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RESEARCH ARTICLE

Regulation of HERG (KCNH2) potassium channel surface expression by diacylglycerol

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Abstract The HERG (KCNH2) channel is a voltage-sensitive potassium channel mainly expressed in cardiac tissue, but has also been identified in other tissues like neuronal and smooth muscle tissue, and in various tumours and tumour cell lines. The function of HERG has been extensively studied, but it is still not clear what mechanisms regulate the surface expression of the channel. In the present report, using human embryonic kidney cells stably expressing HERG, we show that diacylglycerol potently inhibits the HERG current. This is mediated by a protein kinase C-evoked endocytosis of the channel protein, and is dependent on the dynein–dynamin complex. The HERG protein was found to be located only in early endosomes and not lysosomes. Thus, diacylglycerol is an important lipid participating in the regulation of HERG surface expression and function.

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Introduction

Potassium channels are expressed in virtually all cell types regulating a wide variety of physiological functions including heart rate, release of neurotransmitters and insulin secretion. Ion channels are modified by posttranslational changes such as channel phosphorylation [1]. The human ether á-go-go-related gene (HERG1 or KCNH2) was originally cloned from a human hippocampal cDNA library by homology to *Drosophila* EAG [2]. HERG encodes the α-subunit of a potassium channel with a structure resembling that of other voltage-gated potassium channels, i.e. six transmembrane domains, one of which acts as a voltage sensor, intracellular N (amino) and C (carboxy) termini, and a pore loop linking the S5 and S6 domains. The HERG channel was then found to underlie the rapid component of the cardiac delayed rectifier current (I_{Kr}) and hence plays a central role in mediating repolarization of the cardiac action potential [3]. Mutations in the HERG channel that cause a loss- or gain-of-function can result in type 2 long QT syndrome (LQTS) and one form of short QT syndrome, respectively, which are associated with cardiac arrhythmias and a risk of sudden death [4, 5]. Of more common occurrence are drug-induced LQTS and torsade de pointes tachyarrhythmia resulting primarily from HERG channel inhibition by class III antiarrhythmics, as well as by a wide range of non-cardiac drugs, including antihistamines, antimicrobial and psychiatric drugs [6].

HERG expression is, however, not limited to cardiac tissue. The channel has also been found in the hippocampus

regulating the excitability of neurons [7], in jejunal smooth muscle controlling electrical and contractile activities [8] and in pancreatic β-cells regulating insulin secretion [9]. Furthermore, a growing number of studies show that HERG is selectively up-regulated in several animal and human tumours and tumour cell lines [10, 11].

Diacylglycerol (DAG) is one of the key lipid second messengers generated from phosphatidyl inositol 4,5-biphosphate (PIP_2) by the action of receptor-activated phospholipase C, PLC. In addition to its recognized role in protein kinase C (PKC) activation, DAG also targets protein kinase D (PKD) [12], DAG kinases and chimaerins [13] among others. Both DAG and its precursor PIP_2 have been shown to regulate a number of potassium channels. DAG inhibits the voltage-gated Kv1.3 current of human T lymphocytes [14] and in *Xenopus* oocytes inhibits the minK current resembling the cardiac I_{Ks} current [15]. The HERG current is regulated by PIP_2 resulting in an increase in current amplitude, acceleration of activation and slowing of inactivation [16]. Another lipid second messenger, ceramide, whose diverse signalling includes PKC activation, has also been shown to regulate the HERG channel. In rat pituitary GH_3 cells, an ERG current is inhibited by ceramide [17], while we have previously shown that ceramide reduces the HERG current by decreasing the amount of membrane-bound HERG protein [18]. The actions of these lipids on the HERG channel appear devoid of PKC involvement. However, investigations have shown that PKC may indirectly [19, 20], or by direct phosphorylation [21], decrease HERG current. As both ceramide and DAG activates PKC, and both lipids seem to modulate HERG channel function, it was of interest to further investigate the mechanism of action of DAG on the channel. In this report, we show that DAG evokes a time-dependent decrease in HERG current. The underlying mechanism was found to be a PKC-mediated decrease in plasma-membrane HERG protein expression, due to a rapid endocytosis of the channel.

Materials and methods

Cell culture

HEK293 cell line stably expressing HERG in the pcDNA3.1 expression vector was selected for using G418 (A. G. Scientific, CA, USA). The cells were cultured in DMEM supplemented with 10% foetal calf serum, penicillin–streptomycin (BioWhittaker Cambrex Bio Science, Verviers, Belgium) and G418 (0.2 mg/ml). SHSY5Y cells were cultured in DMEM:Ham's F12 medium (1:1) supplemented with 10% foetal calf serum and penicillin–streptomycin.

Patch-clamp recording

Whole-cell recordings of HEK293 cells expressing HERG were performed using an EPC-9 amplifier and Pulse/Pulsefit software (Heka, Lambrecht, Germany) as previously described [18]. HERG-like tail currents present in SHSY5Y neuroblastoma cells were recorded using a modified version of a previously described protocol [22]. Briefly, cells were clamped to a holding voltage of –0 mV and tail currents were evoked by a 200-ms step to −120 mV applied every 5 s. At the beginning of the experiment, cells were perfused with a 5.4-mM K^+ and then with a 50.4-mM K^+ solution to enhance K^+ carried inward currents. The first trace of each experiment recorded in 5.4-mM K^+ solution was subtracted from subsequent sweeps. For illustration purposes, the residual current amplitudes were normalized against the largest response obtained in the presence of 50.4 mM K^+ (given the value of −1). For statistical analysis, the logarithms of absolute current values (A pF−1) were used. Logarithm values were normalized against respective controls (taken as 100%) to prepare diagrams.

The electrodes had resistances of 2–6 MΩ when filled with 150 mM KCl, 2 mM $MgCl_2$, 5 mM BAPTA, 5 mM Mg_2ATP_3 and 10 mM HEPES, pH 7.2. The standard extracellular solution contained 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. The high K^+ solution was otherwise identical, but 45 mM NaCl was replaced with KCl, ending to a $[K^+]_0$ of 50.4 mM. 1,2–dioctanoyl-*sn*-glycerol, DAG (Sigma, St. Louis, MO, USA) and *m*-3M3FBS (Merck, Darmstadt, Germany) were dissolved in DMSO and added to the extracellular solution (the final vehicle concentration was 0.1%). All experiments were carried out at room temperature. The whole-cell recordings capacitance was compensated for, as was series resistance by at least 70%.

Labelling of cell surface proteins

Cell surface proteins were biotinylated with a water-soluble biotinylating reagent, sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-biotin). Cells were washed twice with PBS and cell surface proteins were labeled with 1 mg/ml Sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL, USA) in PBS (30 min, +4°C). After two washes, non-reacted biotinylation reagent was quenched with 100 mM glycine in PBS (20 min, +4°C), and after washes cells were lysed and HERG protein was immunoprecipitated (see "Immunoprecipitation"). The precipitated HERG proteins were subjected to 6% SDS-polyacrylamide gel electrophoresis, and biotin-labeled HERG was detected by horseradish peroxidase-conjugated strept-avidin (1:500; Pierce).

Western blot analysis

Membrane fractions were prepared as previously explained [23]. Briefly, cells were incubated with 10 μM DAG for 60 min, and then scraped from the plates and broken by sonication in a buffer (200 mM NaCl, 33 mM NaF, 10 mM EDTA, 50 mM HEPES pH 7.5) supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany). The cells were then ultracentrifuged (100,000*g* for 1 h). Protein concentrations were determined using the Pierce protein assay (Pierce) and equal amounts of protein were loaded on a 6% SDS-PAGE gel, followed by transfer to nitrocellulose membranes (Amersham Biosciences, Buckingham, England). Membranes were blocked with 5% milk–TBS for 1 h at RT followed by incubation with primary antibodies: anti-HERG (1:1000; Alomone Labs, Jerusalem, Israel) and secondary antibody (anti-rabbit, 1:2500; Pierce).

For translocation-studies of the PKC isoforms, the cells were stimulated by 10 μM DAG for the indicated times, and cytosolic and particulate fractions were prepared as described by Kass et al. [24]. The samples were then stored at –20°C. Cytosolic and particulate fractions (15 μg of protein/sample) were subjected to SDS/PAGE (10% polyacrylamide) for PKC analysis. The proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Western blot analysis was performed using isoenzyme-specific PKC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Sigma). The proteins were detected by enhanced chemiluminescence. Densitometric analysis was performed using MCID + software for data acquisition and image analysis (Imaging Research, St. Catherines, Ontario, Canada). Results are expressed as % of PKC in the particulate fraction compared to unstimulated control in 0 min.

Immunoprecipitation

Cells were treated with DAG (10 μM for 60 min). After stimulation, cells were lysed in buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodiumdeoxycholate, pH 7.5) supplemented with protease inhibitors (Roche). Lysates were incubated with anti-HERG (8 μl antibody per 500 μl lysate; Alomone) overnight at +4°C. Immunocomplexes were precipitated using protein G-Agarose (Roche) for 2 h at +4°C and washed three times with washing buffer (250 mM NaCl, 0.1% NP40, 50 mM Tris pH 7.5). The beads were boiled in SDS-PAGE sample buffer and samples separated using a 6% SDS-PAGE gel followed by transfer to nitrocellulose membranes. Membranes were probed with either anti-ubiquitin clone P4G7 (1:2500; Nordic Biosite, Täby, Sweden) or anti-ubiquitin clone FK1 (1:1000; Affiniti Research Products, Exeter, UK), and anti-HERG (1:1000; Alomone) antibodies.

Immunocytochemistry

For immuno-cytochemistry, cells were plated on poly-L-lysine (Sigma) coated coverslips and fixed with methanol-acetic acid (95:5) for 5 min at −70°C. After fixation wells were washed with PBS, permeabilized with 0.1% Triton X-100 for 10 min and blocked for 30 min with 5% normal goat serum. Cells were incubated with anti-HERG (1:200; Alomone) antibody over night in +4°C and washed with PBS. The unspecific sites were blocked with 5% goat serum for 30 min followed by 1 h with goat anti-rabbit FITC-conjugated secondary antibody (1:500, Alexis, Lauflefingen, Switzerland) or Alexa Fluor 594 secondary antibody (1:500; Invitrogen, Eugene, Oregon, USA). For co-localization cells were incubated overnight at +4°C with antibodies against Lamp-1 (1:100; Santa Cruz Biotechnology) or EEA1 (1:50; BD Transduction Laboratories, CA, USA) followed by anti-mouse Cy3-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The cells were examined using either a Nikon Eclipse TE300 microscope equipped with an Ultra View confocal imaging system (Perkin Elmer, Waltham, MA, USA) or a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser (Omnichrome; Melles Griot, Carlsbad, CA, USA). The figures were acquired with Lecia TCS NT-software.

Metabolic labelling

Cells were starved for 1 h in serum-free DMEM without methionine and cysteine, and containing 0.25% BSA. The medium was changed to same DMEM containing [^{35}S]methionine/cysteine (100 μCi/ml; Amersham) and cells were incubated for an additional 1 h. The labeling was stopped by changing to DMEM with unlabeled methionine and cysteine. Cells were then treated with DAG (10 μM for 60 min) and lysed at different time intervals (0, 3, 6 and 24 h). HERG protein was immunoprecipitated with anti-HERG (see "Immunoprecipitation"), subjected to 6% SDS-polyacrylamide gel electrophoresis and ^{35}S- labeled HERG proteins were visualized with autoradiography.

Quantifications and statistics

Quantification of western blots was done using Image-Quant software (BioRad Laboratories, CA, USA) and was based on at least three independent experiments. All data are expressed as mean ± SEM. Comparison of the

difference between two experimental groups was performed using Student's *t* test for unpaired data and ANOVA was used for multiple comparisons in conjunction with the Newman–Keuls test. *p* values of less than 0.05 were considered statistically significant.

Results

DAG modulates HERG currents in HEK293 cells

To investigate whether DAG has an effect on the HERG channel, whole-cell patch-clamp recordings were performed on HEK293 cells, stably expressing HERG, incubated with either vehicle (DMSO; control) or 10 μM DAG in the extracellular solution. After 40 min of incubation with DAG, a decrease of the HERG current was evident both as tail current (Fig. 1a) and as the instantaneous current following the relief of inactivation (Fig. 1b). The instantaneous and tail HERG current densities were significantly smaller with DAG treatment (Fig. 1c, d), the maximum tail current density being 39.9 ± 6.5 pA/pF ($n = 10$) versus 61.4 ± 5.3 pA/pF ($n = 8$; $p < 0.05$) in control. As with ceramide [18], DAG did not affect the half-maximum activation voltage or slope factor of the HERG current but significantly accelerated the deactivation time constants (Table 1). A concentration-dependent effect of DAG was also evident (Table 2).

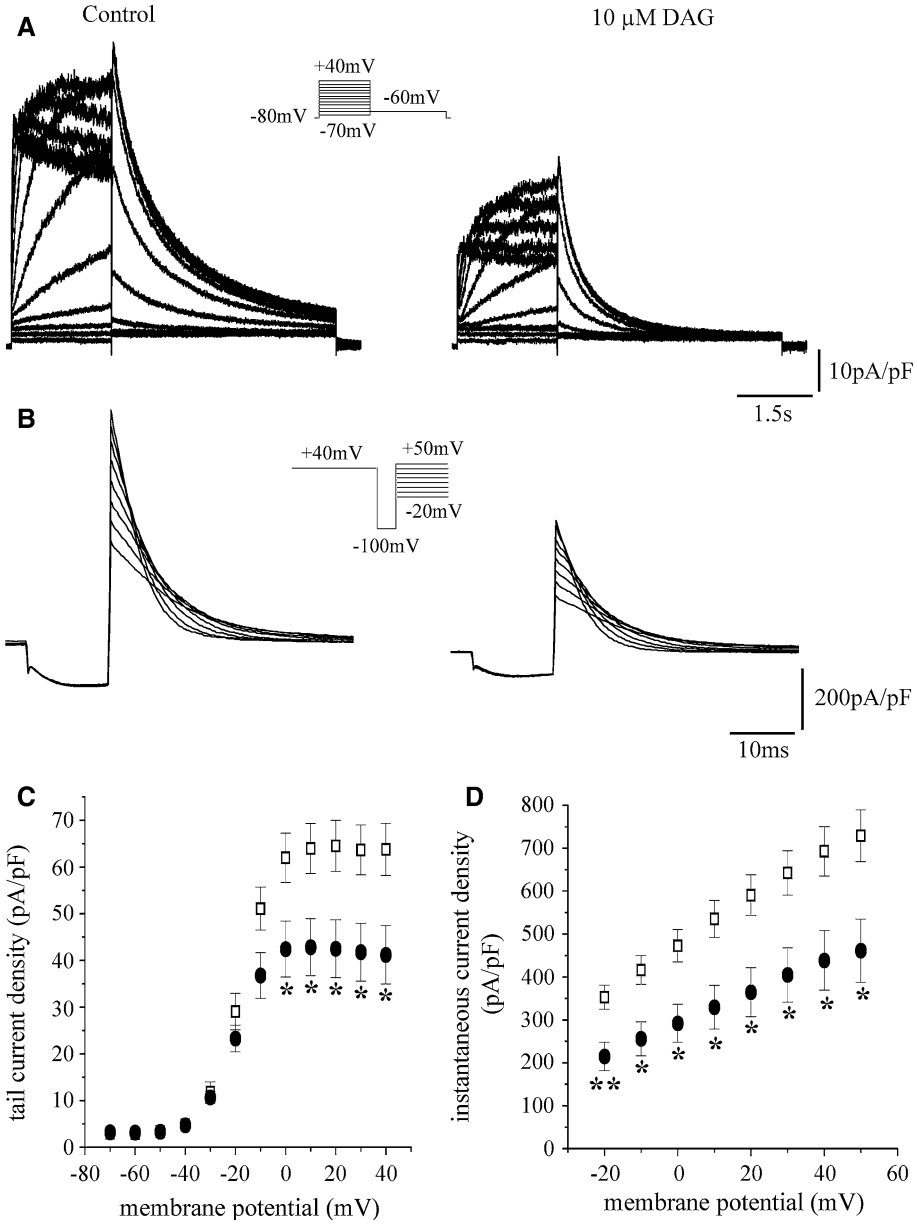

Fig. 1 The effect of DAG on HERG current density. **a** Whole-cell recordings from HEK293 cells stably expressing HERG exposed to either vehicle (*left*) or 10 μM DAG (*right*). The voltage protocol consisted of a 2-s depolarization, from the holding potential of −80 mV, to potentials between −70 and +40 mV with tail currents elicited on repolarization to −60 mV for 4.5 s.**b** From the holding potential, a 2-s depolarization to +40 mV was followed by a brief hyperpolarization step to −100 mV to relieve inactivation and then a step to potentials between +50 and −20 mV. **c–d** Voltage-dependence of HERG current density. The current measured as peak tail current (**c**, as protocol in **a**) and instantaneous current (**d**), i.e. the peak current immediately following the end of the hyperpolarization step as in (**b**). For control (*open square*), $n = 8$ in both **c** and **d**, while for 10 μM DAG (*filled circle*), $n = 10$ and 5 in **c** and **d**, respectively (*$p < 0.05$, **$p < 0.01$)

Table 1 Gating properties of the HERG channel in the presence and absence of 10 μM DAG

	CTRL	DAG
Activation		
$V_{1/2}$	−18.2 ± 1.2 mV	−20.9 ± 1.3 mV
k	6.1 ± 0.1 mV	5.8 ± 0.1 mV
n	8	10
Deactivation		
At –60 mV		
τ_f	294.9 ± 19.1 ms	163.0 ± 14.1 ms ($p < 0.001$)
τ_s	1,337.3 ± 79.1 ms	630.4 ± 63.0 ms ($p < 0.0001$)
At –120 mV		
τ_f	12.0 ± 0.4 ms	8.1 ± 0.2 ms ($p < 0.0001$)
τ_s	81.8 ± 5.2 ms	45.3 ± 4.1 ms ($p < 0.001$)
n	7	5

Values for the voltage-dependence of activation were obtained from Boltzmann fits to the peak tail currents at −60 mV (voltage protocol as in Fig. 1a)

The deactivation time constants were obtained by a double exponential fit to the tail current at −60 and −120 mV after a 2-s depolarization to +40 mV

Table 2 Concentration–response effects of DAG on HERG current

Control	100
3 μM DAG	75.2 ± 7.1
10 μM DAG	64.7 ± 9.7*

Peak tail currents were measured as described in Fig. 1a

The cells were preincubated with the different concentrations of DAG for 60 min. Tail currents at −60 mV were recorded following depolarization to +40 mV for 2 s from the holding potential of −80 mV

The values are normalized to results obtained in control experiments, which have been considered as 100%. The data given are the mean ± SEM of 3–8 separate experiments

*$P < 0.05$

DAG causes a reduction of surface HERG channels in HEK293 cells

Our previous study showed that the ceramide-evoked decrease in the HERG current was due to a decrease in surface expression of the channel [18]. To investigate whether the DAG-evoked decrease in HERG current was also due to the same mechanism, the localization of HERG protein was examined by immunocytochemistry and confocal microscopy. Exposing HERG expressing HEK293 cells, to DAG for different time periods caused a decrease in surface expression already after 15 min. After 60 min, virtually all surface expression had disappeared as shown by immunocytochemistry (Fig. 2a). Clearly, exposure to

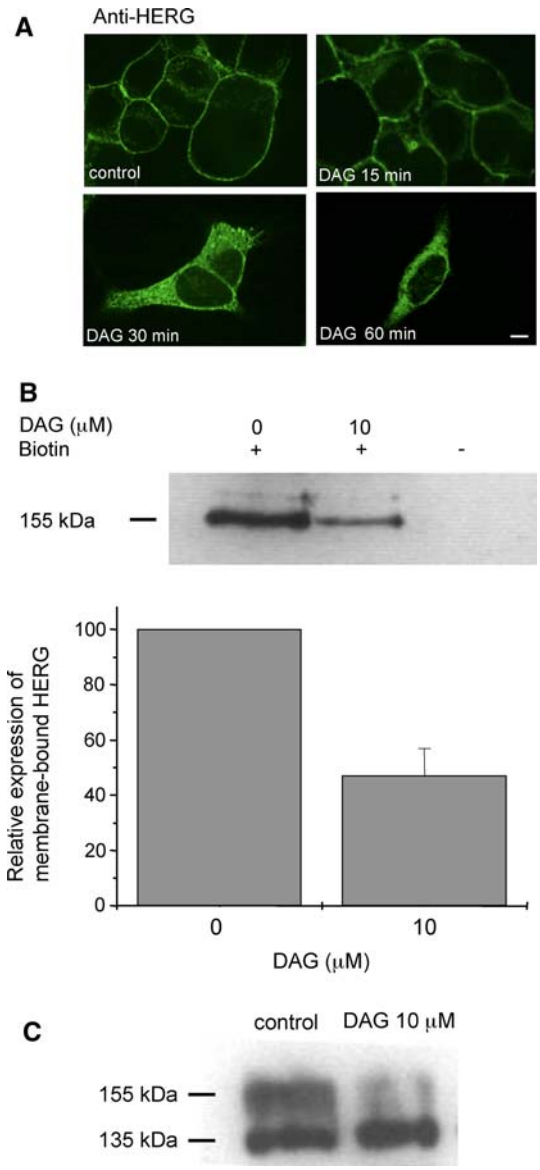

Fig. 2 DAG reduces HERG surface expression. **a** Confocal images of HEK293 cells stably expressing HERG and immunostained for HERG (*green*) after treatment with DAG for 15, 30, and 60 min. DAG induces a time-dependent internalization of HERG peaking at 60 min. *Size bar* 10 μm. **b** The effect of DAG on cell surface expression of HERG can also be seen when analysing specific cell surface expression of HERG protein by biotin labelling. Cells were treated with DAG for 60 min whereupon cell surface proteins were biotinylated with a biotinylating reagent, sulfo-NHS-SS-biotin. HERG protein was immunoprecipitated and the biotinylated HERG channels were detected by horseradish peroxidase conjugated streptavidin. The blot shown is a representative of three separate experiments. **c** Surface expression was analysed by western blot after 60 min incubation with DAG-treated (10 μM). Proteins were separated and HERG was detected with monoclonal HERG antibody. The higher molecular weight (155 kDa) corresponds to the fully glycosylated mature HERG protein and the lower molecular weight band (135 kDa) corresponds to the core-glycosylated immature HERG protein

DAG caused a marked decrease in HERG protein surface expression, with a concomitant appearance of punctuate clusters in the cytosol.

To further examine the level of surface HERG expression, we labeled surface membrane proteins with a biotinylating reagent, sulfo-NHS-SS-biotin, immunoprecipitated the HERG protein with anti-HERG and detected the labeled HERG proteins by streptavidin-HRP. Exposure to DAG for 60 min reduced markedly the amount of biotin-labeled HERG protein to $47 \pm 10\%$ ($p < 0.01$, $n = 3$) compared to control cells (Fig. 2b). Similarly, 1-h exposure to DAG reduced the surface-bound HERG as shown by western blotting (Fig. 2c). These results suggest that DAG decreases the HERG current by inducing an internalization of the channel. Previously, we have reported that ceramide induces HERG channel ubiquitinylation leading to degradation of the protein [18], but, when immunoprecipitating HERG and staining for either mono- or poly-ubiquitin, we were not able to detect any changes after 60 min of exposure to DAG (data not shown).

To rule out the possibility that the DAG-evoked decrease in cell surface expression of HERG was due to defective synthesis or trafficking of the protein, and not a rapid internalization, pulse-chase experiments were performed by metabolic labelling of the newly synthesized proteins. In these experiments, proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine and chased with unlabeled methionine/cysteine for intervals of 0, 3, 6, and 24 h, followed by immunoprecipitation and detection of HERG protein by western blotting. No significant changes in the distribution of the immature versus mature HERG between control and DAG-treated (10 μM, 60 min) cells were, however, detected at any time point (Fig. 3). Thus, treatment of the cells with DAG did not result in a defective synthesis of HERG.

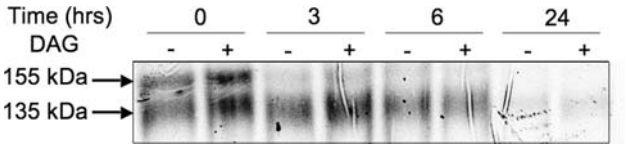

Fig. 3 Pulse-chase analysis of HERG in HEK cells. Cells expressing HERG were labeled with [^{35}S]methionine/[^{35}S]cysteine and chased with unlabeled methionine/cysteine for intervals of 0, 3, 6, and 24 h, followed by immunoprecipitation and detection of HERG protein by western blotting. The cells were treated with 10 μM DAG during the labelling and the first hour of the chase. No significant changes in the distribution of the immature versus mature HERG between control and DAG-treated. The experiment was repeated three times

DAG effects is mediated by dynein

Dynein is required for transport of endosomes along microtubules from the cell surface to the cytosol. To interfere with this transport mechanism, we used the microtubule depolymerizing agent nocodazol. Pretreating cells for 60 min with nocodazole (200 ng/ml; Sigma) inhibited the DAG induced internalization (Fig. 4). Similarly, when pretreating the cells for 60 min with 50 μM of a dynamin inhibitor peptide, DIP (Tocris, Bristol, UK), which has previously been established as a specific inhibitor of endocytosis [25], the effect of DAG on surface HERG expression was inhibited, in the same manner as when cells were pretreated with nocodazol (Fig. 4). A scrambled, inactive control peptide was without an effect. Taken together, these data suggest that DAG stimulates an endocytic uptake of HERG proteins from the cell surface.

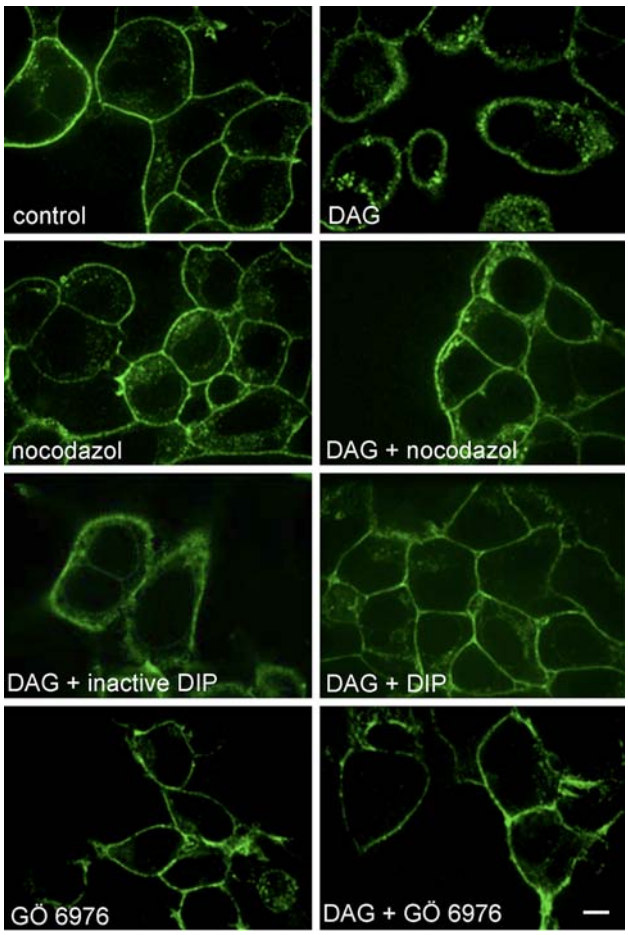

Fig. 4 Mechanisms of internalization of HERG. Confocal images of HEK293 cells stably expressing HERG and immunostained for HERG (*green*) showing that pretreating cells with either nocodazole (200 ng/ml for 60 min), dynamin inhibitor peptide (DPI, 50 μM for 60 min) or PKC-inhibitor GÖ 6976 (GÖ, 1 μM for 60 min) sustains surface expression of HERG and inhibits the DAG-induced internalization, whereas an inactive DIP analogue cannot inhibit DAG-evoked internalization. In all the experiments, the cells were treated with 10 μM DAG for 60 min in the continuous presence of respective inhibitor. *Size bar* 10 μm

DAG- induced endocytosis of HERG is PKC dependent

Since DAG is an effective PKC activator, a plausible pathway for the observed DAG-induced effect would be through PKC. To test this possibility, we used the PKC inhibitor GÖ6976 (Biosource International, CA, USA) known to effectively inhibit the classical PKCα and *β* isoforms, to attenuate the effect of DAG on HERG. Pretreatment with GÖ6976 (1 μM for 60 min) completely inhibited the reductive effect of DAG (Fig. 4). HEK293 cells express both PKCα, *β*I and *β*II (and the PKC*δ* isoform), and to test if one or both isoforms are involved we used HBDDE (Biomol Research Laboratories, Plymouth Meeting, PA, USA), a specific PKCα inhibitor. A 1-h pretreatment with the inhibitor (50 μM) did not significantly inhibit the DAG-induced endocytosis (data not shown), implying that PKCα is not involved in mediating the DAG-induced endocytosis of HERG protein. To confirm the immunocytochemical data, whole-cell patch-clamp recordings were performed on cells preincubated with 1 μM GÖ6976. As can be seen in Table 3, preincubating the cells with GÖ6976 totally abolished the DAG-evoked decrease in the HERG current. We also tested whether 10 μM of DAG was able to evoke a translocation of PKC isoforms from the cytosol to the membrane fraction. We could not observe a significant translocation of any isoform from the cytosolic to the membrane fractions, but a substantial amount of PKC*β*I was detected in the membrane fractions of the cells. Also, PKCα was detected in the membrane fractions (Fig. 5).

To further investigate the regulation of HERG channels, we stimulated the cells with 100 μM carbachol to activate membrane receptors. As can be seen in Fig. 6, in these cells, the HERG current was decreased. Furthermore, the carbachol-evoked decrease in HERG current was attenuated in cells treated with 1 μM GÖ6976. In addition, incubating the cells with 100 μM carbachol caused a marked decrease in HERG protein surface expression (Fig. 7). This decrease was not observed in cells pretreated with GÖ6976.

Table 3 Effects of GÖ6976 on DAG-evoked decrease in HERG current

	*p*A/*p*F
Control	57.8 ± 4.4
DAG	34.5 ± 5.7*
DAG + GÖ	65.0 ± 4.9

Peak tail currents were measured as described in Fig. 1a

The cells were preincubated with 10 μM DAG for 60 min, or with 1 μM GÖ6976 for 60 min and then with 10 μM DAG in the presence of GÖ6976 for another 60 min

Tail currents at −60 mV were recorded following depolarization to +40 mV for 2 s from the holding potential of −80 mV. The data given are the mean ± SEM of 3–12 separate experiments

*$P < 0.05$

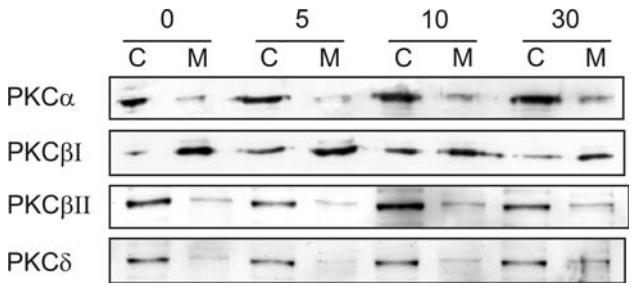

Fig. 5 Lack of translocation of PKC isoforms in response to DAG in HEK293 cells stably expressing HERG. **a** The cells were treated with 10 μM DAG for the indicated times (min), and the cells were fractioned into cytosolic *c* and membrane *m* fractions. The blots show the effect of DAG on the distribution of PKCα. PKC*β*I, PKC*β*II, and PKC*δ*. The experiments were repeated three times

HERG is present in early endosomes

Next, we examined whether the punctuate clusters of HERG appearing in the cytosol, upon exposure to DAG, would be localized in endosomes or lysosomes. As seen in Fig. 8, HERG and the early endosome marker EEA1 co-localizes upon a 60-min exposure to DAG in small clusters close to the cell surface. The co-localization with EEA1 was clearly time-dependent since it was not evident with either a 30-min exposure to DAG, i.e. too short a period, or with a 24-h exposure, too long (data not shown). Co-localization with HERG and the lysosomal marker Lamp-1 was not seen at any of the time points used. These results suggest that DAG targets the HERG channel for endosomes not leading to lysosomal degradation.

DAG causes a reduction of HERG channels in SH-SY5Y cells

The above experiments were made in cells overexpressing HERG channels. To verify our observations in cells endogenously expressing HERG channels, we used SH-SY5Y neuroblastoma cells. These cells express both HERG channels and the truncated HERG1b channels [26]. Incubating the cells with 10 μM DAG for 60 min resulted in a significant decrease in membrane expression of HERG, but not of HERG1b, as measured by western blot. Pretreatment with the PKC inhibitor GÖ6976 completely abrogated the effect of DAG on HERG (Fig. 9a, b).

In patch-clamp experiments, the relative tail currents were markedly suppressed following applications of 10 μM DAG, -0.61 ± 0.09 versus control of -0.94 ± 0.01 ($n = 8, p < 0.05$). In addition, perfusing the cells with

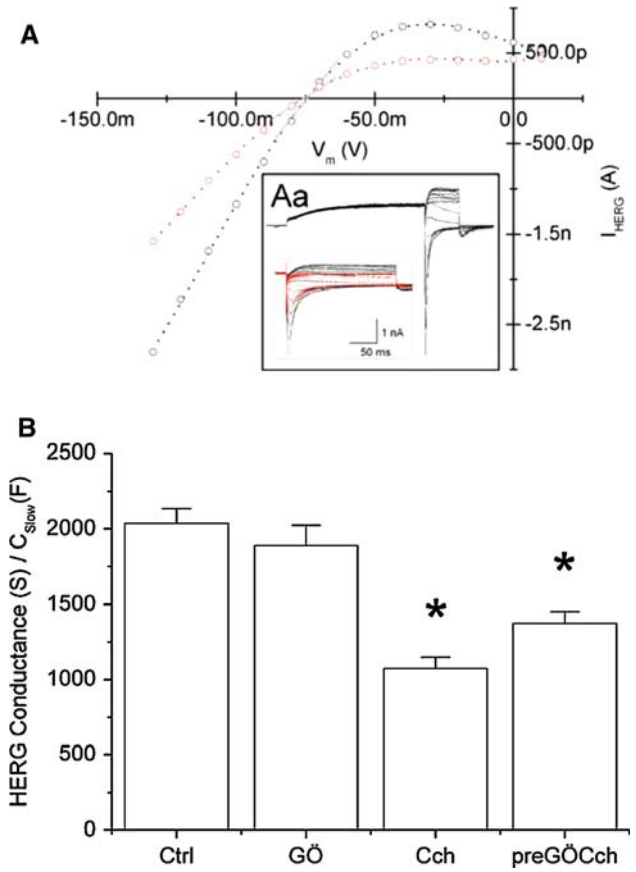

Fig. 6 Carbachol decreases HERG current density. **a** Current–voltage plot of whole-cell HERG currents showing the decrease in HERG conductance in response to Cch incubation. *Dotted lines* are the 4-order polynomial functions fitted to data (*circles*) in control conditions (*black*) or after incubation in 100 μM Cch (*red*). Conductance was measured by a linear fit (*grey line*) to polynomial function, fit range 20 mV centred at E_{HERG}. *Aa* Full-length 1.4-s current traces in response to a voltage protocol used. The protocol constituted of 15 sweeps, each starting from a holding potential of –80 mV. After the 800-ms-long depolarization to +20 mV, the membrane was clamped to a series of potentials between +10 and (−140 mV) for 200 ms and then returned to the holding level. Inside the *grey box* are representative recordings from control cell (*black*) and Cch exposed cell (*red*) in an extended time scale. **b** Summary of HERG conductance measurements done as described in **a**. *Bars* represent the mean ± SEM of membrane conductance related to its capacitance. Control (*Ctrl*) $n = 66$, 1 μM GÖ6976 incubation (GÖ) $n = 31$, 100 μM carbachol incubation (*Cch*) $n = 25$, pre-incubation in GÖ + Cch incubation (preGÖCch) $n = 38$. *$p < 0.001$ using the two-sample independent *t* test. Pre-incubation with GÖ6976 significantly increase conductance compared with Cch-treated cells

50 μM of the phospholipace C activator *m*-3M3FBS significantly decreased the tail current (−0.44 ± 0.04 vs control of −0.88 ± 0.03, $n = 4$, $p < 0.05$). Pretreatment of the cells with GÖ6976 did not inhibit the effect of DAG (data not shown). However, in cells pretreated with 100 nM PMA to downregulate PKC, the effect of DAG on the current was abolished (−0.84 ± 0.04 vs control of −0.89 ± 0.02, $n = 5$, $p = 0.37$) (Fig. 9c). The normalized values are shown in Fig. 9d. Thus, also in cells expressing HERG endogenously, DAG reduces the membrane expression of the channels protein through a PKC-dependent mechanism.

Discussion

This study demonstrates that the current mediated by the voltage-sensitive potassium channel HERG can be depressed by DAG and, for the first time, that the mechanism for the decrease is a PKC-mediated, dynamin-dependent endocytosis of the channel. Our results introduce a new mechanism of HERG channel regulation.

Many G protein-coupled receptors evoke the production of DAG by two mechanisms: first, a rapid and transient production of DAG as a result of PLC activation, followed by a slower and more prolonged DAG production due to activation of phospholipase D (PLD) [27, 28]. The difference in magnitude and duration of the DAG signal may then result in activation of specific PKC-isoenzymes and activation of separate signalling pathways, e.g., phosphorylation of channel proteins or proteins regulating endocytosis. The prolonged increase in DAG thus adds a new level of regulation of channel function, e.g., by activating a PKC-evoked endocytosis of the channel protein. This effect may be of significant importance in the regulation of channel function.

The effect of PKC in regulating HERG channels is controversial. Barros et al. [20] showed that in HERG-expressing *Xenopus* oocytes, thyrotropin-releasing hormone (TRH) through its receptor evoked a decrease in current through a protein kinase C-dependent acceleration of deactivation, and slower time course of activation. The effect was mimicked by phorbol 12-myristate 13-acetate (PMA), and inhibited by blocking PKC. In another study, angiotensin II evoked a pronounced irreversible decrease in I_{kr} in myocytes and in HEK cells expressing HERG [29]. The effect was mimicked by stimulating the cells with PMA, and was attenuated if PKC was blocked. The above results are thus in line with our observations, i.e., that both the DAG-evoked and the carbachol-evoked decrease in HERG current was attenuated when PKC was blocked. However, in GH_3B6 rat pituitary cells, TRH was shown to decrease ERG currents by shifting the voltage dependence of activation and by accelerating the time course of deactivation in a PKC-independent manner [30]. In this study, PMA evoked a PKC-independent shift in voltage dependence of ERG activation and a decrease in the current, but not a decrease in maximal tail current amplitude. Interestingly, PKC was necessary for the recovery of a transient TRH-evoked decrease of rat ERG current in GH_3 rat pituitary cells [31]. If PKC was blocked, the TRH-evoked

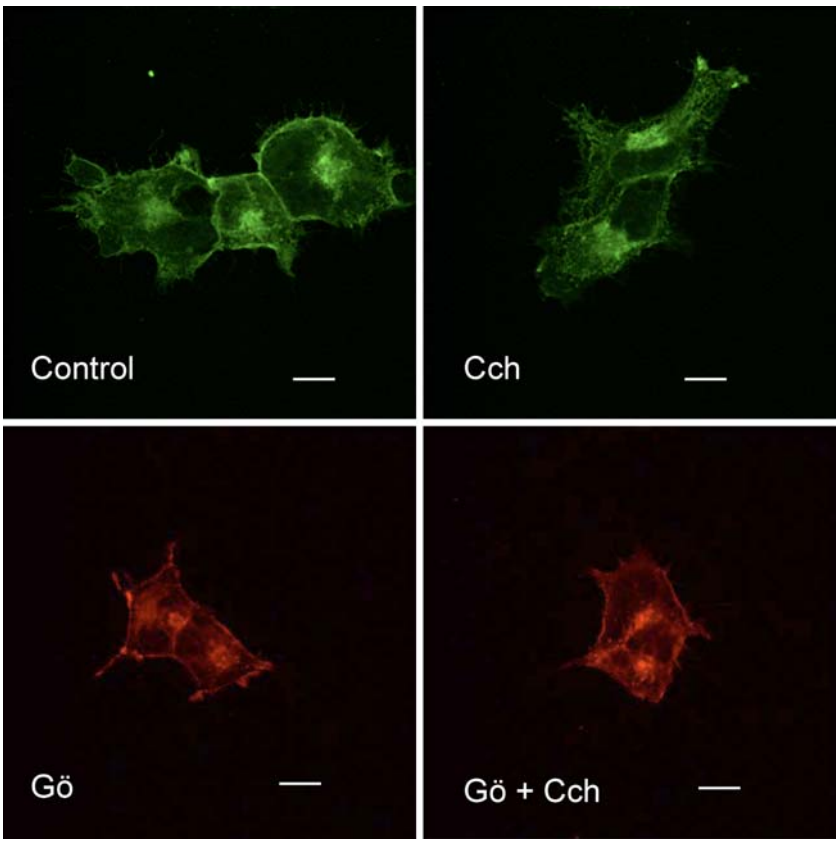

Fig. 7 Carbachol decreases HERG surface expression. Confocal images of HEK293 cells stably expressing HERG and immunostained for HERG (*green*) showing that stimulating the cells with carbachol (100 μM for 60 min) evokes internalization of the channel protein. Pretreatment of the cells with the PKC-inhibitor GÖ6976 (GÖ, 1 μM for 60 min) sustains surface expression of HERG and inhibits the carbachol-induced internalization. *Size bar* 10 μm

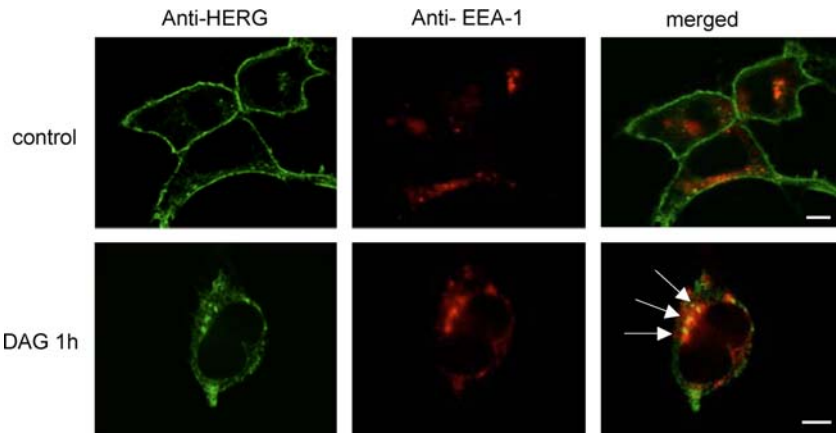

Fig. 8 Internalized HERG is present in early endosomes. HERG (*green*) co-localizes, upon 60 min of DAG treatment (10 μM), with the early endosome marker EEA-1 (*red*). Co-localization of HERG and EEA-1 is indicated by *yellow*. *Size bar* 10 μm

decrease in current became irreversible. Furthermore, in *Xenopus laevis* oocytes heterologously expressing HERG, PMA decreased the current and shifted the voltage-dependence of HERG activation towards more positive potentials. This effect was suggested not to be mediated by a direct PKC-evoked phosphorylation of the channel, as all except one PKC-dependent phosphorylation sites were mutated [19]. However, in a recent investigation, Cockerill et al. [21] showed that stimulating HERG-expressing cells with PMA, OAG or metacholine, PKC decreased HERG current by a PKC-mediated phosphorylation of the pore-forming unit of the channel. Thus, PKC may function either as an enhancer or an inhibitor of the channel function. This discrepancy may, in part, be the result of the different model systems used in the investigations. Different cell systems may express different isoforms of PKC, further complicating the interpretation of the results. It would thus be helpful to investigate which isoforms of PKC that mediate the observed effects. Although a role for either PKCα and β is implicated by the use of the fairly PKCα/β selective inhibitor Gö6976 (Cockerill et al. [21], and our study), more precise results would be obtained by downregulating PKC isoforms using specific siRNA constructs.

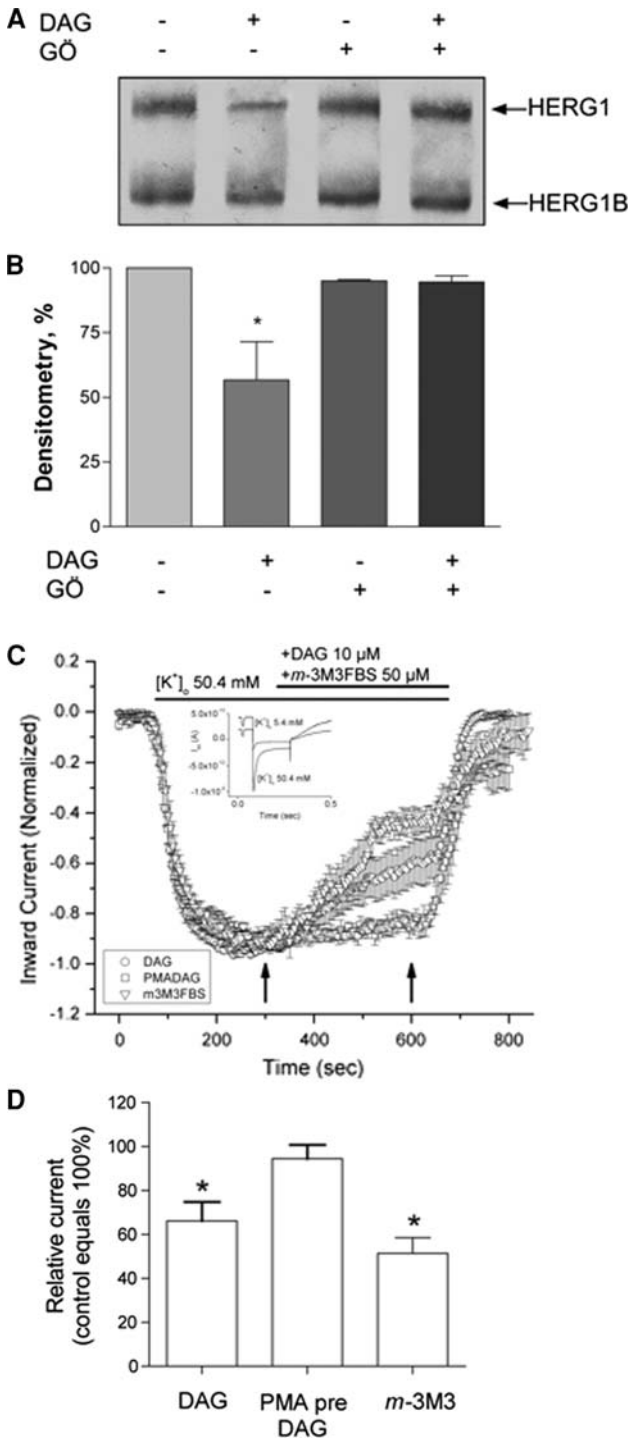

Fig. 9 DAG decreases HERG membrane expression and current in SH-SY5Y cells. **a** Western blot analysis of HERG in membranes from SH-SY5Y cells treated with 10 µM DAG for 1 h. **b** Summary of densitometric analysis of the distribution of HERG in the membrane fraction. The values given are the mean ± SEM of *N* experiments. **c** HERG-like currents present in SHSY5Y cells are reduced in response to treatment with DAG and PLC activation. Tail currents were markedly suppressed following applications of 10 µM DAG (*circle*), $n = 8$. The reductive effect of DAG was prevented by a 3-h pre-incubation with 100 nM PMA (*square*), $n = 5$. Furthermore, 50 µM of the PLC-inhibitor *m*-3M3FBS (*triangle*), $n = 4$, decreased the tail current. A change from 5.4 to 50.4 mM K^+ extracellular solution (control) enhanced tail currents (see *inset*). Amplitudes are presented relative to the largest peak found during control period (considered as −1). The wash-in periods of high $[K^+]_0$ solution and subsequent drug application are indicated with *horizontal lines*. Values at 300 and 600 s time points (marked with *arrows*) were selected to represent control and treated responses, respectively. **d** The currents shown in (**c**) were normalized as described in "Materials and methods". Each *bar* gives the mean ± SEM of 4–8 separate measurements. *$p < 0.05$

A further twist was introduced when it was shown that the PLC-evoked reduction of PIP_2 was the signal that reduced HERG activity [16]. According to this investigation, DAG-mediated activation of PKC was without any effects. A role for PIP_2 in our experiments also seems apparent, as the charbachol-evoked decrease in HERG conductance was only in part restored in cells pretreated with the PKC antagonist GÖ6976. A PIP_2-dependent regulation has also been proposed for the neuronal KCNQ potassium channel, although both calcium and PKC are apparently participating in the fine-tuning of the regulation (see [32] for a review).

In other potassium channels, an effect of PKC is also evident. A rapidly activating delayed rectifier potassium channel was enhanced by PKC through a reduction in C-type inactivation [33], whereas a G-protein gated inwardly rectifying K channel was inhibited for a prolonged period after transient stimulation with carbachol [34]. In these experiments, inhibition of PKC attenuated the effect of carbachol only in part, in a manner similar to our results. Furthermore, transforming growth factor $\beta 1$ regulated Kir2.3 channels via a PKC-mediated down-regulation of the conductance [35], whereas the Kir2.1b channel and a G-protein-coupled inward rectifier channel were inhibited by a direct PKC-mediated phosphorylation of the channel proteins [36, 37]. In contrast, the surface expression of the ROMK1 channel has been shown to be critically dependent on PKC-evoked phosphorylation, as inhibition of PKC or mutating PKC phosphorylation sites in the channel significantly decreased the membrane expression of the channel protein [38]. Thus, different channels are regulated by PKC through different mechanisms.

The expression of proteins on the cell surface, e.g., tyrosine kinases [39], G-protein coupled receptors [40] and some ion channels, can be regulated by endocytosis. The surface expression of the epithelial sodium channel (ENaC), was regulated by an ubiquitin-dependent endocytosis [41]. A few reports indicating the importance of endocytosis as a regulator mechanism of voltage-gated potassium channels have also been published. Suppression of Kir1.1 was regulated by clathrin-dependent endocytosis [42], whereas a tyrosine kinase-induced inhibition of Kv1.2 was mediated by a dynamin-dependent endocytosis [39]. Furthermore, the surface expression of another potassium

channel, Kv1.5, was also regulated by a mechanism dependent on endocytosis [43]. We have previously shown that the ceramide-induced suppression of the voltage-gated potassium channel HERG is regulated by ubiquitin-mediated lysosomal degradation [18].

Our results suggested that PKC evoked dynamin-dependent endocytosis of HERG channels. In both cardiac myocytes and in neurons, PMA evoked a substantial decrease in the ATP-sensitive potassium channel (K_{ATP}) current through a PKC-evoked internalization via dynamin-dependent endocytosis [44]. In these experiments, no phosphorylation of the channel was observed, but there was an activation of the dynein motor complex. In addition, PKC stimulated the endocytosis of $GABA_A$ receptors through a dynamin-dependent pathway [45]. It is thus possible that the prolonged or irreversible decrease in HERG currents observed in previous studies in response to activation of PKC is, at least in part, the result of a substantial internalization of the channel protein, in a manner similar to what we report in the present study.

Treatment with DAG did not affect the surface expression of HERG1b. Presently, we do not know the reason for this. However, in HERG1b, the amino terminus is truncated compared with HERG. According to the results of Cockerill et al. [21], this domain is important for PKC-mediated phosphorylation and inactivation of HERG. Further studies will reveal whether this domain is also important for the internalization of HERG. Interestingly, the PKC inhibitor GÖ6976 attenuated the internalization of HERG evoked by DAG, but was ineffective in attenuating the DAG-evoked decrease in HERG tail current in SH-SY5Y cells. However, treatment with PMA, which down-regulates all classical and novel isoforms of PKC, clearly blocked the effect of DAG. Whether this result is due to different isoforms of PKC in HEK cells and SH-SY5Y or to the presence of HERG1b in the SH-SY5Y cells is presently not known.

It must be pointed out that activation of PKC may also enhance surface expression of channel proteins. Lan et al. [46] and Lin et al. [47] showed that PKC both increased the NMDA channel opening rate and delivered new NMDA channels to the membrane by a mechanism dependent on PKC. The latter mechanism was through SNARE-mediated exocytosis. In addition, a 5-HT3 receptor-mediated current in *Xenopus* oocytes was enhanced by a PKC-mediated increase in the amount of receptor protein on the cell membrane [48].

In the heart, autonomic signalling through α-adrenergic and muscarinergic receptors are important determinants for the regulation of contractility and heart rate [49, 50]. As these receptors couple to the PLC-signalling pathway and the production of DAG and activation of PKC, they are probably of importance in regulating HERG. This is supported by clinical observations in patients with LQTS. These patients develop arrhythmias during physical or emotional stress [51], which would suggest a link between adrenergic stimulus and HERG potassium activity. The β-adrenergic link to HERG is fairly well studied, showing that elevated levels of cAMP regulate HERG channel activity through protein kinase A. The α-adrenergic link is, however, not that well studied. One plausible link could be the DAG-induced suppression of HERG, since it is known that adrenergic activity activates PLC [15] and generates DAG through hydrolyses of PIP_2. It is evident that the relatively slow internalization of HERG observed in our study cannot explain the sudden onset of arrhythmia. However, as receptor-mediated production of DAG and the activity of PKC is biphasic [27, 52], the latter prolonged phase can affect HERG expression on the plasma membrane. This could, at least in part, contribute to the onset of arrhythmia during, e.g., emotional stress, especially in conjunction with the reported inhibitory effect of receptor-mediated decrease in PIP2 on HERG function [16].

In conclusion, DAG is a ubiquitous lipid second messenger strongly associated with PKC activation. This lipid has also been shown to be associated with regulation of ion channels. In addition to the recent observation that HERG can be down-regulated by a direct phosphorylation by PKC [21], we show an additional mechanism for the regulation of plasma membrane expression of HERG, i.e., a DAG-evoked, PKC-mediated removal of the channel from the cell surface. The plausible mechanism of the internalization is a dynamin-dependent endocytosis of the channel.

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