Downregulation of the HERG (KCNH2) K+ channel by ceramide: evidence for ubiquitin-mediated lysosomal degradation

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Summary

The HERG (KCNH2) potassium channel underlies the rapid component of the delayed rectifier current (I_{kr}), a current contributing to the repolarisation of the cardiac action potential. Mutations in HERG can cause the hereditary forms of the short-QT and long-QT syndromes, predisposing to ventricular arrhythmias and sudden cardiac death. HERG is expressed mainly in the cell membrane of cardiac myocytes, but has also been identified in cell membranes of a range of other cells, including smooth muscle and neurones. The mechanisms regulating the surface expression have however not yet been elucidated. Here we show, using stable HERG-expressing HEK 293 cells, that ceramide evokes a time-dependent decrease in HERG current which was not attributable to a change in gating properties of the channel. Surface expression of the HERG channel protein was reduced by ceramide as shown by biotinylation of surface proteins, western blotting and immunocytochemistry. The rapid decline in HERG protein after ceramide stimulation was due to protein ubiquitylation and its association with lysosomes. The results demonstrate that the surface expression of HERG is strictly regulated, and that ceramide modifies HERG currents and targets the protein for lysosomal degradation.

Key words: Sphingolipids, Channels, Internalisation, Ubiquitin, HERG, Ceramide

Introduction

HERG encodes the α-subunit of the potassium channel underlying the rapid component of the cardiac delayed rectifier current (I_{Kr}) (Sanguinetti et al., 1995). This current plays a crucial role in the repolarisation of the myocardium with impairment through mutation of HERG or block by drugs being a common cause of the long-QT syndrome (LQTS) (Curran et al., 1995; Keating and Sanguinetti, 2001; Redfern et al., 2003), which predisposes to cardiac arrhythmia and may occasionally cause sudden death. Recently one form of the short-QT syndrome, a novel inherited arrhythmogenic disorder (Gussak et al., 2000), was identified as arising from a gain-of-function mutation in HERG (Brugada et al., 2004). HERG expression is not limited to cardiac tissue, with HERG current also regulating the excitability of neurones (Chiesa et al., 1997), jejunal smooth muscle (Farrelly et al., 2003) and pancreatic β-cells (Rosati et al., 2000). Furthermore, HERG is present in a variety of tumour cell lines of different histogenesis, as well as in primary human cancers (Pillozzi et al., 2002; Wang et al., 2002; Crociani et al., 2003; Lastraioli et al., 2004).

The HERG channel represents a typical potassium channel with six α-helical transmembrane segments, one of which functions as a voltage sensor, and a highly selective ion conduction pathway in the linker between transmembrane segments S5 and S6 (Tseng, 2001). HERG protein is synthesised in the endoplasmic reticulum (ER), and undergoes N-linked glycosylation, which increases channel protein stability (Gong et al., 2002).

Previous studies suggest that the sphingolipid ceramide, which is synthesised de novo or by agonist-dependent activation of sphingomyelinases, may modulate the HERG current. In rat pituitary GH3 cells an ERG current, identified on molecular, biophysical and pharmacological basis (Barros et al., 1997; Schäfer et al., 1999), is inhibited by ceramide (Wu et al., 2001). Similarly TNF-α, which can induce sphingomyelinase activation and ceramide production, was recently shown to reduce the HERG current via reactive oxygen species (ROS) (Wang et al., 2004). In addition to ROS, ceramide signalling also results in activation of protein kinase C and alteration of protein kinase B activity (Ruvolo, 2001; Ramström et al., 2004) both of which modulate HERG channel function (Barros et al., 1998; Thomas et al., 2003; Zhang et al., 2003). However, little is known of the effects of ceramide on

HERG channel expression, function and trafficking in mammalian cells

With regard to the action of ceramide on cardiac myocytes, it has been shown to mediate the immediate negative inotropic effect produced by the cytokine interleukin-1β in adult rat ventricular myocytes (Schreur and Liu, 1997). This effect occurs mainly by an inhibition of L-type calcium channels by ceramide (Schreur and Liu, 1997; Liu and Kennedy, 2003). Despite this, ceramide enhances cardiac contractile function (Liu and Kennedy, 2003; Relling et al., 2003). Ceramide also induces apoptosis and inhibits proliferation of cardiomyocytes (Levade et al., 2001). Furthermore, levels of ceramide are elevated significantly prior to cardiomyocyte apoptosis induced by ischemia-reperfusion (Bielawska et al., 1997) or TNF-α treatment (Krown et al., 1996). Similarly there is a wealth of information about the role of ceramide as a trigger of apoptosis in cancer cells (Ogretmen and Hannun, 2004) and the induction of cell death by ceramide can occur through activation of a multitude of cellular pathways. On the other hand the importance of HERG to tumorigenesis is increasingly recognised. In tumour cells the modulated expression of full length and truncated isoforms of the HERG protein during the cell cycle determines the resting membrane potential and so progression through the cycle (Crociani et al., 2003). In addition to the role in proliferation (Pillozzi et al., 2002; Wang et al., 2002; Crociani et al., 2003), the HERG channel also regulates tumour cell apoptosis (Wang et al., 2002) and invasiveness (Lastraioli et al., 2004). As both ceramide and HERG has been implicated in the same processes it is of great interest to establish what effects ceramide has on the HERG channel.

In this study, we have shown, using HERG-expressing HEK293 cells, that ceramide evokes a time-dependent decrease in the HERG current and in the surface expression of the HERG channel protein. The underlying mechanism for the observed decrease was shown to be ubiquitylation of the HERG channel upon ceramide stimulation with the targeting of the protein to lysosomes.

Materials and Methods

Cell culture
HEK293 cell line stably expressing HERG in the pcDNA3.1 expression vector was selected 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). For electrophysiological study, cells were harvested and plated on plastic Petri dishes.

Patch-clamp recording
Whole-cell patch-clamp recordings were performed using an EPC-9 amplifier and Pulse/Pulsefit software (Heka, Lambrecht, Germany) as described previously (Paavonen et al., 2003). The electrodes had resistances of 2-4 MΩ when filled with 150 mM KCl, 2 mM MgCl$_2$, 5 mM BAPTA, 5 mM Mg$_2$ATP$_3$ and 10 mM HEPES, pH 7.2. The 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. C$_6$-ceramide and dihydro-C$_6$ (Biomol Research Laboratories, Plymouth Meeting, PA, USA) were dissolved in ethanol and DMSO respectively and added to the extracellular solution (the final vehicle concentration was 0.1%). All experiments were carried out at room temperature (22-24°C). The whole-cell recordings capacitance was compensated, as was series resistance (before which the average was 4.5±0.3 MΩ, n=44) by at least 75%.

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 labelled with 1 mg/ml Sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL) in PBS (30 minutes, +4°C). After two washes non-reacted biotinylation reagent was quenched with 100 mM glycine in PBS (20 minutes, +4°C) and after washes cells were lysed and HERG protein was immunoprecipitated (see Immunoprecipitation methods). The precipitated HERG proteins were subjected to 6% SDS-polyacrylamide gel electrophoresis and biotin-labelled HERG was detected by horseradish peroxidase-conjugated streptavidin (1:500; Pierce).

Western blot analysis
Membrane fractions were prepared as previously explained (Zhou et al., 1998b). Briefly, cells were incubated with C$_6$-ceramide and then scraped from the plates and lysed with 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). Cells were sonicated and then ultracentrifuged (100,000 g for 1 hour). 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 hour at room temperature followed by incubation with primary antibodies: anti-HERG (1:1000; Alomone Laboratories, Jerusalem, Israel) and secondary antibody (anti-rabbit, 1:2500; Pierce).

Metabolic labeling
Cells were starved for 1 hour in serum-free DMEM without methionine and cysteine, and containing 0.25% BSA. The medium was then replaced with the same DMEM but containing [^{35}S]methionine/cysteine (100 μCi/ml; Amersham), in which the cells were incubated for 1 hour, after which the labelling was stopped by changing to DMEM with unlabelled methionine and cysteine. Cells were exposed to C$_6$-ceramide (10 μM) for 2 hours, i.e. during the labelling and then the first subsequent hour. Cells were lysed at different time intervals following ceramide exposure (0, 4, 8 and 24 hours) and HERG protein was immunoprecipitated with anti-HERG (see Immunoprecipitation), subjected to 6% SDS-polyacrylamide gel electrophoresis and ^{35}S-labelled HERG proteins were visualised with autoradiography.

Immunoprecipitation
Cells were treated with either the proteasome inhibitors lactacystin (Calbiochem Merck Biosciences, La Jolla, CA; 5 mg/ml for 24 hours) or MG132 (Calbiochem; 20 mM for 1 hour), or the lysosome inhibitors, bafilomycin A1 (Calbiochem, 0.25 mM for 1 hour) or folimycin (Calbiochem, 1 μM for 1 hour) prior to ceramide treatment (10 μM; 1 hour). After stimulation cells were lysed in buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 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 hours at 4°C and washed three times with washing buffer (250 mM NaCl, 0.1% NP40, 50 mM Tris-HCl 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 anti-ubiquitin clone P4G7 (1:2500; Nordic Biosite Ab, Täby, Sweden), anti-ubiquitin clone FK1 (1:1000; Affiniti Research Products, Exeter, UK) and anti-HERG (1:1000; Alomone) antibodies.

Immunocytochemistry

For immunocytochemistry, cells were plated on poly-L-lysine (Sigma)-coated coverslips and fixed with methanol-acetic acid (95:5) for 5 minutes at $-70°C$. After fixation, wells were washed with PBS, permeabilised with 0.1% Triton X-100 for 10 minutes and blocked for 30 minutes 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 minutes followed by 1 hour with goat anti-rabbit FITC-conjugated secondary antibody (1:500, Alexis Corporation, Lauflefingen, Switzerland). For colocalisation cells were incubated overnight at $4°C$ with antibodies against Lamp-1 (1:100; Santa Cruz Biotechnology, CA), followed by anti-mouse Cy3-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA).

Quantifications

Quantification of western blots was done using ImageQuant software (BioRad Laboratories, CA) and was based on at least three independent experiments. To determine the subcellular distribution of HERG channels, HEK cells were stained before and after ceramide treatment for 30 minutes. Images were obtained using confocal microscopy (Ultra View, Perkin Elmer) with a section thickness of ~0.5 µm. Labelled cells were excited at 480 nm and observed at >515 nm to detect FITC-conjugated secondary antibody. Immunofluorescence levels at plasma membrane, cytosol and nucleus were quantified from at least five cells in each confocal image using NIH-image computer program. Only healthy looking cells with normal HEK293 cell morphology were analysed.

Statistics

All data are expressed as mean±s.e.m. 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
Ceramide modulates the HERG channel

To study the role of ceramide in the regulation of the HERG channel, stably expressing HEK293 cells were incubated for 1 hour with 10 µM ceramide, 10 µM dihydro-ceramide (an inactive analog) or vehicle (control) in the extracellular solution, and then patched. In the whole-cell configuration HERG channels were activated by sequential depolarising steps and characteristically exhibited inward rectification, owing to fast inactivation, with the subsequent repolarising pulse yielding large tail currents (Zhou et al., 1998b) (Fig. 1A). The HERG current (I_{HERG}) was decreased by incubation with ceramide, whereas dihydro-ceramide had no effect (maximum tail I_{HERG} density in control and dihydro-ceramide-treated cells was 54.7±7.1 pA/pF and 69.7±7.7 pA/pF respectively, $n=4$ for both, $P>0.05$) (Fig. 1A). Step (Fig. 1B) and tail (Fig. 1C) I_{HERG} densities, as well as the instantaneous I_{HERG} density from the relief of inactivation (Fig. 1D), were significantly smaller with ceramide exposure. Over a physiologically relevant concentration range (Hannun, 1996) a dose-dependent

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

		Control	C_6
Activation	$V_{1/2}$	$-15.8±1.0$ mV	$-14.9±1.1$ mV
	k	$7.4±0.1$ mV	$7.1±0.1$ mV
	n	12	9
Inactivation	$V_{1/2}$	$-79.2±2.1$ mV	$-75.2±1.6$ mV
	k	$23.4±0.8$ mV	$23.2±0.5$ mV
	n	6	6
Deactivation (at -60 mV)	τ_f	$263±12$ mseconds	$176±9$ mseconds ($P<0.001$)
	τ_s	$1635±69$ mseconds	$944±63$ mseconds ($P<0.001$)
	n	12	9
Inactivation (at 0 mV)	τ	$12.5±0.5$ mseconds	$12.5±0.4$ mseconds
	n	7	7

Values for the voltage-dependence of activation obtained from Boltzmann fits to the peak tail currents at -60 mV (voltage protocol as in Fig. 1A). Steady-state inactivation values were determined by Boltzmann fits to peak currents recorded at $+20$ mV following a 13 msecond step to potentials between $+20$ mV and -120 mV. Prior to this hyperpolarising step HERG current was activated and inactivated during a 2 second pulse to $+40$ mV. The deactivation time constants were obtained by a double exponential fit to the tail current at -60 mV after depolarisation to $+20$ mV (voltage protocol as in Fig. 1A). Inactivation time constant was determined by a single exponential fit to the inactivating current at 0 mV following the hyperpolarisation step to -100 mV (protocol as Fig. 1D) (Paavonen et al., 2003). The numbers given are the mean±s.e.m.

decrement of the instantaneous I_{HERG} density at 0 mV was observed, though a significant difference was obtained only with 10 µM ceramide (333.6±24.9 pA/pF versus 434.5±27.6 pA/pF in control, $n=7$ for both; Fig. 1E).

The time course of the I_{HERG} decline induced by 10 µM ceramide was investigated with the whole-cell mode (Fig. 2A). After the establishment of the I_{HERG} baseline the perfusate was switched from control to 10 µM ceramide or continued in control. A significant reduction in the peak tail I_{HERG} density (compared to rundown of control I_{HERG} density) was observed (Fig. 2B).

Modulatory mechanisms of ceramide action on the HERG channel

The ceramide-induced decrease in I_{HERG} could not be explained by a change in the gating properties of the HERG channel (Table 1). After 1 hour in the presence of 10 µM ceramide, no significant alteration in the half-maximum activation voltage or slope factor was exhibited, nor was there an observable shift in steady-state inactivation compared to the control. Furthermore, there was no change in the inactivation rate, but a significant acceleration of the deactivation rate time constants with ceramide was seen.

Changes in HERG protein cell surface expression were, however, apparent. In western blots of HEK293 cells, HERG protein appears as two bands, a precursor core glycosylated (cg) endoplasmic reticulum located form (135 kDa) and a fully glycosylated (fg) mature cell surface located form (155 kDa) (Zhou et al., 1998a; Zhou et al., 1998b). Exposure to ceramide (10 µM, 60 minutes) decreased the intensity of fg HERG, but not cg HERG (Fig. 3A). Quantitative densitometric analysis showed that the plasma membrane band intensity was reduced

by 30±7% when compared to control ($P<0.05$, $n=9$). In contrast, no statistically significant alteration of the intensity of the band was observed after exposure to dihydro-ceramide

(results not shown). To further study the level of cell surface HERG expression, we labelled surface membrane proteins with a biotinylating reagent, sulfo-NHS-SS-biotin,
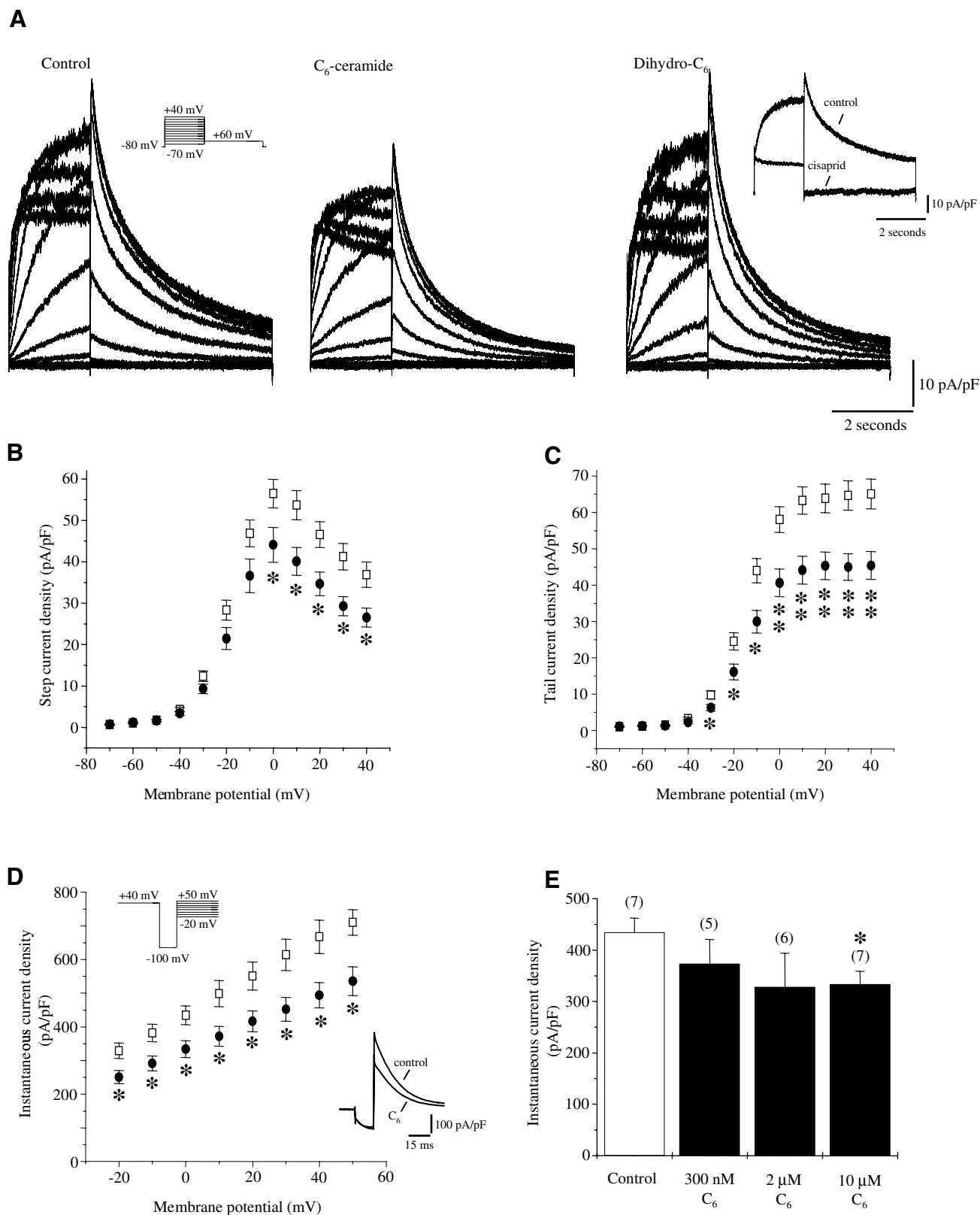
A

B

C

D

E

Fig. 1. See next page for legend.

immunoprecipitated HERG proteins with anti-HERG and detected them by streptavidin-HRP. Exposure to ceramide (10 μM, 60 minutes) reduced markedly the amount of biotin-labelled HERG (Fig. 3B). When densitometrically quantified, the intensity of the band was reduced by 42±7% ($P<0.001$, $n=3$) compared to the control.

We then performed immunostainings to determine the subcellular localisation of the HERG protein in these HEK cells. In control cells, HERG protein labelling was most intense at the cell membrane with homogenous distribution in the perinuclear region (Fig. 4B). In cells exposed to ceramide, surface staining was considerably weaker, reduced by 56% after 30 minutes and 62% after 180 minutes ($P<0.01$ for both time points), with the appearance of punctuate clusters in the cytosol (Fig. 4B). The intensity of the cytosolic HERG immunofluorescence increased by 219% after 30 minutes ($n=7$; $P<0.05$) of ceramide treatment. The change in the pattern and intensity of HERG immunofluorescence is shown in Fig. 4A. Clearly, incubation with ceramide caused a significant decrease of HERG protein at the plasma membrane and an increase of cytosolic HERG protein in conjunction with an overall loss of HERG staining. For initial investigations, using immunostainings, of this ceramide-induced decrease in HERG protein we used the lysosomal blocker bafilomycin A1, the proteosomal blocker MG132 (Alwan et al., 2003), and low-temperature (16°C) treatment. The use of low-temperature treatment, to curtail endocytosis, or bafilomycin blocked the reduction of cell surface HERG protein whereas inhibition of the proteosome had no effect (Fig. 4B).

Ubiquitylation and degradation of HERG

The turnover of the HERG channel occurs at a rather slow rate (approximately 11 hours) (Ficker et al., 2003). To exclude the possibility that the ceramide-induced decrease in the cell surface expression of the HERG channel was due to defective synthesis or trafficking of the protein we performed pulse-chase experiments. Newly synthesised proteins were labelled

with [35S]methionine and [35S]cysteine and chased with unlabelled methionine/cysteine for up to 24 hours. HERG protein was immunoprecipitated and detected by western blotting. In these experiments HERG protein was initially synthesised as the immature 135 kDa form, which was gradually converted, reaching a peak at 8 hours, to the mature 155 kDa form (Table 2). In order to separate any effects of ceramide on HERG protein synthesis from those on degradation, ceramide (10 μM) was only present for a 2 hour time period (during the protein labelling and 1 hour immediate post labelling) hence precluding direct interaction with cell surface 35S-labelled HERG protein. At each time point no significant changes were detected between control and ceramide-treated cells of the levels of neither the immature nor mature HERG protein (Table 2), indicating that ceramide does not affect HERG protein synthesis or trafficking.
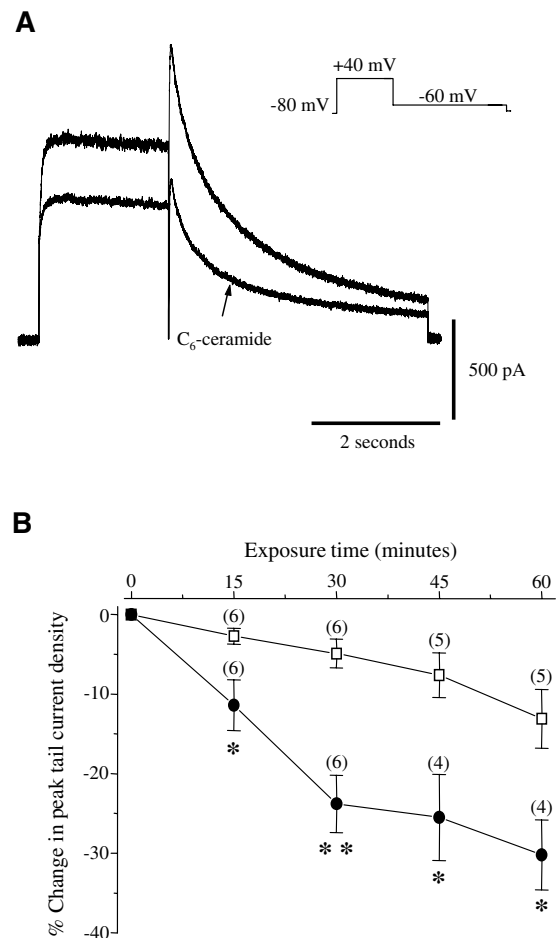
Fig. 2. Time course of I_{HERG} decline induced by 10 μM ceramide. (A) Typical recording obtained from a cell in control solution and then after 1 hour exposure to 10 μM ceramide. Tail currents at –60 mV were recorded following depolarisation to +40 mV for 2 seconds from the holding potential of –80 mV. The protocol was repeated every 12 seconds. (B) Plot of change in I_{HERG} density from baseline against time. A baseline was established during 5-10 minutes of perfusion with control solution and then solutions were changed to 10 μM ceramide (●) or control (□). Protocol as in A with I_{HERG} taken as the peak tail current. The number of cells tested are given in parenthesis (*$P<0.05$, **$P<0.001$).

Fig. 1. The effect of ceramide on I_{HERG} density in stably expressing HEK293 cells. (A) Whole-cell current recordings of I_{HERG} in the absence and presence of 10 μM ceramide or dihydro-ceramide. From a holding potential of –80 mV cells were stepped to potentials between –70 mV and +40 mV for 2 seconds with tail currents observed on repolarisation to –60mV for 4.5 seconds. Inset, Identification of I_{HERG} by the use of the inhibitor cisapride (1 μM) with the current evoked by a depolarising step to 0 mV. (B,C) Voltage-dependence of I_{HERG} density with I_{HERG} measured at the end of the depolarising steps (B) and as peak tail current (C). □, control , $n=12$; ●, 10 μM ceramide, $n=9$. Significant differences in current density were observed (*$P<0.05$, ** $P<0.001$) compared to levels in the control. (D) Instantaneous I_{HERG} density-voltage plot. Insets show the effect of 10 μM ceramide on the instantaneous I_{HERG} density at 0 mV and the voltage protocol used, which consisted of a 2 second activating step to +40 mV, a 13 msecond step to –100 mV to relieve inactivation and then a step to potentials between +50 mV and –20 mV. I_{HERG} was measured immediately following the end of the brief hyperpolarising step ($n=7$ for both control (□) and 10 μM ceramide (●); *$P<0.05$). (E) The effect of ceramide concentration on the instantaneous I_{HERG} density at 0 mV (voltage protocol as in D). The number of cells tested are given in parentheses (*$P<0.05$ compared to control current).

The most plausible mechanism for the rapid alterations in expression of the HERG channel by ceramide is that ceramide affects the internalisation and degradation of the channels at the cell surface. We investigated protein degradation and HERG protein ubiquitylation as targets for the action of ceramide in this system. After exposure to ceramide immunoprecipitations with anti-HERG antibodies were performed followed by analysis using anti-ubiquitin antibodies. In these experiments, multiubiquitylated HERG protein was observed as a high molecular weight smear (Fig. 5B) using the antibody recognising mono- and polyubiquitylated chains, P4G7. The intensity of the ubiquitylated bands was increased by ceramide treatment, although the total amount of HERG protein immunoprecipitated was reduced (Fig. 5B) so resulting in a
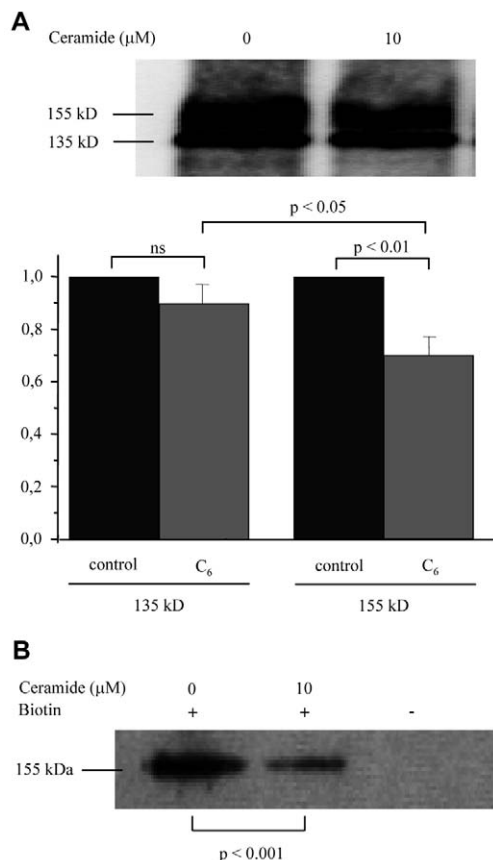

Fig. 3. Effect of ceramide on HERG protein expression. (A) HERG protein expression was analysed by western blot. Ceramide-treated (10 µM for 60 minutes) cells were lysed and ultracentrifuged as described in Materials and Methods and proteins were separated by 6% SDS-PAGE. HERG protein was detected with monoclonal rabbit anti-HERG (1:1000). The higher molecular weight band (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. Results of the densitometric analysis are shown in the lower panel. The results represent the mean±s.e.m. of nine separate experiments. (B) Specific cell surface expression of HERG protein was analysed by biotin labelling. After incubation with ceramide 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.
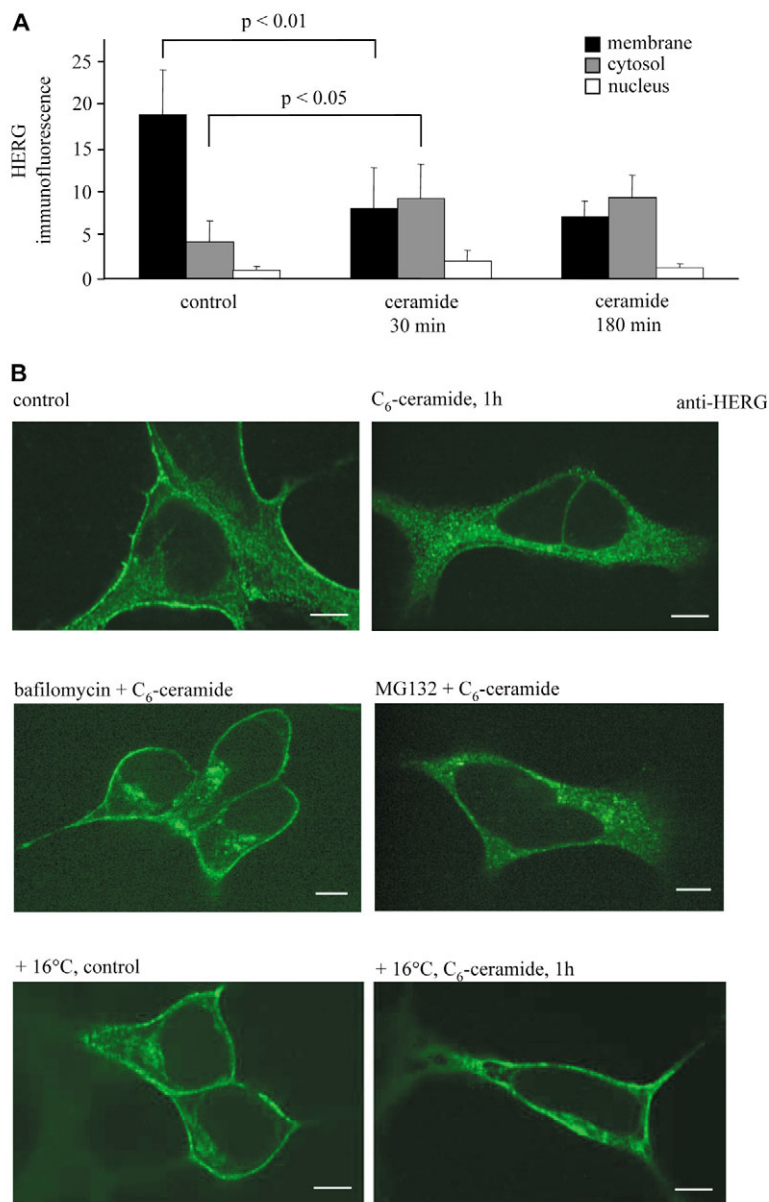

Fig. 4. Effect of ceramide on HERG cell surface expression. (A) Ceramide-induced internalisation of HERG channels in HEK293 cells. In quantitative analysis of anti-HERG immunoreactivity statistically significant changes in plasma membrane and cytosolic immunofluorescence levels were observed after 30 minutes of ceramide treatment. Results are shown relative to nuclear fluorescence levels at baseline. Each bar gives the mean±s.e.m. of seven separate experiments. (B) Confocal sections of HEK cells labelled with anti-HERG antibody. Ceramide treatment for 60 minutes induced a clear internalisation of immunoreactivity from plasma membrane to cytosol. This internalisation was inhibited by the lysosomal inhibitor bafilomycin and by low-temperature treatment (16°C, 45 minutes), which curtails endocytosis. In contrast, the ceramide-induced internalisation was not inhibited by the proteosomal inhibitor MG132. The figures shown are representative cells of three to ten separate experiments. Bar, 10 µM.

significant increase of the ubiquitin to HERG protein ratio (Fig. 5A).

Treatment of cells with MG132 increased the level of ubiquitylated HERG protein in control cells significantly (Fig. 5B). Most interestingly, the effect of MG132 was abolished when the cells were treated with both MG132 and ceramide (Fig. 5B). Similar results were seen with lactacystin, another proteosome inhibitor. In sharp contrast, incubation with either lysosomal inhibitor, bafilomycin A1 (Fig. 5B) or folimycin (data not shown) significantly enhanced the ceramide-induced

increase of ubiquitylated HERG protein. We then performed double-labelling experiments of HEK cells using antibodies for HERG and the lysosomal-associated membrane protein Lamp 1, a marker for late endosome/lysosomes (Brannvall et al., 2003). There was a significant colocalisation of HERG with Lamp 1 in these cells (Fig. 5C), supporting the view that ceramide induces HERG channel internalisation and targeting to lysosomes.

Discussion

The results presented here show that the sphingolipid ceramide modulates the HERG potassium channel in HEK293 cells by targeting the protein for lysosomal degradation. The mechanism for the decrease in the HERG channel observed at the cell membrane was shown to involve ubiquitylation of the protein with translocation into the cytosol and subsequent degradation. To our knowledge this is the first study to describe an effect of a sphingolipid on the HERG channel.

Previously, we and others have shown that ceramide regulates a number of potassium conductances (Wu et al., 2001; Ramström et al., 2004; Gulbins et al., 1997; Hida et al., 1998; Yu et al., 1999; Li et al., 1999; Chik et al., 2001). The addition of I_{HERG} to this list appears to be expected as a related inwardly rectifying potassium current of lactotrophs and neuroblastoma cells is also inhibited by ceramide (Wu et al., 2001). The inhibitory mechanism of ceramide on the $I_{K(IR)}$ of GH$_3$ lactotrophs was unresolved and disparities exist though from our present findings, or those of Wang et al. (Wang et al., 2001). Specifically a robust inhibition within one minute of ceramide application (~70% with 10 µM C$_2$) was shown and a +10 mV shift of the activation curve (Wu et al., 2001). These differences probably emanate from the cellular systems used, where such contributing factors could include
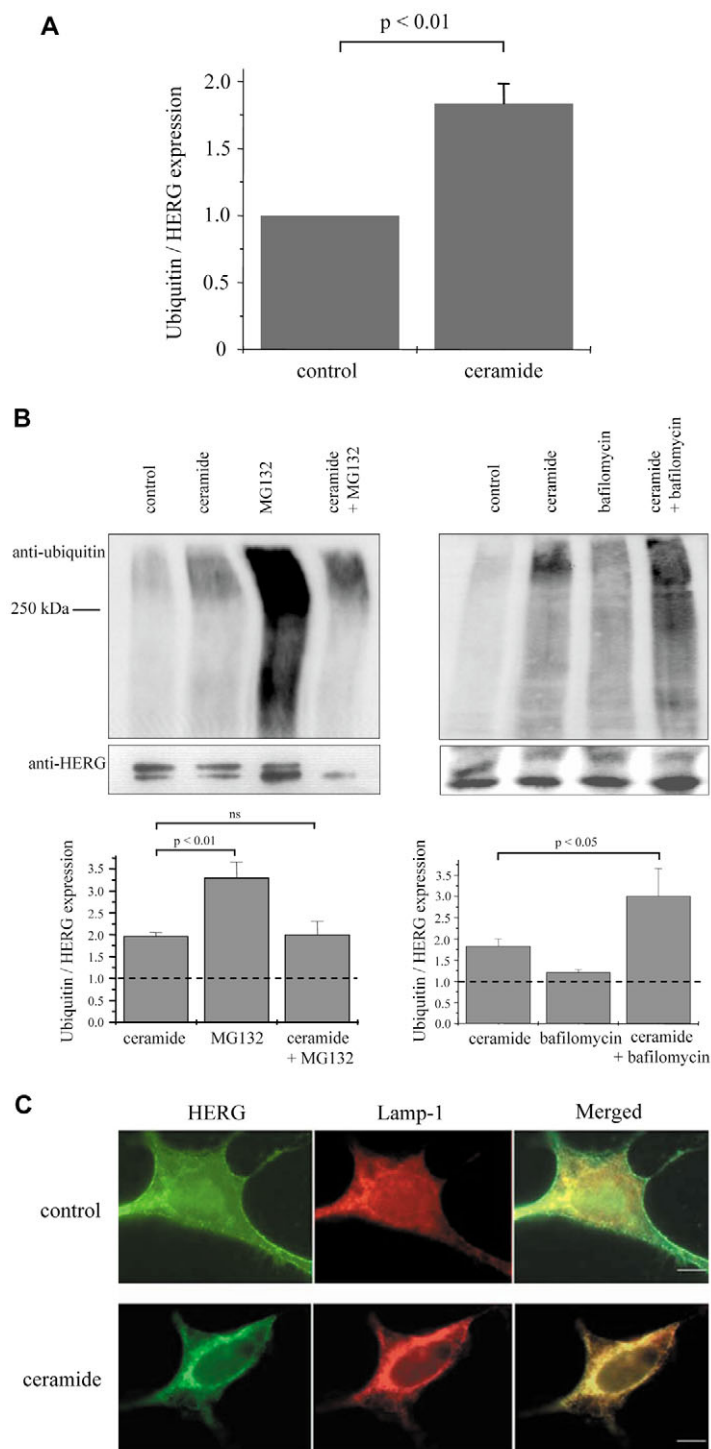

Fig. 5. Ubiquitylation of the HERG channel in HEK293 cells. (A) HERG-expressing HEK293 cells were exposed to ceramide 10 µM (60 minutes) and the HERG protein was immunoprecipitated. The amount of ubiquitin in HERG protein was determined by western blotting using an anti-ubiquitin antibody (clone P4G7) recognising both poly- and monoubiquitylated proteins. The same blot was also stained for HERG showing the levels of HERG protein in the cell lysates. The intensity of the bands was analysed and the bars show the ratio of ubiquitin and HERG in control conditions and in cells stimulated with 10 µM ceramide for 1 hour. The normalised values represent the mean±s.e.m. of five experiments. (B) Prior to treatment with 10 µM ceramide (60 minutes), HERG-expressing HEK293 cells were exposed for 60 minutes to either 20 µM MG132 or 0.25 µM bafilomycin. HERG protein was immunoprecipitated and the amount of ubiquitin was determined by western blot using an anti-ubiquitin antibody (clone P4G7). The bar graphs show the intensity of the bands as a ratio of ubiquitin and HERG. The normalised values represent the mean±s.e.m. of three experiments. (C) Downregulation of HERG through the lysosomal pathway. Control cells and ceramide-treated cells (10 µM for 60 minutes) were co-stained with antibodies for the lysosomal marker Lamp-1 (red) and HERG protein (green). The colocalisation is seen as yellow. The figures shown are representative cells of three separate experiments. Bar, 10 µM.

Table 2. Pulse-chase analysis of HERG

Time post C$_6$ (hours)	135 kDa		155 kDa	
	Control	C$_6$	Control	C$_6$
0	100	89±7	100	94±4
4	31±5	33±1	109±7	117±5
8	27±8	26±7	142±3	169±34
24	10±0	10±1	63±28	63±28

HEK cells stably expressing HERG were labelled with [^{35}S]methionine/cysteine for 1 hour and chased with unlabelled methionine/cysteine. Cells were treated with C$_6$-ceramide (10 μM) during the labelling and the first hour of the chase. The cells were lysed at the indicated times after ceramide treatment, then HERG was immunoprecipitated and subjected to electrophoresis on SDS-polyacrylamide gel for analysis, and the amount of mature and immature HERG was visualised and quantified by autoradiography and densitometric analysis. The results for both 135 kDa and 155 kDa are shown as percentage of the values at time point 0, respectively. Statistical analysis showed no differences between control and ceramide of either the mature (155 kDa) or the immature (135 kDa) forms. The data shown are mean±s.e.m. of three separate experiments.

heterogeneous ERG current properties owing to the presence of non-cardiac ERG family member subunits (Schäfer et al., 1999; Wimmers et al., 2002), and diversity of signalling (Barros et al., 1998; Schäfer et al., 1999). However, using HEK293 cells stably expressing HERG, as here, Wang et al. (Wang et al., 2001) observed that ceramide had no significant effects on I_{HERG}. The reason for this discrepancy is presently not known, but may arise from the time-dependent nature of the effect evident here, and from differences in the concentration of ceramide used.

The regulation of the cell surface stability of the HERG channel per se has not been widely studied. For the first time we provide evidence of inducible downregulation of the HERG channel. The present study shows that ceramide induces a rapid downregulation of HERG protein at the cell surface, through internalisation and degradation. The mechanism for this was due to ubiquitylation of the HERG channel by ceramide. Previous studies on epidermal growth factor and platelet-derived growth factor receptors have shown that these cell surface receptors are ubiquitylated upon ligand binding (Haglund et al., 2003; Mosesson et al., 2003; Marmor and Yarden, 2004) with subsequent endocytosis and degradation by the activity of lysosomes and/or the proteasome (Alwan et al., 2003; Duan et al., 2003). There is also a distinction as to whether a protein is mainly mono- or polyubiquitylated. The latter modification mainly targets the protein for proteasome degradation whereas monoubiquitylation can regulate protein trafficking, involving endosomes, in addition to other cellular functions (Hicke, 2001; Korhonen and Lindholm, 2004). In our experiments we observed that HERG protein is readily ubiquitylated using an antibody against mono- and polyubiquitylated chains, with further studies required to distinguish the type of ubiquitylation. In our study, we observed that ceramide treatment in the presence of bafilomycin, a blocker of lysosomes, prevented the decrease in surface HERG protein staining and increased the steady state level of ubiquitylated HERG protein suggesting an involvement of lysosomes in HERG channel degradation. Consistent with this was the colocalisation of immunoreactive HERG protein with lysosomes and late endosomes using the antibody Lamp1 (Fig. 5C). So, in HEK293 cells, the HERG
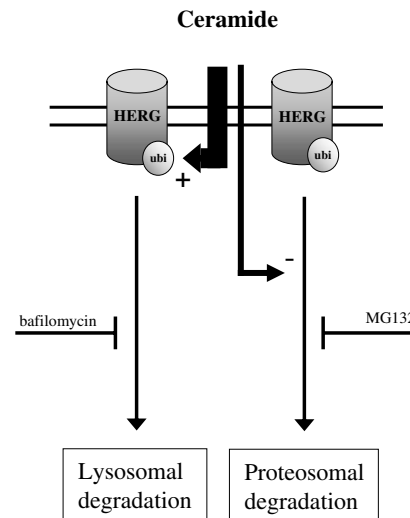

Fig. 6. Effect of ceramide on HERG channel ubiquitylation and degradation in HEK cells. As shown in this study, ceramide leads to HERG channel ubiquitylation and subsequent degradation via lysosomes. However, ceramide also inhibits to some extent the basal turnover of HERG protein through proteosomes. Bafilomycin is a lysosome inhibitor whereas MG132 and lactacystin inhibit proteosomal degradation. See Discussion for further details.

channel is mainly degraded in lysosomes after stimulation with ceramide (Fig. 6).

However, there is evidence that the HERG channel is also subject to basal ubiquitylation and degradation by the proteosomal pathway (Ficker et al., 2003). In line with this, MG132, a blocker of proteasome activity, increased the levels of ubiquitylated HERG protein in control cells (Fig. 5B; Fig. 6). Ceramide was able to decrease HERG protein ubiquitylation in MG132-treated cells, suggesting that the compound may rapidly target all HERG to the lysosomes once the proteasomes are inhibited. Apart from the HERG channel, constitutive regulation of the surface expression by ubiquitylation is observed with other ion channels including the epithelial and cardiac (Na$_v$1.5) sodium channel (Staub et al., 1997; van Bemmelen et al., 2004).

Although ceramide is capable of initiating the ubiquitin/proteosome pathway (Ogretmen and Hannun, 2004) its action on ion channels is recognised as being mediated primarily by kinase activity, in particular PKC (Ramström et al., 2004; Hida et al., 1998; Chik et al., 2001). PKC activation is reported to result in the internalisation of the ATP-sensitive potassium channel (Hu et al., 2003) and the sodium channel, Na$_v$1.7 (Yanagita et al., 2000). The activation of PKC is associated with attenuation of I_{HERG} but via a shift in the activation curve to more positive voltages (Barros et al., 1998; Thomas et al., 2003). This property is unaltered in this study as is the decrement of I_{HERG} in the presence of the PKC inhibitor calphostin C (our unpublished results), implying that the downregulation of the HERG channel by ceramide does not involve PKC. Another possible candidate is the phosphatidylinositol 3-kinase (PI3K)/PKB pathway. This can be inhibited by ceramide (Ruvolo, 2001) and reportedly promotes the translocation of ion channels to the plasma membrane (Lhuillier and Dryer, 2002; Viard et al., 2004). Additionally PI3K/PKB regulates I_{HERG}

density in stably expressing HEK cells (Zhang et al., 2003), though the underlying mechanism was not elucidated.

The ceramide-induced inhibition of the GH_3 lactotroph ERG current was abolished by the reducing agent dithiothreitol (Wu et al., 2001). It remains to be clarified whether the effect of ceramide was a consequence of ROS production. ROS mediated the TNF-α suppression of I_{HERG} of stably expressing HEK cells and canine cardiomyocyte I_{Kr}; though this was apparently not associated with alteration of the HERG protein levels (Wang et al., 2004). However it is important to note that this conclusion was drawn from western blot analysis of the 135 kDa band only, with no reference made to the surface located 155 kDa band, and therefore is analogous to the results obtained here (Fig. 3A). The I_{HERG} decrement was attributed to unspecified changes at the functional level (Wang et al., 2004). ROS modulation of I_{HERG} was shown to occur through changes in the voltage dependence of activation and inactivation, with ROS generation enhancing I_{HERG} (Taglialatela et al., 1997; Han et al., 2004). I_{HERG} kinetics were unchanged by TNF-α (Wang et al., 2004), whereas previously ROS was demonstrated to accelerate I_{HERG} deactivation (Taglialatela et al., 1997). The acceleration of the HERG channel deactivation rate by ceramide (Table 1) may result from the action of ROS, or other effectors such as PKC (Thomas et al., 2003), or from the binding of ubiquitin (or associated proteins) to particular channel domains fundamental to the retardation of this process such as the N-terminus or the S4-S5 linker (Tseng, 2001). The determination of the components in the signalling cascade initiated by ceramide and the resulting loss of surface HERG channels, as well as the alteration of deactivation, requires further investigation.

The maximal concentration of 10 μM ceramide used in this study equates to an intracellular concentration of 10-100 pmol/nmol lipid; levels that are reached following physiological and pathophysiological stimulation (Hannun, 1996). The identification of the HERG channel as a target for ceramide raises important questions as to the contribution of this interaction to different physiological and pathophysiological processes. Alterations of I_{kr} functioning as well as HERG protein levels are associated with disease conditions of the heart such as myocardial infarction, heart failure and atrial fibrillation (Tseng, 2001; Tsuji et al., 2000; Brundel et al., 2001). Moreover, in tumours given the significant role of the HERG channel and the present findings it is noteworthy that the ceramide content of tumours is decreased and can be correlated with the degree of malignant progression (Ogretmen and Hannun, 2004).

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