Modulation of hERG potassium currents in HEK-293 cells by protein kinase C. Evidence for direct phosphorylation of pore forming subunits


Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, UK

The human *ether-a-go-go* related gene (hERG) potassium channel is expressed in a variety of tissues including the heart, neurons and some cancer cells. hERG channels are modulated by several intracellular signalling pathways and these provide important mechanisms for regulating cellular excitability. In this study, we investigated muscarinic modulation of hERG currents and direct phosphorylation of channel subunits expressed in HEK-293 cells at physiologically relevant temperatures by protein kinase C (PKC). Activation of G\(_{\alpha q/11}\)-coupled M\(_3\)-muscarinic receptors with methacholine, reduced current amplitudes at all potentials with minor effects on the voltage dependence of activation and inactivation. The response to methacholine was insensitive to intracellular BAPTA, but was attenuated by either acute inhibition of PKC with 300 nM bisindolylmaleimide-1 (bis-1) or chronic down-regulation of PKC isoforms by 24 h pretreatment of cells with phorbol 12-myristate 13-acetate (PMA). Stimulation of PKC with 1-oleoyl 2-acetylglycerol (OAG), an analogue of diacylglycerol (DAG), mimicked the actions of muscarinic receptor stimulation. Direct phosphorylation of hERG was measured by \(^{[32P]}\)orthophosphate labelling of immunoprecipitated protein with an anti-hERG antibody. Basal phosphorylation was high in unstimulated cells and further increased by OAG. The OAG dependent increase was abolished by bis-1 and down-regulation of PKC, but basal levels of phosphorylation were unchanged. Deletion of the amino-terminus of hERG prevented both the modulation of channel activity and the increase of phosphorylation by OAG. Our results are consistent with calcium and/or DAG sensitive isotypes of PKC modulating hERG currents through a mechanism that involves direct phosphorylation of sites on the amino terminus of hERG.

(The resubmitted 23 October 2006; accepted after revision 13 March 2007; first published online 15 March 2007)

Corresponding author J. S. Mitcheson: University of Leicester, Department of Cell Physiology and Pharmacology, Maurice Shock Medical Sciences Building, University Road, Leicester LE1 9HN, UK. Email: jm109@leicester.ac.uk

The *ether-a-go-go* related gene (ERG) channel belongs to the *ether-a-go-go* (EAG) family of voltage gated potassium channels (Sanguinetti et al. 1995; Trudeau et al. 1995). In mammals, the ERG subfamily comprises three genes, *erg1*, *erg2* and *erg3*. The human ERG1 channel (hERG or Kv11.1) mediates the rapidly activated delayed rectifier K\(^+\) current (\(I_{Kr}\)), which makes a vital contribution to repolarization of the cardiac action potential. Dysfunction of hERG, either because of inherited mutations of the channel or block by any of a variety of medications, causes long QT syndrome, which is characterized by abnormalities of repolarization that predispose to cardiac arrhythmias and an increased risk of sudden death (Keating & Sanguinetti, 2001; Sanguinetti & Mitcheson, 2005). The hERG channel is also expressed in a wide variety of non-cardiac tissues. Furthermore, there is mounting evidence that hERG channels are expressed in various types of human cancers (Arcangeli, 2005) and may regulate several aspects of cancer cell biology (Wang, 2004; Arcangeli, 2005). How hERG channels mediate these effects remains largely unknown.

ERG channels are also expressed in neuronal tissue (Papa et al. 2003; Guasti et al. 2005) and may contribute to the maintenance of the resting membrane potential and cellular excitability. Pharmacological inhibition of ERG currents in neuroblastoma cells abolishes spike frequency adaptation during long lasting depolarizations (Chiesa et al. 1997; Selyanko et al. 1999) consistent with slow ERG current activation providing a progressively increasing repolarizing influence. In this regard, ERG currents may limit repetitive firing in a similar manner to M-currents. Indeed, ERG channels are thought to contribute to M-like currents in the brain (Meves et al. 1999; Selyanko et al. 1999) and thus neurotransmitter-mediated modulation
of ERG current amplitudes may be important for regulating neuronal excitability. In addition, there is considerable evidence that modulation of ERG channels by thyrotropin-releasing hormone (TRH) results in membrane depolarization that increases the rate of action potential firing and secretion of prolactin (reviewed in Schwarz & Bauer, 2004). Thus ERG channels are expressed in a variety of tissues and receptor-mediated modulation of activity is vital to their physiological function.

There have been several studies on TRH receptor modulation of ERG (Barros et al. 1998; Schwarz & Bauer, 1999; Schledermann et al. 2001; Storey et al. 2002; Bauer et al. 2003; Gomez-Varela et al. 2003b), but relatively little is known about modulation of ERG channels by other G-protein coupled receptors (Selyanko et al. 1999; Kagan et al. 2002; Hirdes et al. 2004; Thomas et al. 2004). Receptor stimulation tends to cause a reduction in maximal current amplitude, a positive shift of activation and acceleration of deactivation, with little or no effect on inactivation. However, there are divergent reports on the underlying signalling mechanisms and the importance of channel phosphorylation. TRH receptor and M1 muscarinic receptor mediated current inhibition has been reported to be largely insensitive to either kinase inhibitors or cell dialysis with non-hydrolysable analogues of ATP (Schledermann et al. 2001; Storey et al. 2002; Hirdes et al. 2004), suggesting phosphorylation is not required. On the other hand, IKr and hERG current modulation by α1A and β adrenoceptor (AR) stimulation is blocked by inhibitors of protein kinases (Heath & Terrar, 2000; Karle et al. 2002; Thomas et al. 2004). Elevating cAMP to directly activate protein kinase A (PKA) causes a positive shift of activation that is removed when four consensus PKA phosphorylation sites on hERG are mutated (Thomas et al. 1999; Cui et al. 2000). Thus, PKA stimulation alters channel function by a mechanism that requires direct phosphorylation of hERG subunits. The situation with protein kinase C (PKC) dependent modulation is less straightforward. Modulation by phorbol ester activation of PKC remains when 17 of 18 consensus PKC sites on hERG are mutated (Thomas et al. 2003). Although this may indicate that PKC dependent modulation is indirect, perhaps involving PKC phosphorylation of an auxiliary channel subunit or signalling molecule (Thomas et al. 2003), mutation of the 18th consensus PKC site (Thr74) produces a non-functional channel – highlighting the importance of this residue and leaving the distinct possibility of direct PKC-mediated phosphorylation at this site.

In the present study, we investigated the modulation of hERG channels by M1 muscarinic receptor stimulation, elevation of the intracellular \([\text{Ca}^{2+}]\) ([\text{Ca}^{2+}]_i), and analogues of diacylglycerol that directly activate PKC. In all cases hERG currents were reduced in a PKC-dependent manner. Direct measurements of subunit phosphorylation indicate that basal phosphorylation is high and is further increased by PKC stimulation. Our results are consistent with receptor-mediated modulation of channel activity by direct PKC phosphorylation of a site on the amino-terminus of hERG.

**Methods**

**Cell culture and transfection**

HEK-293 cells stably expressing hERG (hERG-HEK cells) were a kind gift from Dr Craig January (University of Wisconsin) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax-1, sodium pyruvate, glucose and pyridoxine, supplemented with 10% fetal bovine serum, 400 μg ml\(^{-1}\) geneticin and 50 μg ml\(^{-1}\) gentamycin. Muscarinic receptor modulation of hERG was investigated by transiently expressing wild-type (WT) hERG in HEK-293 cells stably expressing M1 muscarinic receptors (HEK-M3 cells; Tovey & Willars, 2004). hERG mutants (see later) were investigated by transient expression in WT HEK-293 cells. For all transient transfections, 2–5 μg of hERG cDNA was cotransfected with 0.2–0.5 μg EGFP (pEGFP-N1, Clontech laboratories, UK) using Lipofectamine 2000 (Invitrogen, UK), following the manufacturer’s instructions. Transfection media were replaced with culture media 6 h after transfection and experiments performed 1–2 days later. Cells were maintained in MEM Alpha Medium without nucleosides (Gibco, UK), supplemented with 10% fetal bovine serum and 100 units ml\(^{-1}\) penicillin and streptomycin (Gibco, UK).

**Cloning and mutagenesis of hERG channels**

The WT hERG expression construct in pCDNA3.0 and the NTK-hERG expression construct (N-terminus between amino acids 2–354 deleted) in pSP64 were kindly provided by Dr M. Sanguinetti (University of Utah). 4M-hERG, ΔPKC-hERG and 18M-hERG constructs in pSP64 were a generous gift from Dr D. Thomas (University of Heidelberg). ΔPKC-hERG lacks 17 of 18 PKC consensus phosphorylation sites, whereas 18M-hERG lacks all 18 (Thomas et al. 2003). 4M-hERG has all four consensus PKA phosphorylation sites mutated. 4M-hERG, ΔPKC-hERG and 18M-hERG were subcloned into pCDNA3.0 for expression in HEK-293 cells. Thr74 mutations were generated from WT hERG using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. All mutations and subcloning reactions were confirmed by DNA sequencing.

**Electrophysiology**

Whole cell voltage clamp recordings of hERG currents were made using an Axopatch 200B amplifier and Clampex

© 2007 The Authors. Journal compilation © 2007 The Physiological Society
the recording chamber within 60 s and recordings were
switching system was used to fully exchange solutions in
required concentrations in extracellular solution.
of compounds on hERG current amplitudes was to
fitted with a Boltzmann function to obtain half-maximal
peak tail currents upon repolarization to test potentials between
activation was investigated with 5 s depolarizations to
5 mV. Tail currents were evoked with a conventional three-pulse protocol. Cells were
depolarized to 0 mV. The protocol was repeated
54 mV, applied in 10 mV increments. Peak tail currents upon repolarization to
Normalizing to −50 mV were normalized to maximal amplitudes in
control solution, plotted against test pulse potential and fitted with a Boltzmann function to obtain half-maximal activation
2007 The Authors. Journal compilation C

−
50 mV . The protocol was repeated
+50 mV, applied in 10 mV increments. Peak tail currents upon repolarization to
Normalized against the maximal current in each condition. Data
potentials between −140 and +20 mV applied. Current through non-inactivated channels was measured at the
beginning of a third pulse to +40 mV, and normalized against the maximal current in each condition. Data
were plotted against test pulse potential and fitted with a Boltzmann function to obtain half-maximal inactivation
(V_{0.5,act}) values and slope factors. Electrophysiological recordings were analysed using Clampsfit (Molecular Devices) and GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

Western blotting of PKC isoforms

hERG-HEK cells were grown to ∼70% confluency in
6-well plates. Down-regulation of PKC isoforms by chronic application of phorbol esters was investigated by incubating cells in either culture medium containing 0.1% DMSO (control) or medium containing 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h. In some experiments the inactive 4α-PMA analogue was substituted for PMA. Cells were washed once with
phosphate-buffered saline (PBS), then lysed for 10 min on ice in solubilization buffer containing (mm) Tris 10, EDTA 10, NaCl 500, 1% Nonidet P-40, 0.5% deoxycholate (pH 7.4), cleared by centrifugation and protein concentrations measured using the Lowry protein assay using bovine serum albumin as a standard (Lowry et al. 1951). Total protein was equal in cells from all wells, including PMA treated cells. For each experimental condition, lysate containing 360 μg total protein was loaded into a full width well of an 8% SDS-PAGE mini gel and proteins separated by electrophoresis, transferred to nitrocellulose and blocked overnight at 4°C in blocking solution containing 5% (w/v) skimmed powdered milk and 0.1% TWEEN-20 in 0.137 M Tris-buffered saline. Multi-channel blotting apparatus (Mini Protean Multi-Screen, Bio-Rad Laboratories, CA, USA), which divides the nitrocellulose blot into 10 channels (each with equal amounts of protein), was used to simultaneously probe for different PKC isotypes. This system allows detection of multiple PKC isotypes from the same lysate without having to cut the blot into strips. PKC isoform specific antibodies were applied for 1 h at room temperature following manufacturer’s instructions (BD Biosciences, NJ, USA; cat. no. 611421). Anti-mouse secondary antibody (Sigma, cat no. A4416), diluted to 1:1000, was applied for 1 h at room temperature.
Protein detection was carried out using ECL detection (Amersham Biosciences, UK). Control and PMA treated cell samples were prepared in pairs to allow relative levels of each PKC isotype to be compared. To further confirm equivalent protein loading, blots were stripped for 30 min at 50°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7), washed in 0.137 M Tris-buffered saline, blocked for 1 h in blocking solution and re-probed using β-actin antibody (Sigma, cat. no. A5441) for 1 h at room temperature.

hERG subunit phosphorylation

Channel phosphorylation in intact cultured cells was carried out using previously described methods (Budd et al. 1999). Briefly, hERG-HEK cells were plated at equal densities onto 6-well plates and grown to ∼90% confluency. Cells were washed once with phosphate-free Krebs buffer, containing (mm): NaCl 118, KCl 4.3, MgSO 4.7H 2O 1.17, CaCl 2 .2H 2O 1.3, NaHCO 3 0.34, glucose 11.7, Heps 10 (pH 7.4). Then 5 μCi [32P]orthophosphate (Amersham Biosciences, UK), was added to each well, and the cells incubated for 1 h at 37°C. To stimulate PKC, cells were incubated for 5 min with 10 μM 1-oleoyl 2-acetylglycerol (OAG). In some experiments, PKC was inhibited by incubating cells in bisindolylmaleimide-I (bis-1) for 15 min prior to OAG stimulation. Bis-1 was at 3 μM used for most experiments, to obtain a rapid and complete PKC inhibition, but
we subsequently confirmed in further experiments that 300 nM bis-1 was also sufficient. Alternatively, certain PKC isotypes were down-regulated by culturing cells in medium contained 1 μM PMA for 24 h prior to OAG stimulation. Reactions were terminated by aspiration and addition of 1 ml solubilization buffer. Lysates were cleared by centrifugation and solubilized proteins incubated on ice with 5 μg anti-hERG serum for 90 min. The anti-hERG antibody serum was raised in rabbit against the sequence TCNPLSGAFSGVSNIF (C-terminus hERG residues 983–998) by Pepceuticals, Ltd (Leicester, UK). A previously well characterized pan-hERG1 antibody (Roti et al. 2002), kindly provided by Dr Gail Robertson (University of Wisconsin), was used for immunoprecipitation of transiently expressed WT and NTK-hERG channels. Isolation of immunocomplexes was carried out using protein A- Sepharose beads (Amersham Biosciences, UK). Samples were washed with TE buffer containing (mm): Tris-HCl 10, EDTA 10, β-glycerol phosphate 20, pH 7.4. Proteins were then resolved on 8% SDS-PAGE gels and stained with Coomassie blue (0.2 % w/v) stain to check for equal antibody loading (and therefore equivalent immunoprecipitation between lanes). Gels were dried and subjected to autoradiography at −80°C for 16–24 h. Phosphorylation was quantified by densitometric measurements of autoradiograph bands using the Alpha Imager 3400 system (Alpha Innotech Corporation, San Leandro, CA, USA). Background was subtracted and values in treated samples were normalized to basal levels in untreated cells from the same experiment.

hERG subunit biotinylation

These experiments were carried out to enable visualization of both the amount of hERG protein and the extent of hERG protein phosphorylation in the same immunoprecipitate. Cells were washed twice with PBS before incubating for 30 min at 37°C in PBS containing 0.5 mg ml−1 sulfo-NHS-LC-biotin (Pierce Biotechnology, Inc., Rockford, IL, USA). Cells were washed and incubated in 10 μCi [32P]orthophosphate per well and the phosphorylation assay performed as described above with the exception that lysates were also precleared by incubation on ice with 5 μg rabbit serum (from rabbits in which antibodies have been raised to unrelated protein; mouse muscarinic M3 receptor) and proteins were resolved on a 6% rather than 8% SDS-PAGE gel before transferring to nitrocellulose. The nitrocellulose was blocked overnight, washed and incubated for 30 min at room temperature in 0.5 μg ml−1 streptavidin conjugated with horseradish peroxidase (Pierce) in 0.3 M TBS with 0.1% Tween-20. Excess streptavidin was washed off before protein detection with ECL+. To measure phosphorylation levels of hERG, the nitrocellulose was stripped as described for PKC Western blots, and subjected to autoradiography at −80°C for 48 h.

Phospho-peptide separation by 2D electrophoresis

Isolation of radiolabelled hERG was carried out as described above, with the exception that the amount of [32P] orthophosphate was increased to 200 μCi per well. Following separation on SDS gel, and electroblotting onto nitrocellulose, hERG protein was visualized by autoradiography. The appropriate area of the membrane containing hERG protein was excised and blocked for 30 min at 37°C with 0.5 % polyvinylpyrrolidone-K 30 (Aldrich) solution containing 0.6 % acetic acid, followed by three washes in water and one wash in ammonium bicarbonate solution (50 mM NH4HCO3 and 0.5 mM CaCl2). Control samples from WT HEK cells not expressing hERG were run alongside samples from cells stably expressing hERG and the equivalent area of membrane excised and handled as described above. The samples were then digested with 10 μg ml−1 trypsin in ammonium bicarbonate solution at 30°C for 23 h, and following speed-vac drying and resolubilization in electrophoresis pH 1.9 buffer (formic acid (88 %)/acetic acid/water, 25 : 78 : 897 v/v), the cleaved peptides were subjected to two dimensional phospho-peptide separation (Boyle et al. 1991) on a cellulose-coated thin layer chromatography plate (Merck, Darmstadt, Germany, 20 x 20 cm). Phospho-peptides were resolved in the first dimension by electrophoresis in pH 1.9 buffer at 2000 V for 30 min using a Hunter HTLE-7002 System (C.B.S. Scientific Company Inc., Del Mar, USA), followed by overnight ascending chromatography in isobutyric acid buffer (isobutyric acid/n-butanol/pyridine/acetic acid/water, 1250 : 38 : 96 : 58 : 558 v/v) in the second dimension. The phospho-peptides were visualized with a Storm phosphorimager after an exposure period of 10 or more days.

Measurement of [Ca2+]i

[Ca2+]i was monitored as previously described (Rodrigo et al. 2002). Cells were loaded with 2 μM fura-2-AM (Molecular Probes) for 20 min at room temperature. Cells were alternately excited at 340 and 380 nm with light from a monochromator and emitted light collected at > 520 nm at 2 s intervals from 8 to 16 cells simultaneously using a video imaging system (Photon Technology International, Birmingham, NJ, USA). Data acquisition was performed using PTI Imagemaster software and results expressed as 340/380 ratio of fura-2 emission intensities. Measurements were taken at 35–37°C.

Reagents

Cell culture reagents were purchased from Gibco except for geneticin and gentamycin, which were from Sigma.
Ionomycin, methacholine, BAPTA and OAG were all purchased from Sigma. PMA, 4α-PMA, bis-1 and Gö6976 were all purchased from Calbiochem.

**Statistical analysis**

Data values in text and figures represent means ± S.E.M. Error bars are not plotted in figures when smaller than symbols. To test for the significance of reagent effects against untreated cells, Student’s t test for paired data was used for biophysical parameters and for unpaired data for other simple comparisons. A one-way ANOVA with post hoc Dunnett’s test was used for multiple comparisons. P values less than 0.05 were considered significant.

**Results**

The main focus of this study was the modulation of hERG by PKC. We first examined the hERG current response to activation of G_{αq/11}-coupled M_{3}-muscarinic receptors stably expressed in HEK-293 cells. Activation of M_{3}-muscarinic receptors stimulates phospholipase C, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-phosphate and 1,2-diacylglycerol (DAG). Inositol 1,4,5-phosphate rapidly elicits Ca^{2+} release from the endoplasmic reticulum, whereas DAG initiates longer lasting and slow effects, including the activation of conventional and novel isotypes of PKC. Figure 1 shows the response of hERG currents in HEK-M_{3} cells to maximal muscarinic receptor

![Figure 1. Suppression of hERG currents by M_{3} muscarinic receptor stimulation](image-url)

hERG channels were transiently expressed in HEK-293 cells stably expressing M_{3} muscarinic receptors. A, representative currents elicited with 5 s depolarizations to the indicated test potentials before (control) and during application of a maximal concentration (1 mM) of the agonist methacholine (Mch). Tail currents were obtained with repolarization to −50 mV. B, time course of response to methacholine in cells dialysed for 10 min via the patch pipette with intracellular solutions containing 0 or 5 mM BAPTA. Cells were repetitively depolarized for 5 s to 0 mV and peak tail currents measured upon repolarization to −50 mV. Values were normalized to peak tail currents before methacholine application and mean values plotted as a function of time. C, activation curves before and during application of methacholine. The V_{0.5,ac} before and after muscarinic receptor stimulation was −17 ± 2 mV and −14 ± 2 mV, respectively (n = 5). Slope factors were 8 ± 2 mV and 9 ± 2 mV before and during methacholine application, respectively. D, muscarinic receptor stimulation had no significant effects on voltage-dependent inactivation. E, representative intracellular Ca^{2+} response to methacholine assessed by fura-2 imaging of a single cell. The time course of the Ca^{2+} response is notably different from the hERG current response in B and shows the characteristic initial transient rise in Ca^{2+} followed by the more slowly declining component of the response. F, comparison of time-course effects of ionomycin in cells dialysed for 10 min with 0 or 5 mM BAPTA. Values were normalized to peak tail currents before ionomycin application and mean values plotted as a function of time.
stimulation with 1 mM methacholine. hERG currents in the presence of methacholine were smaller at all test potentials (Fig. 1A). The decrease of current amplitude was relatively slow to develop and reached a new steady state level of 73 ± 2 % of control after 4 min (n = 8, P < 0.005; Fig. 1B). Deactivation of tail currents upon repolarization to −50 mV was significantly faster (P < 0.05, n = 8); fast and slow time constants (τf and τs) were 233 ± 6 ms and 1241 ± 45 ms, respectively, in control solution compared to 170 ± 23 ms and 1021 ± 34 ms in the presence of methacholine. Analysis of voltage-dependent properties revealed a small, but statistically significant (P < 0.05, n = 5), positive shift in the voltage dependence of activation of 5 ± 1 mV (Fig. 1C), but there was no significant shift in the voltage dependence of inactivation (Fig. 1D). Since hERG is susceptible to block by a wide variety of pharmacological agents, including several G-protein coupled receptor agonists and antagonists, we tested for direct block by methacholine. However, no inhibition of current in hERG-HEK cells (not over-expressing M3-muscarinic receptors) was observed (data not shown), indicating that methacholine acts via a receptor mediated pathway.

Stimulation of M3-muscarinic receptors not only activates PKC, it also raises [Ca2+], with the ionophore, ionomycin, also inhibited hERG current to 59 ± 4% of control (n = 7), with a similar time course to methacholine and with no significant changes in the voltage dependence of activation and inactivation. Thus, the effects of ionomycin and muscarinic M3-receptor stimulation were qualitatively similar. However, several lines of evidence indicate that modulation of hERG is not due to Ca2+ regulation of channel activity. (1) Monitoring of [Ca2+], with fura-2 showed that the time course of the Ca2+ response was markedly different from the hERG current response. Whereas hERG tail current amplitudes in the presence of methacholine decreased with a slow, mono-exponential transient increase that lasted less than 8 s and a second longer-lasting, but lower component (Fig. 1E). (2) The time course of the Ca2+ and current amplitude responses also did not correlate in ionomycin stimulated cells (data not shown). (3) hERG current modulation by methacholine was unaffected by buffering intracellular Ca2+ with the fast Ca2+ chelator BAPTA. Cells were dialysed via the patch pipette with intracellular solution containing 5 mM BAPTA for 10 min after the whole cell configuration was achieved. We have previously shown that 5 mM BAPTA is sufficient to buffer the rapid release of Ca2+ from intracellular stores by caffeine (Cockerill & Mitcheson, 2006). It was also sufficient to attenuate the current response to ionomycin (Fig. 1F). Nevertheless, BAPTA had no significant effect on the muscarinic modulation of hERG current (Fig. 1B). Together, these results suggest that the Ca2+ transient was not required for the muscarinic modulation of hERG current and, furthermore, that an increase in [Ca2+] does not directly regulate hERG channel activity.

Modulation of hERG by ionomycin and methacholine is dependent on PKC activity

The results described above are consistent with ionomycin activating conventional PKC (cPKC) isotypes and muscarinic receptor stimulation modulating hERG via either conventional or novel PKC isotypes. To investigate whether reduction of PKC activity alters the ability of muscarinic receptor stimulation to regulate hERG we first tested the PKC-selective inhibitor bis-1. High concentrations of bis-1 directly inhibited hERG; 1 μM bis-1 reduced hERG current by > 30%. Similar effects have been reported previously (Thomas et al. 2004). Therefore, we used a concentration of 300 nM bis-1, which did not significantly block hERG channels. Currents were elicited by repetitive pulsing to 0 mV, with peak tail currents recorded at −50 mV; 300 nM bis-1 was applied extracellularly for 3 min and then co-applied with methacholine. In the presence of 300 nM bis-1, the effects of methacholine were substantially attenuated; 85 ± 4% current remained after 3 min of methacholine application, which is not significantly different from current reductions due to rundown in untreated cells over the same time period (Fig. 2). Bis-1 at 300 nM also significantly reduced the response to ionomycin (P < 0.05, n = 5; see Fig. 2B). Therefore, the results with bis-1 suggest a role for PKC in hERG modulation by ionomycin and methacholine.

Since kinase antagonists are not entirely selective (Davies et al. 2000) and bis-1 directly inhibits hERG current, we used an alternative approach and down-regulated PKC by long-term incubation with PMA. To investigate which isotypes were down-regulated in our HEK-293 cell line, PKC protein levels were quantified by Western blotting. Untreated hERG-HEK cells expressed α, β, δ, ε, φ, and δ PKC isotypes (Fig. 3C). In cells pretreated with 1 μM PMA for 24 h, isotypes α and β were almost undetectable, and δ protein levels were consistently reduced compared to untreated cells. No changes to isotype expression were observed with 4α-PMA, a PMA analogue that does not bind to or stimulate PKC (Fig. 3C). Thus, PKC down-regulation is PMA specific and reduces the levels of Ca2+ and DAG activated isotypes of PKC.

Using this PKC down-regulation approach, we investigated if hERG current modulation by ionomycin and methacholine was decreased in PMA treated cells. Following a 3 min exposure to methacholine, hERG currents were 93 ± 2 % of control currents (Fig. 3D), which was not significantly different from rundown
in untreated cells. A 2 min ionomycin application also failed to evoke a response in chronically PMA stimulated cells. However, chronic PMA pretreatment did not alter the intracellular Ca\(^{2+}\) response to either ionomycin or methacholine (data not shown), indicating receptor and phospholipase C function is unchanged. Chronic PMA treatment also had no effect on forskolin dependent modulation of hERG currents (Fig. 2D), suggesting that cAMP and PKA dependent pathways are also conserved. These results strongly suggest that the effects of both ionomycin and methacholine are mediated through the activation of PKC rather than Ca\(^{2+}\) or PKA. One or more

---

**Figure 2. hERG current modulation by methcholine and ionomycin can be attenuated by inhibition or down-regulation of PKC**

* A, mean hERG current in response to methacholine (Mch) in HEK-M3 cells following inhibition of PKC with 300 nM bis-1. The protocol and tail current analysis are the same as described in Fig. 1B. Bis-1 was applied for 3 min before co-application with methacholine. B, hERG current modulation is attenuated by 300 nM bis-1. Mean percentage values for tail current remaining in control conditions after 5 min (untreated), and upon application of the following: 300 nM bis-1 for 3 min; 1 \(\mu\)M bis-1 for 3 min; methacholine (Mch) for 2 min; 3 min perfusion with bis-1 followed by co-application of bis-1 with Mch for 2 min; 5 \(\mu\)M ionomycin (IM) for 2 min; 3 min perfusion with bis-1 followed by co-application of IM for 2 min. C, representative Western blots showing PKC isotype expression in hERG-HEK cells treated with 0.1% DMSO (control), 1 \(\mu\)M PMA, or 1 \(\mu\)M 4\(\alpha\)PMA for 24 h. Control and PMA blots were performed in pairs to enable direct comparisons to be made. 4\(\alpha\)PMA blots were performed separately. Similar results were observed in 4 other experiments. The lower panels show the blots after being stripped and re-probed for \(\beta\)-actin to show protein loading across all lanes. D, down-regulation of PKC isotypes abolishes hERG current modulation by Mch and IM. hERG current modulation in untreated cells and cells incubated in 1 \(\mu\)M PMA for 24 h (PMA). Tail current amplitudes were measured after > 2 min application of Mch, IM (5 \(\mu\)M) or forskolin (40 \(\mu\)M) and expressed as a percentage of current before test compound application. Experiments with Mch activation of muscarinic receptors were performed in HEK-M3 cells transiently expressing hERG and all other experiments were performed on hERG-HEK cells. **Significantly different from untreated \((P < 0.01)\); ‡significantly different from Mch-only response \((P < 0.01)\); †significantly different from IM-only response \((P < 0.05)\). Note, for clarity not all significant differences are presented.

© 2007 The Authors. Journal compilation © 2007 The Physiological Society
of the \(\alpha, \beta\) or \(\delta\) isotypes are implicated, since the hERG current responses were abolished when these PKC isoforms were down-regulated.

**Modulation of hERG by OAG**

A more direct method to investigate modulation of hERG current by PKC without activation of other signalling pathways is to stimulate PKC with the DAG analogue OAG (Fig. 3). hERG currents were elicited with repetitive depolarizations to 0 mV and OAG perfused onto the cells. OAG decreased current amplitudes in a concentration dependent manner (pIC\(_{50}\) = 5.9 ± 0.1, \(n \geq 4\)). OAG at 10 \(\mu\)M reduced hERG to 56 ± 3% (\(n = 9\)) of control after 5 min. Deactivation time constants were significantly accelerated by OAG, from 247 ± 41 ms and 1229 ± 150 ms for \(\tau_f\) and \(\tau_d\), respectively, in control conditions, to 144 ± 15 ms and 782 ± 36 ms, respectively, in the presence of OAG (\(n = 6\)). As with methacholine, there was also a small, but statistically significant shift of the voltage dependence of activation by OAG of 6 ± 2 mV (\(n = 6\)), but steady state inactivation was not significantly shifted (data not shown). The decrease of hERG current amplitudes, shift of activation and acceleration of deactivation by OAG could all be abolished by chronic pretreatment of cells with PMA (but not 4\(\alpha\)-PMA). The modulation by OAG could also be blocked with 100 nM G\(\alpha\)6976 (Fig. 3C), which is selective for \(\alpha\) and \(\beta\)1 PKC isoforms. A 3 min application of G\(\alpha\)6976 alone had no effect, indicating the compound did not directly block the channel pore. However, it significantly reduced the action of OAG (\(P < 0.001, n = 5\)), suggesting that it is \(\alpha\) and/or \(\beta\)1 PKC isoforms that modulate hERG.

**Does the regulation of hERG by OAG depend upon direct phosphorylation by PKC?**

hERG contains 18 putative PKC phosphorylation sites and a number of other serine, threonine and tyrosine residues on intracellular domains that could potentially be phosphorylated to alter channel function. To measure direct phosphorylation of hERG channel subunits, cells were incubated with \[^{32}\text{P}\]orthophosphate for 1 h and the amount of radiolabelled phosphorylated hERG quantified. In lysates from cells expressing hERG, immunoprecipitation with an anti-hERG antibody identified two phospho-proteins that were highly phosphorylated under basal conditions and were absent in cells not transfected with hERG (Fig. 4A). These phospho-proteins have the molecular masses predicted for core (135 kDa) and fully glycosylated (155 kDa) hERG subunits and correspond with immature and mature, surface-expressed hERG subunits, respectively (Zhou et al. 1998). Tryptic digests of \[^{32}\text{P}\]orthophosphate labelled hERG and the separation of phospho-peptides following 2D electrophoresis suggest that hERG is phosphorylated on a large number of sites (Fig. 4B). Each ‘hot spot’ represents phosphorylated fragments of hERG since identically prepared samples from WT HEK cells produced no detectable phospho-proteins.

Stimulation of cells with 10 \(\mu\)M OAG for 5 min significantly (\(P < 0.001\)) increased hERG subunit phosphorylation (Fig. 4C, D and E). The increase compared to the basal level in the same number of untreated cells was relatively small (18 ± 2%, \(n = 4\)), but consistent across experiments. Coomassie blue staining of proteins within the gels confirmed equal loading of lysate and antibody. Biotinylation experiments on intact, viable cells revealed a 155 kDa band that was immunoprecipitated by the hERG antibody, consistent with fully glycosylated hERG subunits (Fig. 4D, upper panel). Furthermore, biotinylation experiments confirmed equal loading of hERG protein irrespective of whether the cells

---

**Figure 3. Activation of PKC mimics modulation of hERG currents by \(M_3\)-muscarnic receptor stimulation**

A, representative currents at a test potential of 0 mV from hERG-HEK cells in control conditions and during application of 10 \(\mu\)M OAG. B, OAG-dependent inhibition of hERG tail current was concentration dependent. The mean results were fitted with a Hill function with a pIC\(_{50}\) of 5.9 ± 0.1 (\(\sim 1 \mu\)M). C, summary of mean results for OAG effects on hERG tail current amplitudes. hERG current suppression by 10 \(\mu\)M OAG could be significantly reduced by 100 nM G\(\alpha\)6976 (\(P < 0.01\) compared to OAG, \(n = 5\)), a selective antagonist of \(\alpha\) and \(\beta\)1 PKC isoforms, and by 24 h incubation with 1 \(\mu\)M PMA (OAG & PMA), but not 4\(\alpha\)-PMA (OAG & 4\(\alpha\)-PMA). **Significantly different from untreated (\(P < 0.01\)); †significantly different from OAG (\(P < 0.01\)). Not all comparisons are shown.
had been untreated or challenged with OAG (P > 0.19, n = 6). Indeed, in experiments in which cell surface proteins were biotinylated and the cells labelled with \[^{32}\text{P}\]\text{orthophosphate}, OAG did not affect the amount of immunoprecipitated hERG protein but did increase the level of phosphorylation (Fig. 4D). Thus, the increase in \[^{32}\text{P}\]\text{orthophosphate} labelling in hERG immunoprecipitates following treatment of cells with OAG is due to a substantial increase in channel phosphorylation and is not a consequence of an altered efficiency of immunoprecipitation.

The OAG dependent increase of phosphorylation could be abolished by incubating cells with bis-1 for 15 min or by chronic pretreatment of cells with PMA (Fig. 4E, F and G). Interestingly, bis-1 and chronic pretreatment with PMA had no effect on phosphorylation levels in untreated cells. One conclusion from this may be that PKC does not have a role in basal hERG phosphorylation; however, we cannot entirely discount the possibility that changes in basal phosphorylation at PKC sites are not detectable because of a high level of background phosphorylation by other kinases. These results demonstrate that hERG is constitutively phosphorylated within intact cells and that OAG increases direct phosphorylation of the channel in a PKC dependent manner.

The N-terminus of hERG is required for hERG current modulation and phosphorylation by PKC

Previous mutagenesis studies have shown that PKC-dependent modulation of hERG currents in Xenopus oocytes is not ablated in \(\Delta\text{PKC}\)-hERG, a channel in which 17 of the 18 putative phosphorylation sites have been mutated to Ala (Thomas et al. 2003). OAG also inhibited \(\Delta\text{PKC}\)-hERG and \(4\text{M}\)-hERG (PKA phosphorylation sites mutated) currents expressed in HEK-293 cells (Fig. 5A). The percentage inhibition in both mutants was similar to WT hERG. Thus, mutation of all the PKA phosphorylation sites and 17 of 18 PKC sites failed to prevent the channel modulation by OAG.

18M-hERG, a channel in which all 18 PKC phosphorylation sites are mutated to alanine, failed to give measurable currents in voltage clamp studies or detectable bands in Western blots. To further investigate the role of the 18th site (Thr74), we generated three additional Thr74 mutants: a mutation to Val that has a similar size to Thr but cannot be phosphorylated, and substitutions to negatively charged (Asp or Glu) residues to mimic phosphorylation. Unfortunately, all of these mutants were also non-functional when expressed in Xenopus oocytes and HEK-293 cells. Indeed, coexpression of the Thr74 mutants with WT hERG caused profound dominant-negative suppression of hERG currents (data not shown). Clearly, position 74, which is within the Per-Arnt-Sim (PAS) domain of the N-terminus is a functionally important residue and highly sensitive to mutation. We also attempted to study phosphorylation of \(\Delta\text{PKC}\)-hERG or 18M-hERG (all 18 PKC sites mutated to Ala). Four different anti-hERG antibodies, including the anti-hERG serum and pan-hERG1 antibody, were unable to immunoprecipitate phospho-proteins or detect bands of the anticipated molecular mass in Western blots. Evidently, the mutations prevent the antibodies recognizing their epitopes.

As an alternative to mutating Thr74 and other putative phosphorylation sites, we investigated PKC-dependent modulation and phosphorylation of NTK-hERG channels in which amino acids 2–354 (including the PAS) domain have been removed (Spector et al. 1996). NTK-hERG transiently expressed in HEK-293 cells gave functional currents with rapid deactivation kinetics (Fig. 5B). However, whereas WT channel currents recorded on the same experimental day were inhibited by 37 ± 3\% (n = 5), a 5 min application of 10 \(\mu\text{M}\) OAG failed to significantly inhibit NTK-hERG currents (Fig. 5A and B). The loss of functional effects on NTK-hERG currents correlated with an inability of OAG to alter the phosphorylation state (Fig. 5C). OAG at 10 \(\mu\text{M}\) failed to increase NTK-hERG phosphorylation above basal levels, although WT hERG subunit phosphorylation, measured at the same time, increased by 15 ± 2\% (n = 5). These results indicate that an intact N-terminus is required for PKC-dependent phosphorylation and modulation of hERG currents, suggesting that critical residues for PKC phosphorylation reside on the N-terminus. The observation that hERG channels that lack an intact N-terminus are not phosphorylated or functionally modulated by PKC activation provides further evidence for the link between direct PKC-dependent phosphorylation of hERG subunits and the regulation of channel activity.

Discussion

We have studied the modulation of hERG current by intracellular \(\text{Ca}^{2+}\) and \(M_{3}\)-muscarinic receptor stimulation in a mammalian cell expression system. We find that the main effect is to reduce hERG current amplitudes at all potentials, with small effects on the voltage dependence of activation and no effects on the voltage dependence of inactivation. Deactivation is significantly faster with muscarinic receptor stimulation. In our experiments, the inhibition of hERG currents is significantly reduced by a relatively low concentration (300 \(\text{nm}\)) of the PKC inhibitor bis-1 and is also abolished by preincubation of cells with PMA, which down-regulates \(\alpha, \beta\), and to a reduced extent \(\delta\) isotypes of PKC. The effects of ionomycin and muscarinic receptor stimulation are also closely mimicked by the DAG analogue, OAG,
Figure 4. Phosphorylation of hERG is increased by activation of PKC

Phospho-proteins were immunoprecipitated from WT-HEK cells and hERG-HEK cells using anti-hERG antibody. A, upper panel, representative autoradiograph showing phospho-proteins were only pulled down from hERG-expressing cells. Samples were run in duplicate to demonstrate reproducibility of results. The molecular masses of the two bands correspond with the immature (135 kDa) and mature (155 kDa) forms of hERG. Both hERG proteins are clearly phosphorylated in untreated cells. The lower panel shows Coomassie blue staining of the gel before drying and demonstrates there is equal antibody loading and immunoprecipitation across lanes. B, representative phospho-peptide maps following tryptic digest and separation by 2D electrophoresis of phospho-proteins immunoprecipitated from untreated hERG-HEK cells and WT-HEK cells. The hERG-HEK cell plate was exposed for 10 days and the WT HEK cell plate for longer (14 days). Phospho-peptides can only be detected from hERG expressing cells. C, representative autoradiograph showing the increase of phosphorylation in cells treated with 10 μM OAG for 5 min. D, quantification of biotinylation and phosphorylation of immunoprecipitated proteins. Protein samples transferred to nitrocellulose were first probed with streptavidin (upper panel), and subsequently exposed to autoradiography film for phospho-protein detection (lower panel). The streptavidin labelling was equivalent in OAG treated cells (lane 1) and untreated cells (lane 2), indicating equal amounts of immunoprecipitated hERG in each lane. Comparison of hERG phosphorylation levels in lanes 1 and 2 indicate there was a 17% increase over basal levels with OAG stimulation. No phospho-proteins or streptavidin labelled proteins were detected if anti-hERG serum was not added to the lysates (lane 3). Faint streptavidin-labelled bands were
and can be attenuated by bis-1 and preincubation with PMA. Therefore, our findings are entirely consistent with muscarinic-receptor stimulation being coupled to activation of DAG-sensitive isotypes of PKC.

Buffering intracellular Ca\(^{2+}\) with BAPTA does not attenuate the hERG current response to M\(_1\)-muscarinic receptor stimulation suggesting that a rise of Ca\(^{2+}\) does not directly regulate the channel and also is not obligatory for the PKC mediated modulation. Despite this, the ionomycin response is abolished by BAPTA. The most likely explanation is that cPKC isotypes (activated by DAG in methacholine-treated cells and Ca\(^{2+}\) in ionomycin treated cells) are responsible for the hERG channel modulation. This is further supported by experiments with the \(\alpha\) and \(\beta\)1 PKC isotype-selective inhibitor G66976, which abolished the inhibition of hERG currents by OAG stimulation of PKC. Nevertheless, a role for novel isotypes such as \(\delta\)PKC cannot be discounted.

Previous studies on the M\(_1\)-muscarinic receptor modulation of ERG channels expressed in mammalian cells have also shown the same principal effect – a partial reduction in available current with minimal effects on the voltage dependence of activation and inactivation (Selyanko et al. 1999; Hirdes et al. 2004). M\(_1\) and M\(_3\) muscarinic receptors are coupled to phospholipase C by the G\(_{q/11}\) family of G proteins. A recent, detailed study by Hirdes et al. (2004) concluded that although rat ERG1 current modulation by muscarinic receptor stimulation was mediated by phospholipase C, its classical downstream messengers, Ca\(^{2+}\) and particularly PKC, were not involved. Thus, 1 \(\mu\)M bis-1 or stauroporine was ineffective at preventing muscarinic receptor-mediated current inhibition. Despite this evidence suggesting a lack of effect of PKC, the PKC inhibitor calphostin C partially reduced the effects of muscarinic receptor stimulation. Furthermore, the current rundown in the experiments with bis-1 and stauroporine was particularly fast (see Hirdes et al. 2004, Fig. 7), making it difficult to precisely quantify the extent of inhibition. The concentrations of bis-1 and stauroporine used in these experiments were sufficient to block ERG channel conduction and it is possible that the decrease of current described as rundown might actually be use dependent accumulation of block. In the study of Hirdes et al. (2004), dialysis of cells via the patch pipette with a non-hydrolysable analogue of ATP to prevent kinase-mediated phosphorylation events was also ineffective at preventing muscarinic receptor-mediated regulation of ERG1. However, in such protocols it is difficult to verify that levels of ATP are sufficiently depleted within the local vicinity of channels. Interestingly, Cayabyab et al. (2002) did observe more rapid rundown of ERG currents in cells dialysed with 0 mM than 2 mM ATP, suggesting that ATP was required to maintain phosphorylation and normal channel function. The apparent disparity in receptor–channel coupling mechanisms observed in the two studies may also be associated with specific differences in muscarinic receptors (M\(_1\) versus M\(_3\)), channel (rat compared to human ERG) or recording temperatures (35–37°C in this study). In the present study, evidence for the role of PKC comes not only from pharmacological manipulation of PKC activity, but also from direct measurements of changes to phosphorylation.

**PKC mediated modulation of hERG**

Whether hERG is directly modulated by PKC or not has been controversial. Most studies have activated PKC by acute application of PMA with variable results. In one study in tsA-201 cells, 500 nM PMA had little effect (Hirdes et al. 2004). In other studies, PMA caused a positive shift in the voltage dependence of activation and an acceleration of deactivation, with little reduction in maximal current (Barros et al. 1998; Kiehn et al. 1998; Schledermann et al. 2001). Interestingly, the response to PMA is not abolished by mutating 17 of the 18 consensus PKC phosphorylation sites (see later) and is relatively insensitive to PKC inhibitors (Schledermann et al. 2001). Thus 1 \(\mu\)M bis-1 has little effect (Kiehn et al. 1998; Schledermann et al. 2001) and 10 \(\mu\)M bis-1 is required, with long incubation times, to significantly reduce the response to PMA (Barros et al. 1998; Thomas et al. 2003). At these high concentrations there is direct block of the channel pore and inhibition may not be kinase specific (Davies et al. 2000). Indeed, PKA
antagonists and broad spectrum protein kinase inhibitors are also able to block the actions of PMA in some *Xenopus* oocyte studies (Kiehn et al. 1998; Thomas et al. 2003). This suggests that in addition to PKC, PKA may also be part of the signalling cascade that leads to PMA-mediated hERG current modulation. PMA may also exert its action through other molecules with a DAG binding site.

In the present study we used the DAG analogue OAG to activate PKC. We observed a decrease of maximum available current with little change in voltage dependence of activation or deactivation kinetics. The response to OAG was identical in mutant (4M-hERG) channels without PKA phosphorylation sites, ruling out a role for PKA. The response to OAG mimicked ionomycin and muscarinic receptor stimulation, could be blocked by a relatively low concentration of bis-1 (300 nM), and was also abolished in cells chronically pretreated with PMA. These results strongly suggest a PKC-mediated modulation of hERG current without any contribution from PKA.

**Is PKC-dependent modulation due to direct phosphorylation of hERG?**

Previous studies have suggested that PKC-mediated modulation of hERG occurs through an indirect mechanism involving an intermediate protein or auxiliary channel subunit that is phosphorylated and consequently alters the gating behaviour of the channel. This model was proposed on the basis that the hERG current response to PMA and $\alpha_1$-adrenoceptor stimulation is retained in the $\Delta$PKC-hERG mutant in which 17 of 18 consensus PKC phosphorylation sites have been removed (Thomas et al. 2003, 2004). Nevertheless, a limitation of the mutagenesis approach is that PKC phosphorylation could still occur at the 18th consensus PKC site (Thr74), which cannot be mutated without losing functional expression. In addition, PKC phosphorylation at atypical phosphorylation sites cannot be ruled out. In this study we obtained direct measurements of phosphorylation by [$^{32}$P]orthophosphate labelling of hERG in intact cells. OAG increased hERG subunit phosphorylation in a PKC-dependent manner. The increase of phosphorylation above basal levels was abolished by incubating cells with bis-1 and chronic pretreatment with PMA. Thus, our results strongly suggest that hERG subunits are directly phosphorylated in response to PKC activation. Deleting the N-terminus of hERG prevents the OAG-mediated modulation of hERG and the increase of phosphorylation, consistent with a role for Thr74. Unfortunately, mutation of Thr74 to Val, Asp or Glu also caused a loss of hERG channel function, indicating that this position on a surface loop of the PAS domain is important for channel function.

A possibility that we cannot exclude is that the kinase that phosphorylates hERG is not PKC, but an
intermediary kinase that requires PKC activation. Regardless of the precise position of the phosphorylation site(s) and kinase, our results indicate that hERG channel subunits are directly phosphorylated by a PKC-dependent mechanism and an intact N-terminus is required for modulation of channel function (Gomez-Varela et al. 2003a). The N-terminus could be required for PKC binding or be necessary for allosteric regulation of phosphorylation.

**Phosphorylation is an important mechanism for regulating hERG channel activity**

Our studies reveal that hERG channel subunits in intact cells are highly phosphorylated under basal conditions. Phospho-proteins with molecular masses corresponding to both core glycosylated and fully glycosylated channels are immunoprecipitated indicating that surface and intracellularly located hERG channels are phosphorylated. Thus, phosphorylation is not restricted to surface localized channels alone. More than 17 phospho-proteins can be isolated in hERG protein that has been purified and digested with trypsin (phospho-peptide separation), indicating that hERG is phosphorylated at multiple sites. The change of phosphorylation we observed with OAG stimulation was modest. This may arise because only a fraction of channels are phosphorylated by PKC. A more likely explanation, supported by the phospho-peptide analysis, is that the relatively small increase of total phosphorylation by PKC is masked by the high basal level of phosphorylation. Thus, a much larger proportion of the channels may well be phosphorylated by PKC than is indicated by the percentage change in total phosphorylation.

Recently two kinases, protein kinase B (Zhang et al. 2003) and Src tyrosine kinase (Cayabyab & Schlichter, 2002), have been reported to tonically modulate hERG in a manner that would be consistent with basal phosphorylation of the channel. It is becoming apparent that endogenously expressed ERG can exist in a signalling complex in which channel function can be regulated by both tyrosine kinases and phosphatases (Cayabyab & Schlichter, 2002; Cayabyab et al. 2002). Two observations suggest that PKC does not have a profound effect on basal phosphorylation of hERG. First, acute application of 300 nM bis-1 to patch-clamped cells does not significantly alter hERG current amplitudes. Second, the amount of basal phosphorylation is not reduced by 3 μM bis-1 or chronic PMA pretreatment of cells. Thus, significant changes of basal phosphorylation are not obtained with either short or long-term decreases to PKC activity.

This study provides strong evidence that hERG is phosphorylated by PKC (or a kinase dependent on PKC activity), and this leads to inhibition of channel function. Mutagenesis studies indicate that the same applies for PKA, since mutation of four consensus PKA sites prevents the rightward shift of activation and decrease of current density in response to PKA stimulation (Thomas et al. 1999; Cui et al. 2000). Phosphorylation by PKA and PKC provides the potential for hERG to be modulated by a variety of G-protein coupled receptors, and this has important implications for regulating the activity of excitable cells and needs to be further investigated in vivo in cardiac myocytes and neurons. It is becoming apparent that there are many signal transduction pathways that can modify hERG channel function. Our study also demonstrates constitutive phosphorylation at multiple sites under basal conditions. A challenge for the future is to identify the sites of phosphorylation and determine their functional importance in tissues in which hERG is endogenously expressed.

**References**


**Acknowledgements**

Financial support for this work came primarily from a project grant (PG/2001083) to J.S. from the British Heart Foundation. J.S. is also supported by a Career Establishment Award from the UK Medical Research Council. We are also grateful to Dr Jules Hancox (University of Bristol) for assistance with preliminary experiments on hERG regulation by intracellular Ca$^{2+}$ and Dr Harry Witchel (University of Bristol) for advice and help with cellular and molecular reagents.