca^{2+} concentration on the order of 10^{-8} M in guinea-pig cardiac myocytes (Hool, 2000). In some experiments, peptide translocation inhibitor of PKC isoform βC2-4 or εV1-2 (Biomol) was added to the pipette solution and was present throughout the recording period (Ron *et al*., 1995; Johnson *et al*., 1996).

Whole-cell patch-clamp technique and data analysis

Whole-cell membrane currents (Hamill *et al*., 1981) were recorded with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany), and data were low-pass filtered at 1 kHz, acquired at 5 kHz through an LIH-1600 analog-to-digital converter (HEKA) and stored on hard disc drive, using PATCHMASTER software (HEKA). Patch electrodes had a resistance of 1.8–2.5 MΩ when filled with pipette solutions, and approximately 10 min was allowed to elapse to dialyze the interior of cells with test reagents (βC2-4 or εV1-2) in pipette solution. Cells were superfused constantly at 1–2 ml min^{-1} with extracellular solution at 36 ± 1°C in a 0.5 ml bath chamber.

I_{Ks} was activated by depolarizing voltage steps given from a holding potential of −50 mV to various levels, under conditions where the Na^+ current was inactivated by setting the holding potential to −50 mV, and $I_{Ca,L}$ and the rapid component of delayed rectifier K^+ current (I_{Kr}) were, respectively, blocked by nisoldipine (0.4 μM) and E-4031 (5 μM) added to the extracellular solution for the measurement of I_{Ks} (Ding *et al*., 2004). The effect of external application of test reagents on I_{Ks} was investigated after the initial rundown of I_{Ks} within 3–5 min of patch rupture was allowed to reach a steady-state level, and the initial rundown of I_{Ks} is not shown in the figures. The period of exposure to various reagents is denoted by the horizontal bar in the figures, and the original current traces recorded at time points indicated by numerals are also illustrated in the inset.

The zero-current level is indicated to the left of current traces by the horizontal line.

Voltage-dependent activation of I_{Ks} was assessed by fitting the normalized *I–V* relationship of the tail currents to a Boltzmann equation: $I_{Ks,tail} = 1/(1+\exp((V_{1/2}-V_m)/k))$, where $I_{Ks,tail}$ is the tail current amplitude normalized with reference to the maximum value measured at $+50$ mV, $V_{1/2}$ is the voltage at half-maximal activation, V_m is the test potential and k is the slope factor. Concentration–response relationship for the potentiation of I_{Ks} by phenylephrine was drawn by least-squares fit of a Hill equation: $R = R_{max}/(1+(EC_{50}/[agonist])^{nH})$, where R_{max} represents the maximal degree of potentiation expressed as a percentage, EC_{50} is the concentration giving half-maximal potentiation and n_H is the Hill coefficient.

Statistical analysis

All the averaged data are presented as mean ± s.e.m. with the number of experiments given in parentheses. Statistical comparisons were evaluated using either Student's *t*-test or analysis of variance (ANOVA) with Student–Newman–Keuls (SNK) *post hoc* analysis, as appropriate. Differences were considered to be statistically significant if a *P*-value <0.05 was obtained.

Results

Potentiation of I_{Ks} *by phenylephrine via* α_1*-adrenoceptor in guinea-pig atrial myocytes*

We first characterized the stimulatory action of phenylephrine on I_{Ks} in guinea-pig atrial myocytes. Figure 1a demonstrates a representative example for the time course of I_{Ks} response to 30 μM phenylephrine. I_{Ks} was repetitively (once every 20 s) activated by depolarizing voltage steps (2 s in duration) applied from a holding potential of -50 mV to a test potential of $+30$ mV, and the effect of phenylephrine on I_{Ks} was evaluated by measuring the amplitude of the tail current elicited upon return to the holding potential, which reflects the degree of I_{Ks} activation at the preceding depolarizing test potential. In a total of 16 myocytes, bath application of 30 μM phenylephrine evoked a marked (97.1 ± 11.9%) increase in the amplitude of I_{Ks} in a reversible manner (see also Figures 2d and 4).

Figure 1b shows superimposed current traces of I_{Ks} elicited by depolarizing voltage steps given from a holding potential of -50 mV to various test potentials between -40 and $+50$ mV, before and during exposure to 30 μM phenylephrine. As is evident in Figure 1c, phenylephrine markedly increased the amplitude of I_{Ks} tail currents at all test potentials. To elucidate whether phenylephrine affects the voltage dependence of I_{Ks} activation, the amplitude of tail current at each test potential was normalized with reference to its maximal value at $+50$ mV and was then fitted by a Boltzmann equation (Figure 1d). The data points were reasonably well fitted by a Boltzmann equation, yielding $V_{1/2}$ of 8.6 ± 2.4 mV and k of 12.7 ± 0.7 mV for control, and $V_{1/2}$ of 5.0 ± 3.2 mV and k of 12.9 ± 0.7 mV for phenylephrine ($n=4$). There were no significant differences in the values of $V_{1/2}$ ($P=0.133$) and k ($P=0.850$) between control and phenylephrine groups, thus suggesting that phenylephrine has little effect on the voltage dependence of current activation.

In the experiments demonstrated in Figure 2a, the effect of phenylephrine on I_{Ks} was examined in the presence of the selective α_1-adrenoceptor antagonist prazosin. The atrial myocytes were initially exposed to 1 μM prazosin for ~5 min, and then to 30 μM phenylephrine in the presence of prazosin. The stimulatory action of phenylephrine on I_{Ks} was almost totally abolished by the presence of prazosin (3.7 ± 3.3% increase, $n=6$ versus 97.1 ± 11.9% increase, $n=16$; Figure 2b). It should be noted that the amplitude of I_{Ks} was only slightly decreased (by 5.3 ± 2.5%, $n=6$) during exposure to 1 μM prazosin alone (Figure 2a). This observation confirms the view that the action of phenylephrine on I_{Ks} is mediated through the α_1-adrenoceptor. The potentiation of I_{Ks} by phenylephrine was examined at concentrations ranging from 0.3 to 100 μM. Figure 2c illustrates representative examples for the effect of 1 and 100 μM phenylephrine (left and right panels, respectively). Figure 2d depicts the concentration–response relationship for the stimulatory action of phenylephrine on I_{Ks}, which could be reasonably well fitted with a Hill equation with EC_{50} of 5.4 μM and n_H of 1.03.

We also evaluated the effect of phenylephrine (30 μM) on the time course of I_{Ks} activation during depolarizations in the absence and presence of prazosin. For this purpose, the time to obtain half-maximal activation ($T_{1/2}$, Stengl *et al*., 2003; Terrenoire *et al*., 2005) during 2-s depolarizing voltage steps was compared before and during exposure to phenylephrine. In the absence of prazosin (refer to experiments shown in Figure 1b), $T_{1/2}$ at test potentials of $+10$, $+30$ and $+50$ mV were, respectively, measured to be 484 ± 56, 342 ± 40 and 305 ± 33 ms ($n=5$) before phenylephrine and 432 ± 26, 346 ± 34 and 307 ± 44 ms during exposure to phenylephrine. There were no significant differences in the value of $T_{1/2}$ obtained at each test potential ($+10$, $+30$ or $+50$ mV) before and during exposure to phenylephrine. Similarly, in the presence of 1 μM prazosin (refer to experiments shown in Figure 2a), $T_{1/2}$ at a test potential of $+30$ mV (322 ± 10 ms, $n=5$) was insignificantly affected by further addition of phenylephrine (317 ± 8 ms). These observations are in contrast to an increase in the rate of channel activation during β-adrenergic potentiation of I_{Ks} (Han *et al*., 2001; Stengl *et al*., 2003; Volders *et al*., 2003; Terrenoire *et al*., 2005). Taken together with the results using prazosin (Figure 2b), these data also support a predominant role of α_1-adrenoceptors in potentiation of I_{Ks} by phenylephrine.

Functional role of novel PKC isoform PKCε in potentiation of I_{Ks} *by phenylephrine*

We then evaluated a role for PKC and its isoforms in the potentiation of I_{Ks} via α_1-adrenoceptors. For this purpose, the stimulatory action of phenylephrine was measured at its maximally effective concentration (30 μM, Figure 2d) in the presence of various pharmacological (bath) and peptide inhibitors (pipette) for PKC isoforms. Figure 3a illustrates a

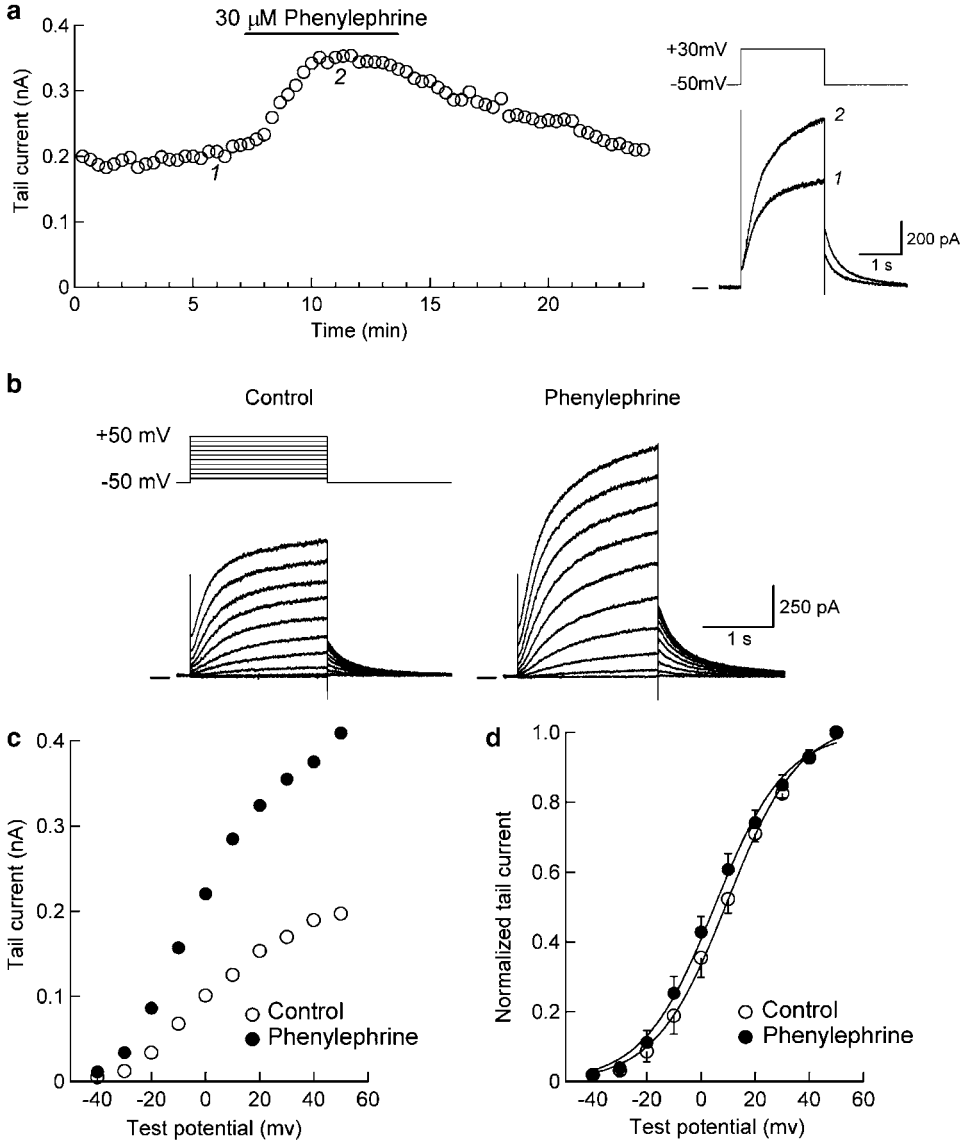

Figure 1 Potentiation of I_{Ks} by phenylephrine in guinea-pig atrial myocytes. (**a**) Time course of changes in the amplitude of I_{Ks} tail current during exposure to 30 μM phenylephrine. I_{Ks} was repetitively (every 20 s) activated by 2-s depolarizing steps to +30 mV from a holding potential of −50 mV. (**b**) Superimposed current traces of I_{Ks} activated at test potentials of −40 to +50 mV, before and ~5 min after exposure to 30 μM phenylephrine. **a** and **b** were obtained from different myocytes. (**c**) *I–V* relationships for I_{Ks} tail currents recorded before and during exposure to phenylephrine, obtained from the records in **b**. (**d**) *I–V* relationships for mean values ($n=4$) of normalized I_{Ks} tail currents. Continuous curves through the data points show the least-squares fit of a Boltzmann equation.

typical example for the action of the general (nonisoform-specific) PKC inhibitor BIS-I (Toullec *et al*., 1991), which at submicromolar concentrations potently inhibits most PKC isoforms, including conventional (α, βI, βII and γ), novel (δ and ε) and atypical (ζ) isoforms (Martiny-Baron *et al*., 1993). In a total of six myocytes, phenylephrine (30 μM) potentiated the amplitude of I_{Ks} by 38.1±12.1% in the presence of BIS-I (100 nM), which is significantly smaller than the degree of I_{Ks} potentiation under control conditions (97.1±11.9% increase, $n=16$; $P<0.05$; Figure 4). A similar degree of I_{Ks} potentiation by phenylephrine (30 μM) was observed in atrial myocytes pretreated for 5–10 min with higher concentration (1 μM) of BIS-I (40.3±6.1% increase, $n=5$; Figure 4). These results support an involvement of PKC activation in the potentiation of I_{Ks} via α_1-adrenoceptors in guinea-pig atrial myocytes. Bath application of BIS-I at concentrations of 100 nM and 1 μM alone reduced the amplitude of basal I_{Ks} by 10.1±3.2 ($n=6$) and 11.1±3.0% ($n=5$), respectively, which appears to be consistent with a recent report showing a modest (12%) reduction of I_{Ks} by exposure to BIS-I (1 μM) in guinea-pig ventricular myocytes (Missan *et al*., 2006).

Immunoblotting study with the use of isoform-specific antibody has detected the expression of at least five different PKC isoforms, namely α, βII, γ, ε and ζ in guinea-pig heart (Takeishi *et al*., 1999). To elucidate which isoform(s) of PKC is involved in the I_{Ks} response to phenylephrine, we used three isoform-selective pharmacological inhibitors of PKC, namely Gö-6976 (Martiny-Baron *et al*., 1993), Gö-6983 (Gschwendt *et al*., 1996) and rottlerin (Gschwendt *et al*., 1994). Figure 3b illustrates a representative example of the I_{Ks} response to

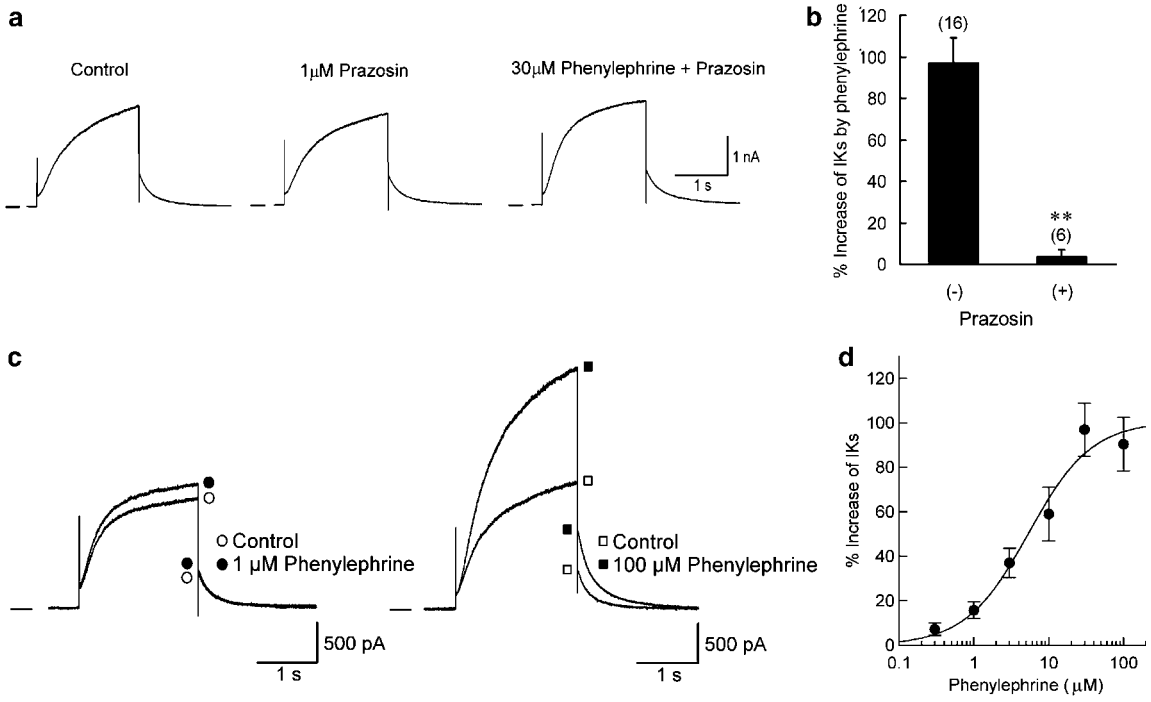

Figure 2 Potentiation of I_{Ks} by phenylephrine through α_1-adrenoceptor. (**a**) I_{Ks}, recorded in response to 2-s depolarization to $+30$ mV from a holding potential of -50 mV (the same voltage-clamp protocol as in Figure 1a), under control conditions (left panel), 5 min after exposure to 1 μM prazosin (middle) and 5 min after subsequent addition of 30 μM phenylephrine (right). (**b**) Percentage increase in I_{Ks} tail current, evoked by 30 μM phenylephrine in the absence and presence of 1 μM prazosin. $^{**}P<0.01$ compared with value in the absence of prazosin. (**c**) Superimposed current traces of I_{Ks} recorded using the same voltage-clamp protocol as in Figure 2a, before and $\sim$5 min after exposure to phenylephrine at concentration of 1 (left panel) or 100 μM (right). These records were obtained from different myocytes. (**d**) Concentration–response relationship for the potentiation of I_{Ks} by phenylephrine. Percentage increase in I_{Ks} tail current was measured for each concentration (0.3–100 μM) of phenylephrine. Each data point represents mean values$\pm$s.e.m. of 3–6 myocytes and is fitted with a Hill equation, yielding EC_{50} of 5.4 μM and n_H of 1.03. Only one concentration of phenylephrine was examined in a given myocyte to exclude possible complications of desensitization.

phenylephrine in the presence of the indolocarbazole Gö-6976, which largely inhibits Ca^{2+}-dependent (α, β and γ) but not Ca^{2+}-independent (δ, ε and ζ) isoforms when used at a concentration of 100 nM (Martiny-Baron *et al*., 1993). The potentiation of I_{Ks} was not significantly influenced by the addition of 100 nM Gö-6976 (78.4$\pm$9.0% increase, $n=6$; Figure 4) when compared with control, suggesting that conventional PKC isoforms (α, β and γ) were little, if at all, involved in the response. Similarly, the stimulatory action of phenylephrine was not significantly affected by the bisindolylmaleimide Gö-6983 at 100 nM (Figures 3c and 4; 79.8$\pm$11.6% increase, $n=6$), which at this concentration inhibits conventional (α, β and γ), novel (δ) and atypical (ζ) PKC isoforms (Gschwendt *et al*., 1996). In addition, I_{Ks} response to phenylephrine was practically insensitive to 10 μM rottlerin (Figures 3d and 4; 84.9$\pm$8.2% increase, $n=6$), which potently inhibits PKCδ but not PKCε at the concentration tested (Gschwendt *et al*., 1994). Based on these differences in the sensitivity of phenylephrine action to pharmacological inhibitors, PKCε isoform appears to be involved in mediating the stimulatory effect of phenylephrine on I_{Ks} in guinea-pig atrial myocytes.

We further tested the effects of the peptide translocation inhibitors of PKC isoforms βC2-4 (Ron *et al*., 1995) and εV1-2 (Johnson *et al*., 1996) on the stimulatory action of phenylephrine on I_{Ks}. It is generally accepted that upon activation, each PKC isoform translocates and binds to the specific anchoring proteins referred to as RACKs (receptors for activated C kinases) within a cell (Mochly-Rosen and Gordon, 1998). The interaction between conventional PKC isoforms and their RACKs is mediated in part by the C2 domain, whereas the binding of the C2-less novel PKC isoforms to their RACKs is via the V1 domain (Mochly-Rosen and Gordon, 1998). The peptide fragment βC2-4, which comprises nine amino acids (SLNPEWNET) derived from the C2 region of PKCβ(218–226), specifically inhibits the translocation of C2-containing conventional PKC isoforms (α, β and γ) but not that of C2-lacking novel PKC isoforms (δ and ε; Ron *et al*., 1995). On the other hand, the peptide εV1-2 is composed of eight amino acids (EAVSLKPT) that are derived from the V1 region of PKCε and specifically prevents translocation of PKCε to its RACKs (Johnson *et al*., 1996). Previous patch-clamp studies have demonstrated that intracellular application of these peptide inhibitors at a concentration of 100 nM is effective at significantly reducing the action of PKC isoform(s) on ion channels in mammalian cardiac myocytes (Zhang *et al*., 1997; Hool, 2000; Xiao *et al*., 2001). After allowing $\sim$10 min for the pipette solution containing βC2-4 or εV1-2 to diffuse into the cell inside, the effect of phenylephrine on I_{Ks} was tested. As demonstrated in Figure 3e and f, the stimulatory action of phenylephrine was not appreciably influenced by 100 nM

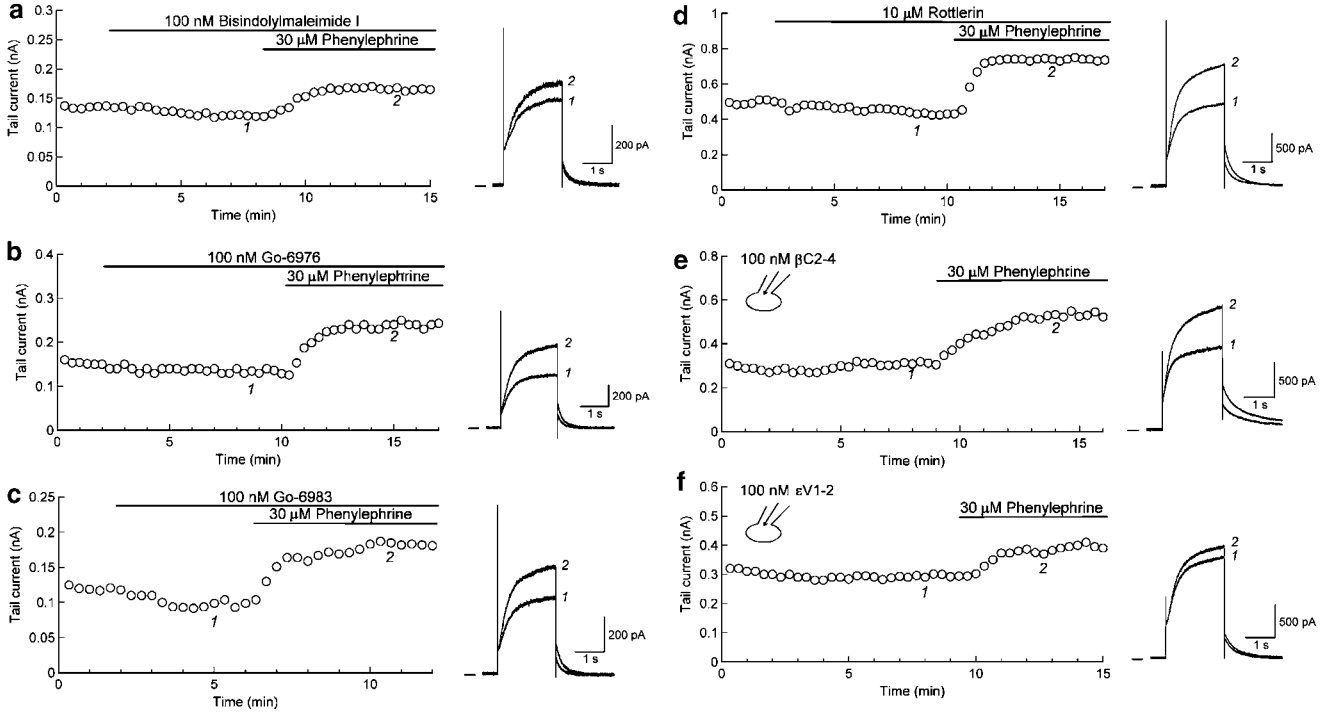

Figure 3 Effect of PKC isoform inhibition on the stimulatory action of phenylephrine on I_{Ks}. (**a**, **b**, **c** and **d**) Atrial myocytes were initially exposed to 100 nM BIS-I (**a**), 100 nM Gö-6976 (**b**), 100 nM Gö-6983 (**c**) or 10 μM rottlerin (**d**) for 5–10 min and then to 30 μM phenylephrine, as indicated by the horizontal bars. (**e** and **f**) Atrial myocytes were dialyzed with a pipette solution containing 100 nM βC2-4 (**e**) or 100 nM εV1-2 (**f**) for approximately 10 min before exposure to 30 μM phenylephrine. I_{Ks} was repetitively (every 20 s) activated by depolarizing steps to +30 mV from a holding potential of −50 mV, and amplitude of the tail current, measured upon return to the holding potential was plotted.

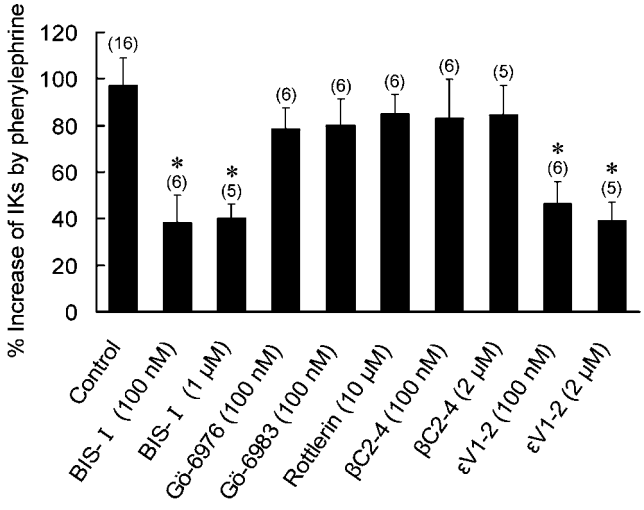

Figure 4 Summary bar graph showing the percentage increase in the amplitude of I_{Ks} tail current by phenylephrine in control and in the presence of pharmacological and peptide inhibitors of PKC isoforms. Effect of each inhibitor was compared with control (*$P<0.05$ vs control).

βC2-4 (83.0±16.6% increase, $n=6$; Figure 4), but was significantly reduced by 100 nM εV1-2 (46.4±9.2% increase, $n=6$; Figure 4). Similarly, I_{Ks} response to phenylephrine was significantly reduced by εV1-2 (39.2±7.7% increase, $n=5$) but not by βC2-4 (84.5±12.5% increase, $n=5$), when applied at higher concentration (2 μM, Figure 4). These experiments with PKC isoform-selective peptide inhibitors again support a significant role for PKCε in mediating the potentiation of I_{Ks} by phenylephrine.

PKCε also mediates the potentiation of I_{Ks} *by phorbol ester*

In the next series of experiments, we explored which isoform(s) of PKC was primarily involved in the I_{Ks} response to PMA, by examining the effects of βC2-4 and εV1-2 added to a pipette solution. Figure 5a illustrates a representative time course for the potentiation of I_{Ks} by bath application of PMA (100 nM) under control conditions. A peak response was typically attained ~5–10 min after starting the application of PMA and was sustained for at least 5 min even after washout of the compound. In a total of six myocytes, bath application of 100 nM PMA increased I_{Ks} by 64.2±1.3% (Figure 5g), when evaluated by the amplitude of tail current at a test potential of +30 mV. This stimulatory action of PMA (100 nM) was almost totally blocked by pretreatment with 100 nM BIS-I (2.7±1.3% increase, $n=6$; Figure 5b and g), supporting the view that activation of PKC mediates potentiation of I_{Ks} by PMA. Figure 5c demonstrates superimposed current traces of I_{Ks} recorded during 2-s depolarizing steps to potentials between −40 and +50 mV, before and after ~10 min exposure to 100 nM PMA. As illustrated in Figure 5d, PMA increased the amplitude of I_{Ks} tail currents at all potentials tested. The voltage dependence of I_{Ks} activation was little affected by exposure to PMA (control, $V_{1/2}=5.0\pm2.2$ mV, $k=11.9\pm1.4$ mV; PMA, $V_{1/2}=5.9\pm1.8$ mV, $k=12.5\pm1.5$ mV, $n=4$).

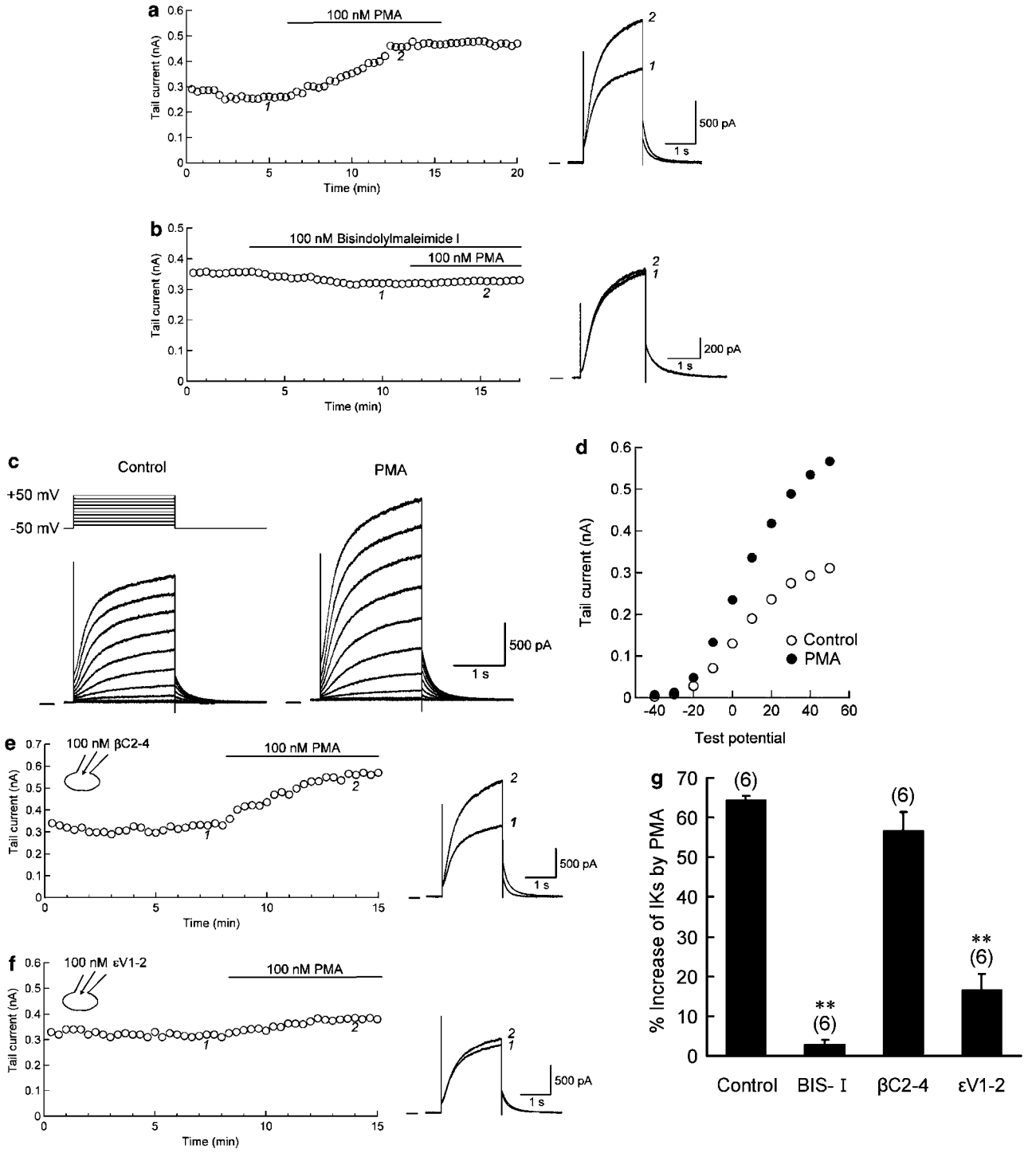

Figure 5 PKCε is primarily involved in the stimulatory action of PMA on I_{Ks}. (**a** and **b**) Time course of changes in the amplitude of I_{Ks} tail current during exposure to 100 nM PMA without (**a**) or with (**b**) pretreatment with 100 nM BIS-I. I_{Ks} was repetitively (every 20 s) activated by 2-s depolarizing steps to +30 mV. (**c**) Superimposed current traces of I_{Ks} activated at test potentials of −40 to +50 mV, before and ~10 min after exposure to 100 nM PMA. (**d**) *I–V* relationships for I_{Ks} tail currents, obtained from the experiments shown in (**c**). (**e** and **f**) Time courses in the changes in I_{Ks} tail currents recorded from an atrial myocyte dialyzed with a pipette solution containing 100 nM βC2-4 (**e**) or 100 nM εV1-2 (**f**). (**g**) Summary bar graph showing the percentage increase in the amplitude of I_{Ks} tail currents by PMA, in control and in the presence of BIS-I (100 nM, bath), βC2-4 (100 nM, pipette) or εV1-2 (100 nM, pipette). Effect of each inhibitor was compared with control (**$P<0.01$ vs control).

Figure 5e and f, respectively, shows a typical example of I_{Ks} response to 100 nM PMA in an atrial myocyte dialyzed with a pipette solution containing βC2-4 (100 nM) and εV1-2 (100 nM). The stimulatory effect of PMA was minimally affected by βC2-4 (56.7±4.7% increase, $n=6$) but was significantly attenuated by εV1-2 (16.5±4.0% increase, $n=6$), compared with the control value (64.2±1.3% increase, $n=6$; Figure 5g). These results indicate that PKCε is

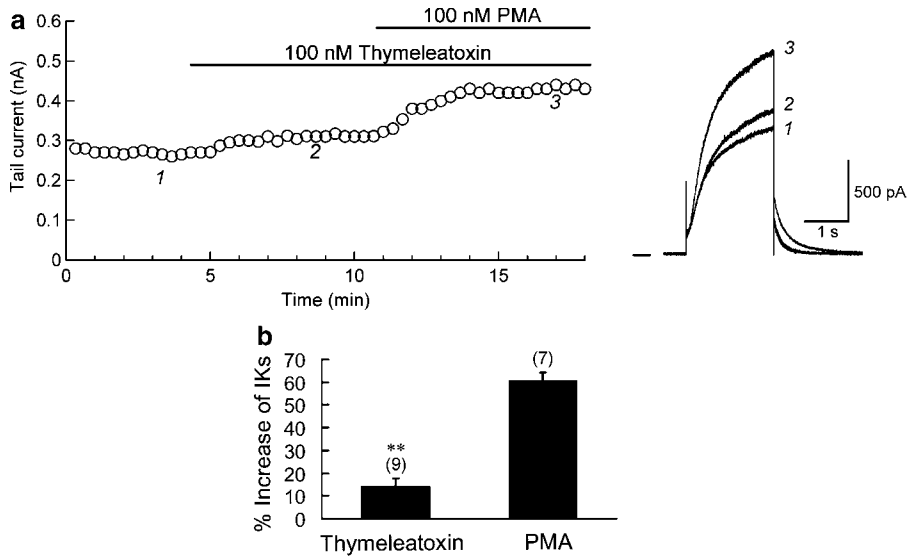

Figure 6 Effects of thymeleatoxin and PMA on I_{Ks}. (**a**) Time course of changes in the amplitude of I_{Ks} during exposure to PMA in the presence of thymeleatoxin. (**b**) Summary bar graph showing the percentage increase in the amplitude of I_{Ks} tail currents, evoked by thymeleatoxin (100 nM) alone, and PMA (100 nM) in the presence of thymeleatoxin. The values are calculated with reference to the baseline amplitude of I_{Ks} tail current. $^{**}P<0.01$ compared between these two groups.

also predominantly involved in the potentiation of I_{Ks} by PMA-induced PKC activation in guinea-pig atrial myocytes.

The experimental results obtained using pharmacological and peptide inhibitors for PKC isoforms indicate that activation of conventional PKCs (α, βI, βII and γ) is not primarily involved in the potentiation of I_{Ks} by the α_1-adrenergic agonist phenylephrine and the phorbol ester PMA in guinea-pig atrial myocytes. To substantiate this view, we examined the response of I_{Ks} to thymeleatoxin, a phorbol derivative which predominantly activates conventional PKC isoforms (Ryves *et al*., 1991). Bath application of 100 nM thymeleatoxin only slightly increased the amplitude of I_{Ks} by $14.1\pm3.4\%$ ($n=9$; Figure 6a and b), which was usually attained within 5–10 min after starting the application. However, subsequent addition of 100 nM PMA in the continued presence of 100 nM thymeleatoxin caused an additional significant increase in I_{Ks} by $60.8\pm3.6\%$ ($n=7$) of the baseline amplitude (Figure 6b). This finding again supports a predominant role of novel PKC (PKCε) over conventional PKCs in the potentiation of I_{Ks} in guinea-pig atrial myocytes.

Discussion

The present study demonstrates that I_{Ks} is markedly (approximately twofold) potentiated by phenylephrine (30 μM) through α_1-adrenoceptors in guinea-pig atrial myocytes (Figure 2). The stimulatory action of phenylephrine was significantly, if not completely, reduced by the general PKC inhibitor BIS-I (Figures 3a and 4), which strongly suggests that PKC activation plays an important role in mediating the α_1-adrenoceptor-induced I_{Ks} potentiation. Western-blot analysis using an antibody specific to PKC isoform has suggested that α_1-adrenoceptor stimulation with phenylephrine causes translocation and thereby activation of both conventional and novel isoforms of PKC in native cardiac myocytes (Ruf *et al*., 2002). On the other hand, in the present study we found that the stimulatory action of phenylephrine on I_{Ks} was significantly reduced by the PKCε-selective inhibitory peptide (εV1-2) but was little affected by the peptide inhibitor (βC2-4) for all conventional PKC isoforms (α, β and γ), which favours a preferential role for the novel isoform PKCε (Figures 3e, f and 4). This view is also supported by the experiments showing that the stimulatory action of phenylephrine was minimally affected by the indolocarbazole Gö-6976 (Figures 3b and 4), which is believed to have the highest selectivity for conventional PKC isoforms among the pharmacological inhibitors currently available (Martiny-Baron *et al*., 1993). The present investigation also confirms a substantial enhancement of I_{Ks} by phorbol ester PMA (Figures 5 and 6), which directly activates both conventional and novel isoforms of PKC, independent of receptor stimulation. This PMA action on I_{Ks} was also significantly reduced by εV1-2 but not by βC2-4 (Figure 5), again supporting a functional role of PKCε in the regulation of cardiac I_{Ks}.

Voltage-clamp study using the *Xenopus* oocyte expression system has clearly demonstrated that both PKCβII and PKCε, but not PKCα, PKCβI, PKCδ and PKCη, are involved in the PMA-induced potentiation of heteromeric KCNQ1/KCNE1 channels (Xiao *et al*., 2003), molecular constituents of human I_{Ks} (Barhanin *et al*., 1996; Sanguinetti *et al*., 1996). On the other hand, it was observed in guinea-pig ventricular myocytes that the delayed rectifier K^+ current, I_K (which seems to largely comprise I_{Ks}), is increased by exogenous application of the type III (α) PKC purified from bovine whole brain (Tohse *et al*., 1990), which resembles the structure of PKCα (Kikkawa *et al*., 1987). These observations appear to be apparently distinct from the present results, concerning the functional role of PKCα or PKCβII in the regulation of I_{Ks}. Individual PKC isoforms (α, βI, βII, δ,

ε and ζ) in native cardiac myocytes have a differential localization before and after stimulation by norepinephrine or PMA (Disatnik *et al*., 1994). Whereas PKCα and PKCβII translocate from the cytosolic region to the perinuclear region and/or cell periphery, PKCε, upon stimulation, typically translocates to cross-striated regions in ventricular myocytes, which places it near the transverse tubules (t-tubules). Evidence has been presented to indicate that minK (KCNE1) proteins, important function-modifying ancillary β-subunit of the I_{Ks} channel, are preferentially localized in the t-tubules in native ventricular myocytes (Furukawa *et al*., 2001). It is probable that the colocalization of activated PKCε with I_{Ks} channel proteins underlies the selective potentiation of I_{Ks} by PKCε in native cardiac myocytes. Further work is required to examine the localization of PKCε and I_{Ks} channel subunits in atrial myocytes, which lack an extensive t-tubule network.

In the present study, BIS-I at 100 nM almost totally abolishes the potentiation of I_{Ks} by PMA (Figure 5b and g) but partially reduces the stimulatory action of phenylephrine on I_{Ks} even when applied at 1 μM (Figure 4). We have previously demonstrated that I_{Ks} in guinea-pig atrial myocytes is increased by intracellular application of anti-phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) antibody but is decreased by intracellular addition of exogenous PtdIns(4,5)P_2 (Ding *et al*., 2004). These observations suggest that endogenous membrane PtdIns(4,5)P_2 exerts an inhibitory action on I_{Ks}. We further showed that stimulatory action of extracellular ATP on I_{Ks} is due, at least partly, to the reduction of membrane PtdIns(4,5)P_2 which is likely to occur following the stimulation of P2Y receptors coupled to a Gq-phospholipase C (PLC) signaling pathway (Ding *et al*., 2004). It is therefore possible that, in addition to PKC activation, the stimulation of Gq-PLC-coupled α_1-adrenoceptors causes other signaling events such as depletion of membrane PtdIns(4,5)P_2, to potentiate I_{Ks} in atrial myocytes. It is of interest to evaluate the relative contribution of PKC activation and membrane PtdIns(4,5)P_2 depletion to the potentiation of I_{Ks} observed during the stimulation of various kinds of Gq-PLC-coupled receptors expressed in the heart.

The maximal response of I_{Ks} to phenylephrine in atrial myocytes (97.1±11.9% increase) appears to be considerably larger than that in ventricular myocytes of same species (29.9±9.1% increase; Tohse *et al*., 1992). On the other hand, it has been demonstrated in guinea-pig ventricular myocytes that the amplitude of I_{Ks} is potentiated by ~45–60% by bath application of phorbol esters PMA (also referred to as 12-*O*-tetradecanoylphorbol 13-acetate, TPA) and phorbol 12,13-dibutyrate (PDBu) (Walsh and Kass, 1988; Tohse *et al*., 1990; Yazawa and Kameyama, 1990). It is thus likely that I_{Ks} can be enhanced by an almost similar degree in atrial and ventricular myocytes of guinea-pigs when PKC is directly activated by phorbol esters. As judged from these observations, it is likely that, following the stimulation of α_1-adrenoceptors, PKC may be more effectively activated to potentiate I_{Ks} in atrial myocytes. Further studies should be conducted to determine the relative efficiency of α_1-adrenoceptor stimulation in potentiating I_{Ks} in atrial and ventricular myocytes under the same experimental conditions, including methods to isolate I_{Ks} from total I_K.

Previous investigators have reported that PKC activation causes a species-specific effect on cardiac I_{Ks}; PKC stimulation increases I_{Ks} in guinea-pigs but decreases the current amplitude in most of the other mammalian species (Varnum *et al*., 1993; Robinson *et al*., 2000). This difference has been accounted for, at least partly, by the sequence variation of minK (KCNE1) protein (Varnum *et al*., 1993); a PKC phosphorylation site (Ser-102), which is responsible for current reduction, is absent in guinea-pigs but is present in other mammalian species including humans. In recent years, however, Kathöfer *et al*. (2003) have demonstrated that PKC activation increases membrane current through the human KCNQ1/KCNE1 channel. Furthermore, these authors detected, using site-directed mutagenesis experiments, the PKC phosphorylation sites (Ser-409, Ser-464, Thr-513 and Ser-577) in a pore-forming α-subunit, KCNQ1 protein, which contributes to PKC-dependent increase in KCNQ1/KCNE1 current. Future studies are required to examine whether the PKCε-mediated I_{Ks} potentiation is present and functions in native cardiac myocytes of various mammalian species including humans.

A number of experimental and/or clinical studies have strongly suggested that, whereas increased expression of PKCβI and/or PKCβII is implicated in the development of cardiomyopathy (Wakasaki *et al*., 1997) and heart failure (Bowling *et al*., 1999), the activation and translocation of PKCε confers cardioprotection that is associated with ischemic preconditioning (Gray *et al*., 1997; Qiu *et al*., 1998; Liu *et al*., 1999). Furthermore, a recent study has found that intracoronary injection of a selective PKCε-activating peptide, ψεRACK, not only reduces infarct size but also suppresses the occurrence of ventricular tachyarrhythmias caused by ischemia–reperfusion in porcine hearts (Inagaki *et al*., 2005). In atrial and ventricular myocardium, I_{Ks} activation plays a major role in determining the action potential duration that controls the amount of Ca^{2+} influx through simultaneously activated $I_{Ca,L}$ (Pennefather and Cohen, 1990). It seems, therefore reasonable to speculate that activation of PKCε and resultant stimulation of I_{Ks} can reduce the amount of Ca^{2+} influx during action potentials and thereby spare energy required for intracellular Ca^{2+} handling, which can be beneficial to the cardiomyocytes, especially in condition where the oxygen supply to cardiac muscle becomes inadequate (hypoxia or ischemia). It will be interesting to elucidate whether PKCε-mediated potentiation of I_{Ks} described here in atrial myocytes can be applicable to the I_{Ks} regulation in cardiac ventricular myocytes of mammalian species including humans and constitutes, at least in part, the cardioprotective action of PKCε during ischemic preconditioning and/or ischemia–reperfusion.

In conclusion, our data indicate that the ε isoform of PKC (PKCε) is predominantly involved in the stimulatory action of PKC on I_{Ks} and contributes to α_1-adrenergic potentiation of I_{Ks} in native guinea-pig atrial myocytes.

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Conflict of interest

The authors state no conflict of interest.

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