

Protein kinase A-mediated phosphorylation of HERG potassium channels in a human cell line

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Objective To investigate the molecular mechanism of human ether-a-go-go-related gene (HERG) potassium channels regulated by protein kinase A (PKA) in a human cell line .

Methods HERG channels were stably expressed in human embryonic kidney (HEK) 293 cells , and currents were measured with the patch clamp technique . The direct phosphorylation of HERG channel proteins expressed heterologously in *Xenopus laevis* oocytes was examined by ^{32}P labeling and immunoprecipitation with an anti-HERG antibody .

Results Elevation of the intracellular cAMP-concentration by incubation with the adenylate cyclase activator , forskolin (10 $\mu\text{mol/L}$) , and the broad range phosphodiesterase inhibitor , IBMX (100 $\mu\text{mol/L}$) , caused a HERG tail current reduction of 83.2% . In addition , direct application of the membrane permeable cAMP analog , 8-Br-cAMP (500 $\mu\text{mol/L}$) , reduced the tail current amplitude by 29.3% . Intracellular application of the catalytic subunit of protein kinase A (200 U/ml) led to a tail current decrease by 56.9% and shifted the activation curve by 15.4 mV towards more positive potentials . HERG WT proteins showed two phosphorylated bands , an upper band with a molecular mass of ≈ 155 kDa and a lower band with a molecular mass of ≈ 135 kDa , indicating that both the core- and the fully glycosylated forms of the protein were phosphorylated .

Conclusions PKA-mediated phosphorylation of HERG channels causes current reduction in a human cell line . The coupling between the repolarizing cardiac HERG potassium current and the protein kinase A system could contribute to arrhythmogenesis under pathophysiological conditions .

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In human cardiac myocytes , activation of the rapid component of the delayed rectifier K^+ current , I_{Kr} , plays a central role in repolarization of the myocardium and termination of the cardiac action potential.¹ Blockade of I_{Kr} prolongs action potential duration and produces a beneficial class III -antiarrhythmic effect.² On the other hand , excessive prolongation of action potentials can cause acquired long QT syndrome , which is known to be associated with ventricular “ torsade de pointes ” arrhythmia , a cause of sudden cardiac death.³

The human ether-a-go-go-related gene (HERG) encodes a K^+ channel with biophysical properties nearly identical to I_{Kr} ,^{4,5} and class III antiarrhythmic drugs have been shown to act via blockade of HERG channels.⁶ Mutations in the HERG gene produce human chromosome 7-linked congenital long QT syndrome (LQT2) , a disorder associated with

delayed repolarization and prolonged electrocardiographic QT intervals.⁷ These patients have a high risk for sudden

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cardiac death because of "torsade de pointes" arrhythmia.⁸

Cyclic AMP-dependent protein kinase (PKA) is a serine/threonine kinase that is involved in regulatory processes in almost all types of cells. PKA can be stimulated by extracellular signals that elevate the intracellular concentration of the second messenger cAMP. Cyclic AMP binds to the regulatory subunit of the enzyme, leading to the dissociation of regulatory and catalytic subunits. The catalytic subunit phosphorylates the substrate, which may be the effector protein or another protein that mediates the effect. Under experimental conditions, the membrane permeable analog of cAMP, 8-bromo-cyclic AMP (8-Br-cAMP), can be used to stimulate PKA directly. An increased intracellular cAMP concentration and subsequently PKA activation can be achieved with the adenylate cyclase activator, forskolin, and with the unspecific phosphodiesterase inhibitor, IBMX (3-isobutyl-1-methylxanthine). Recently, it has been demonstrated that PKA regulates HERG channels heterologously expressed in *Xenopus laevis* oocytes via direct phosphorylation of the channel protein.^{9,10}

The aim of the present study was to investigate the effects of the cAMP-PKA-system on HERG potassium channels expressed in a human cell line (HEK 293 cells). Using different activators of PKA as well as the catalytic subunit of the enzyme, we provide evidence that PKA plays an important role in the regulation of HERG channels in human cells. Finally, we examined radiolabeled HERG channels heterologously expressed in *Xenopus laevis* oocytes and demonstrated phosphorylation of both the immature core-glycosylated and the mature forms of the channel.

METHODS

Cell culture, transfection procedures, and patch-clamp recording technique

The procedures for heterologous expression of HERG cDNA¹¹ in human embryonic kidney cells (HEK 293) was described previously in detail.¹² HERG currents were recorded using the whole-cell patch-clamp configuration according to the protocols published earlier by our group.¹²

Preparation, cRNA injection, and voltage-clamp of *Xenopus* oocytes

Preparation of complementary HERG and HERG 4M (HERG carrying four mutations: S283A, S890A, T895A, S1137A) cRNAs from the corresponding cDNAs,^{10,11} as well as handling and injection of *Xenopus laevis* oocytes was performed as published previously.¹⁰ The two-microelectrode voltage-clamp configuration was used to

record currents from *Xenopus laevis* oocytes.⁶ The measurements were performed in a low K⁺ solution containing (in mmol/L) 5 KCl, 100 NaCl, 1.5 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.4).

Western blot analysis

Methods for western blot analysis have been previously described in detail.¹³ Briefly, the HERG proteins were detected with rabbit polyclonal anti-HERG antibodies (Alomone Labs, Jerusalem, Israel) at a 1:200 dilution. The antibody recognized the C-terminus ([CY] EELPPGAPQLPQEGPT corresponding to residues 1118–1133 of HERG, with the additional N-terminal cysteine and tyrosine). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (diluted 1:6000) served as secondary antibodies.

Immunoprecipitation

Uninjected oocytes and cells injected with HERG or HERG 4M cRNA taken from the same batch were injected with [γ -³²P]-ATP (0.5 μ Ci per oocyte, Amersham Pharmacia Biotech) 6 h or 24 h prior to protein isolation. All steps were performed at 4°C, unless indicated otherwise. Cells were solubilized in lysis buffer containing 1% Triton-X 100, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, one "mini" protease inhibitor cocktail tablet per 10 ml solution (Roche Diagnostics), and 50 mmol·L⁻¹ NaF, 0.2 mmol·L⁻¹ Na₃VO₄ to block protein phosphatases. Homogenized samples were incubated on ice for 1 h and spun at 10 000 × g for 10 min. Supernatant with the HERG proteins was immunoprecipitated by use of rabbit polyclonal antibodies (Alomone Labs) and Dynabeads M-280 sheep anti-rabbit IgG (DynaL, Hamburg, Germany). Beads were collected on a magnetic stand (DynaL), and then washed with lysis buffer. The beads were boiled for 5 minutes in SDS PAGE sample loading buffer to release the immune complexes. Protein complexes were electrophoresed on a 7.5% SDS polyacrylamide gel (extracted protein from 40 oocytes per lane). After this process, the gel was dried and analyzed by autoradiography (3 days or 18 days exposure time).

Chemicals

IBMX (3-isobutyl-1-methylxanthine, Sigma) and forskolin (Sigma) were dissolved in DMSO to stock solutions of 10 mmol/L and 100 mmol/L, respectively, and stored at -20°C. 8-bromo-cyclic AMP (8-Br-cAMP, Sigma) was dissolved freshly every day with bath solution to a final concentration of 500 μ mol/L. Protein kinase A (catalytic subunit; Sigma) was added freshly to the internal pipette solution (activity: 200 U·ml⁻¹). Dofetilide (N-[4-(4-(methanesulfonamino)-phenoxy)]-N-methylethylamino }

ethyl phenyl methanesulfonamide ; Pfizer Central Research , Sandwich , Kent , UK) was dissolved in distilled water , acidified by addition of HCl to make a 10 mmol/L stock solution , and stored at - 20°C . On the day of experiments , aliquots of the stock solution were diluted to the desired concentration with the bath solution .

Data analysis and statistical methods

Activation curves were fitted with a Boltzmann distribution ($G(V) = G_{max} / [1 + \exp((V_{1/2} - V) / k)]$). Statistical data are expressed as mean \pm standard deviation , where n represents the number of experiments performed . An unpaired Student ' s t test was used to compare statistical significance of the results : $P < 0.05$ was considered to be statistically significant .

RESULTS

General description

Transfection of cDNA encoding HERG channels into human embryonic kidney (HEK 293) cells resulted in a potassium current with activation and rectification properties that have been described previously , i.e. fast inactivation at positive potentials and large , slowly decaying tail currents upon repolarization (Fig. 1a) .⁴ Channels were activated by

depolarization and showed inward rectification due to C-type inactivation.¹⁴ Deactivating tail currents obtained during repolarizing steps to - 120 mV were large as a result of rapid recovery from inactivation combined with slow deactivation . A rising phase or " hook " in the tail currents due to recovery from inactivation preceded deactivation . However , in some experiments the outward currents during the test pulse were not distinguishable from endogenous delayed rectifier currents of the HEK 293 cells . The characteristic inward tail currents produced by HERG channels were specific (Fig. 2d , e) and could be used to perform this study .

HERG currents were reduced by increasing cAMP levels via activation of adenylate cyclase and inhibition of phosphodiesterase

To investigate the effects of increased intracellular cAMP levels on HERG potassium channels , we applied the adenylate cyclase activator , forskolin ($EC_{50} = 4.0 \mu\text{mol} \cdot \text{L}^{-1}$) and the broad range phosphodiesterase inhibitor IBMX ($IC_{50} \approx 10 \mu\text{mol/L}$). HERG currents were measured using a two step protocol (Fig. 1a) . A variable first step (test pulse) was applied with different potentials from - 120 mV (- 100 mV for Fig. 2) to + 100 mV (increment 20 mV) for 0.4 seconds followed by a second step at - 120 mV

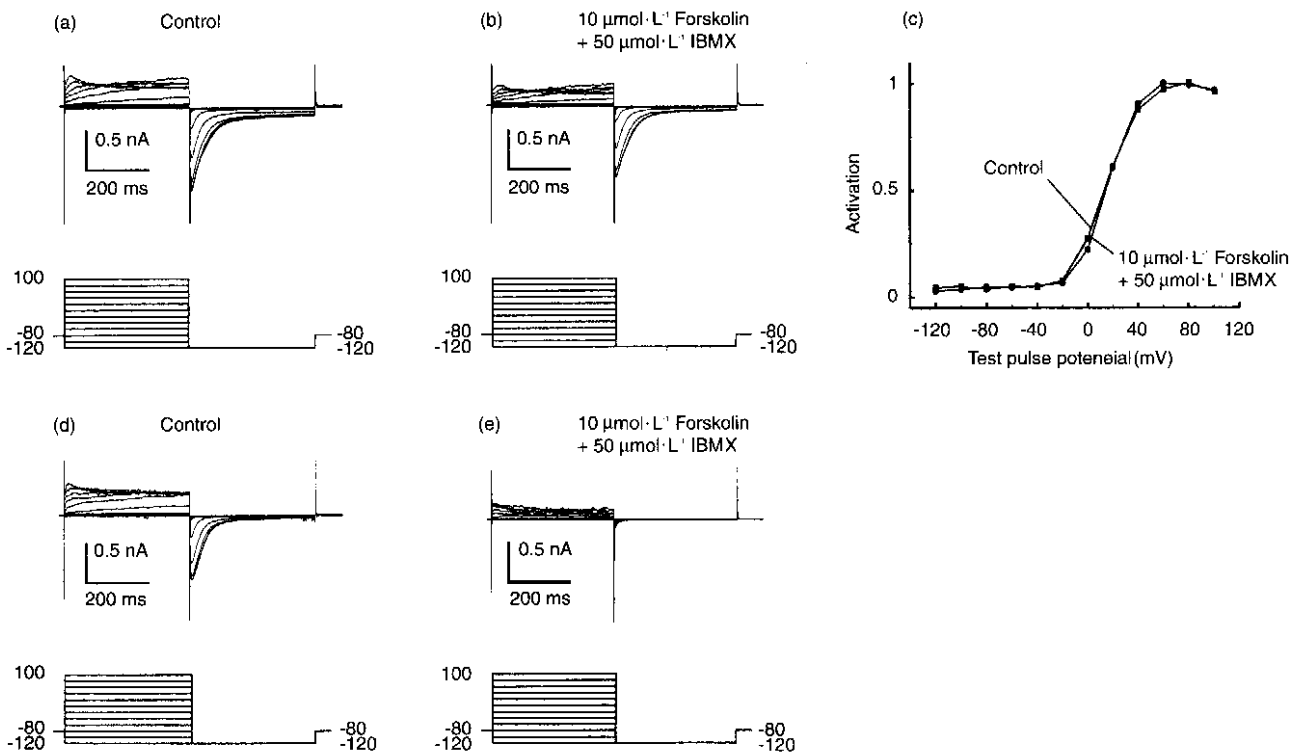


Fig. 1. Activation of PKA by the adenylate cyclase activator forskolin and the broad range phosphodiesterase inhibitor IBMX causes suppression of HERG tail currents recorded from stably transfected HEK 293 cells. The peak inward tail currents were reduced by 15.9% (b) and 85.6% (e) , respectively . Panel c shows activation curves , i.e. the normalized inverted peak tail current amplitude as a function of the test pulse potential during the first step of the protocol in (a) and (b) . The shift of the activation curve was not significant (1.5 mV) .

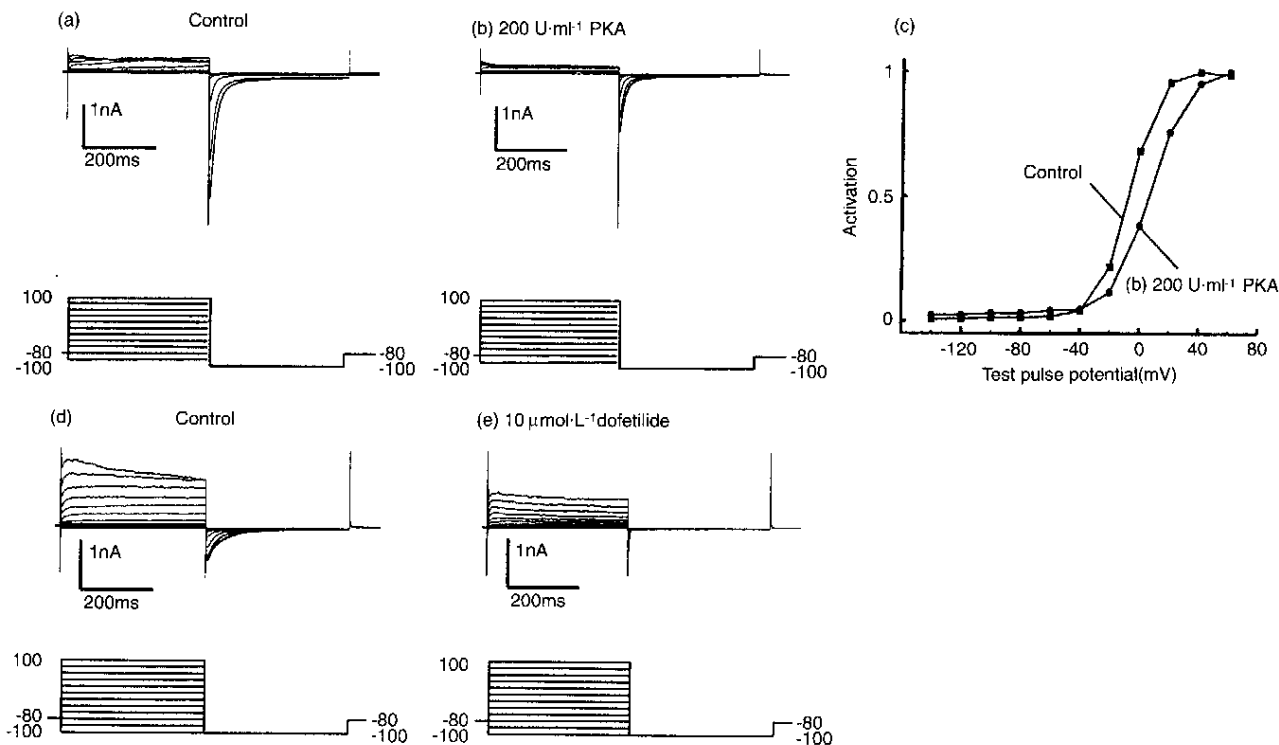


Fig. 2. Intracellular application of the catalytic subunit of PKA leads to a tail current decrease and causes a shift of the activation curve of HERG channels in HEK 293 cells. Panel a displays a control measurement recorded immediately after having obtained whole cell patch clamp configuration. The resulting currents after application of the catalytic subunit of PKA (200 U·ml⁻¹, 15 min) through the pipette are shown in panel b. HERG tail currents were blocked by 53.0% , and the activation curve was shifted by 13.7 mV (c). Panels (d) and (e) : Dofetilide block of HERG channels expressed in HEK 293 cells. Dofetilide (10 μmol·L⁻¹) blocked HERG tail currents almost completely (96.0%), whereas the outward current amplitude at the end of the test pulse was only reduced by 15.6% .

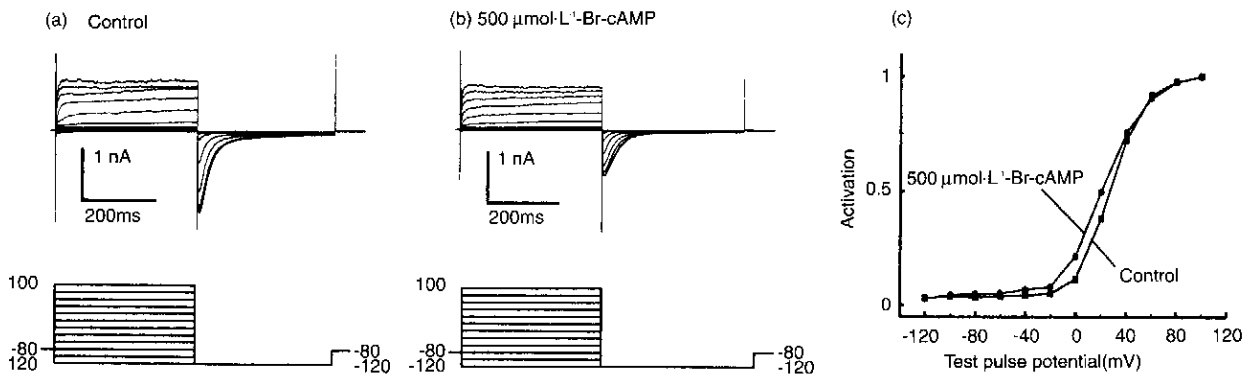


Fig. 3. 8-Br-cAMP (500 μmol·L⁻¹), a membrane permeable cAMP-analog that leads to activation of PKA , reduces HERG potassium currents. Peak tail currents were reduced by 45.0% . The activation curve was not significantly shifted (- 4.7 mV , c).

(0.4 seconds) to measure inward tail currents. The holding potential was - 80 mV in all experiments performed in this study. The tail current amplitude depends on the preceding test pulse and is a measure of channel activation. The normalized tail current amplitude was inverted and displayed as a function of the test pulse potential , which results in the activation curve (Fig. 1 c). When IBMX (50 μmol·L⁻¹ or 100 μmol·L⁻¹) and forskolin (10 μmol·L⁻¹) were washed

into the bath for 20 min , the peak tail current amplitude was reduced (Fig. 1 a , b , d , e). The average reduction was 32.0 ± 21.6% for 50 μmol·L⁻¹ IBMX (n = 6 , not significant) and 83.2 ± 13.0% (n = 7 , significant with P < 0.05) for 100 μmol·L⁻¹ IBMX , respectively. The half-maximal activation voltage V_{1/2} was not significantly altered [13.8 ± 3.1 mV under control conditions versus 11.4 ± 5.7 mV after incubation with 50 μmol·L⁻¹ IBMX and 10 μmol·

L^{-1} forskolin ; Fig. 1c). Due to the small amplitude of the remaining inward tail currents after incubation with $100 \mu\text{mol} \cdot L^{-1}$ IBMX and $10 \mu\text{mol} \cdot L^{-1}$ forskolin , it was not possible to determine $V_{1/2}$ in this set of experiments. In a set of control experiments , a period of 20 min (without forskolin or IBMX) revealed a small rundown of the inward tail current amplitude ($\Delta = -15.1 \pm 10.5\%$; $n = 7$) , while there was virtually no change in the half-maximal activation voltage ($\Delta V_{1/2} = -2.9 \pm 3.3 \text{ mV}$; $n = 6$).

Direct activation of PKA by 8-Br-cAMP causes inhibition of HERG currents

To further elucidate the role of PKA in HERG current reduction in a different experimental approach , 8-Br-cAMP was applied to HEK 293 cells expressing HERG channels. 8-Br-cAMP stimulates protein kinase A by the same mechanism as cAMP under physiological conditions. After having obtained the control measurements , cells were incubated with $500 \mu\text{mol} \cdot L^{-1}$ 8-Br-cAMP for 20 min. The resulting current traces are shown in Fig. 3a and b. Tail currents were reduced by $29.3 \pm 13.7\%$ ($P < 0.05$, $n = 3$) , but the voltage for half-maximal activation (Fig. 3c) was not significantly changed [$V_{1/2 \text{ control}} = 30.0 \pm 4.5 \text{ mV}$, $V_{1/2 \text{ 8-Br-cAMP}} = 33.3 \pm 4.7 \text{ mV}$, difference = $3.3 \pm 6.2 \text{ mV}$, $n = 4$].

HERG channel activation curves were shifted by addition of the catalytic subunit of PKA to the cytoplasm of HEK 293 cells

To address the question whether PKA alone mediates the effects described above , or whether additional upstream factors are necessary , the catalytic subunit of protein kinase A was applied intracellularly. 2 mmol/L potassium adenosine triphosphate ($K_2\text{ATP}$) and 200 U/ml catalytic subunit of PKA which does not require activation by cAMP , were added to the pipette solution. Control measurements were recorded immediately after having obtained whole cell configuration (Fig. 2a). After 15 min of PKA incubation , the inward tail current amplitude was reduced by $56.9 \pm 14.6\%$ ($n = 3$, Fig. 2b) , and the activation curve was significantly shifted towards more positive potentials (Fig. 2c). The average shift in four experiments was $15.4 \pm 2.2 \text{ mV}$ (from $37.4 \pm 5.4 \text{ mV}$ under control conditions to $52.6 \pm 6.9 \text{ mV}$ after incubation with PKA). Subsequently , we also analyzed whether the heat-inactivated catalytic subunit of PKA has an effect on HERG channels. The proteins were boiled for 5 min to inactivate the enzyme before preparing the internal solution , and no other experimental conditions were altered. In this set of experiments , peak tail current amplitude was decreased by $-15.8 \pm 17.4\%$ ($n = 5$, $P > 0.05$) , which is consistent with the rundown observed under control conditions without any drug application. The half-

maximal activation voltage $V_{1/2}$ ($\Delta V_{1/2} = -10.4 \pm 9.8 \text{ mV}$, $n = 5$, $P > 0.05$) was not significantly changed after an incubation period of 15 min.

Inhibitory effects of the class III-antiarrhythmic drug dofetilide on HERG channels expressed in HEK 293 cells

In this set of experiments , HEK 293 cells were incubated with dofetilide ($10 \mu\text{mol} \cdot L^{-1}$) , a specific blocker of HERG potassium channels , to demonstrate that the inward tail currents elicited are pure HERG currents. Under the given experimental conditions , the block of HERG channels by dofetilide is expected to be about 95% .¹⁵ In four experiments , HERG tail currents were blocked almost completely [$94.0 \pm 4.1\%$, Fig. 2d and e] , whereas the outward currents measured at the end of the test pulse were only reduced by $42.2 \pm 39.9\%$ ($P > 0.05$). These results indicate that HERG channels account for the whole tail current amplitude , but only in part for the outward currents. The outward currents in some of the HEK 293 cells used in this study were in part produced by endogenous delayed rectifier channels. In conclusion , the recorded inward tail currents are a correct measure for HERG potassium channel activity.

HERG channels expressed in *Xenopus laevis* oocytes are phosphorylated in the core-glycosylated and mature form of the protein

The *Xenopus oocyte* expression system was used to investigate the biochemical mechanism of HERG channel phosphorylation in more detail. For this study , we used the HERG wild type (WT) and the HERG 4M channel in which all four putative PKA-dependent phosphorylation sites were destroyed by mutating the serine or threonine residues (Ser-283 , Ser-890 , Thr-895 , Ser-1137) to nonphosphorylatable alanines.¹⁰ To perform immunoprecipitation analysis of the channel proteins , HERG WT and HERG 4M channels were expressed in *Xenopus laevis* oocytes. Two-microelectrode voltage clamp recordings were performed to check the expression of HERG channels using a two-step protocol similar to the protocol described above : A variable first step (test pulse) was applied at different potentials from -120 mV to $+100 \text{ mV}$ (increment 10 mV) for 0.4 seconds to evoke outward currents , and inward tail currents were measured during a second step at -120 mV for 0.4 seconds. Oocytes expressing HERG WT channels showed potassium currents with activation and rectification properties that are characteristic for HERG WT channels,⁴ whereas recordings from uninjected oocytes did not reveal any potassium conductance [typical current traces are displayed in Fig. 4a and b]. Recordings from oocytes expressing HERG 4M showed currents with kinetics similar to HERG WT that have been described previously [Fig. 5c].¹⁰ As

shown in Fig. 5a, antibodies immunoprecipitated two phosphorylated bands of HERG WT proteins in cells injected with HERG WT cRNA, a strong upper band with an apparent molecular mass of ≈ 155 kDa and a weaker lower band with a molecular mass of ≈ 135 kDa. Neither band was present in a control group of oocytes that had not been injected with RNA [Fig. 5a]. The upper band represents the phosphorylated complexly N-glycosylated, mature form of the protein, while the lower band is the phosphorylated core-glycosylated, precursor form of the HERG WT channel.¹⁶

Both bands with identical molecular masses could also be observed in Western blot analysis using crude membrane preparations from oocytes expressing HERG [Fig. 5c]. Apparently, HERG WT channel proteins are phosphorylated early during processing of the protein, and also in their mature form, since both the complexly N-glycosylated and the core-glycosylated forms were radiolabeled.

We tested whether application of protein kinase activators could influence the extent of phosphorylation of HERG WT channels. Oocytes were incubated for 30 min with increasing concentrations (1, 10, and 100 $\text{nmol} \cdot \text{L}^{-1}$) of the phorbol ester, PMA, before immunoprecipitation analysis. PMA is a potent although not very specific activator of protein kinase C and other protein kinases, including PKA. This approach revealed no significant differences in HERG phosphorylation compared with control conditions [Fig. 5a]. In addition, incubation of oocytes with the adenylate cyclase activator, forskolin (400 $\mu\text{mol} \cdot \text{L}^{-1}$), for 1 hour did not lead to different results compared with the control measurement [Fig. 5a]. Apparently, phosphorylation of HERG channels is a process that is not mediated via a single protein kinase but more likely depends on various kinases, since application of forskolin or PMA did not lead to significantly higher amounts of phosphorylated proteins.

We also analyzed the phosphorylation of HERG 4M channels. In this approach, we additionally performed Western blot analysis of HERG WT and HERG 4M channel proteins [Fig. 5c] to demonstrate that the total amount of HERG WT and HERG 4M protein used for the immunoblots did not differ significantly. Immunoprecipitation analysis of the HERG 4M channel protein revealed similar amounts of phosphorylation compared to HERG WT channels [Fig. 5b]. The HERG 4M proteins produced two bands, although the PKA-dependent phosphorylation sites are lacking in this construct. Compared with HERG WT, the extent of HERG 4M protein phosphorylation did not differ significantly [Fig. 5b]. In addition, recent electrophysiological studies with HERG WT and HERG 4M channels expressed in *Xenopus oocytes* have revealed that forskolin-induced regulation of HERG currents can be abolished by mutation of all four putative PKA phosphorylation sites.¹⁰ These data suggest that HERG channel phosphorylation in *Xenopus oocytes* is mediated primarily via protein kinases other than PKA.

DISCUSSION

The results presented in this study demonstrate that HERG channels heterologously expressed in human embryonic kidney (HEK 293) cells are regulated by cAMP and PKA. This was investigated by elevating intracellular cAMP levels and secondary activation of PKA with IBMX and forskolin. Furthermore, activation of PKA by the addition of the membrane permeable cAMP analog, 8-Br-cAMP, reproduced this effect. Finally, direct application of the catalytic subunit of PKA to the cytoplasm resulted in peak tail current reduction. In addition to this, the activation curve was shifted towards more positive potentials. Thus, several independent experimental approaches demonstrate that the HERG channel in a mammalian cell line is regulated by PKA.

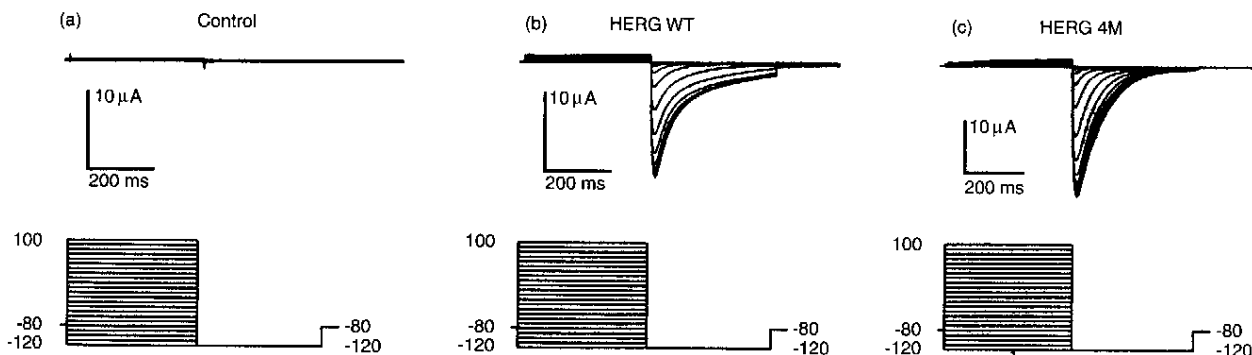


Fig. 4. Typical current recordings obtained from *Xenopus laevis* oocytes using the two-microelectrode voltage clamp technique. a : Uninjected oocyte. b : Oocyte injected with cRNA encoding HERG wild type channels. c : Oocyte expressing HERG 4M channels, where all four PKA phosphorylation sites were mutated.

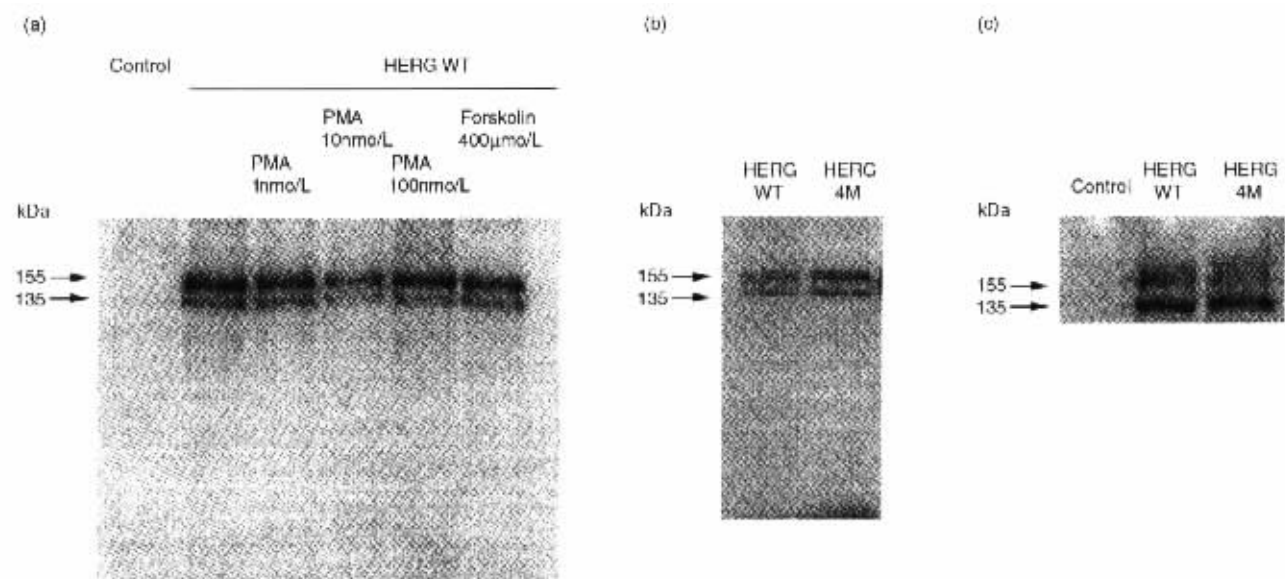


Fig. 5. Immunoprecipitation and Western blot analyses of wild type and 4M mutant HERG proteins heterologously expressed in *Xenopus laevis* oocytes. a : Autoradiogram of ³²P labeled HERG WT protein. Pharmacological treatment did not further affect the high baseline phosphorylation of the HERG protein. b : Autoradiogram of ³²P labeled HERG WT and 4M proteins immunoprecipitated with anti-HERG antibody. c : Western blot analysis showing HERG WT and HERG 4M mutant proteins.

Regulation of HERG channels by PKA has been found in other systems and cell types. It has been shown that activation of PKA leads to current reduction of HERG channels expressed in *Xenopus laevis* oocytes due to a shift in the activation curve.^{9,10} Mutation of all four cAMP-dependent phosphorylation sites abolished this PKA effect. Cui et al. demonstrated that stimulation of PKA reduces HERG currents in HERG-transfected chinese hamster ovary cells.¹⁷ In rat lactotrophs, erg-like potassium currents were inhibited by forskolin-induced activation of adenylate cyclase.¹⁸ In isolated guinea pig cardiomyocytes, the rapid component of the delayed rectifier potassium current, I_{Kr} , that is produced by the HERG potassium channel is decreased by stimulation of protein kinases. This effect is presumably mediated by PKA.⁹ Thus, the regulation of HERG channels may be ubiquitous to all cell types and it may therefore be of physiological relevance. The baseline midpoints of the activation curves [Fig. 1c] of HERG channels expressed in HEK 293 cells and *Xenopus oocytes* are different: $V_{1/2}$ was 13.8 mV for HEK 293 cells and -4.7 mV for the *Xenopus oocyte* expression system under isochronal current recording conditions. This difference indicates that most likely the intracellular environment of each cell type plays an important role in the regulation process of HERG channel activation. Whether phosphorylation by PKA is the factor accounting for the differences exhibited between the heterologous systems will require further experimentation.

In this study, we exclusively investigated PKA-specific

effects particularly with PKA-pathway-specific compounds, such as forskolin, IBMX, 8-Br-cAMP, and the catalytic subunit of PKA. Nonetheless, only the catalytic subunit of the enzyme produced a shift in the HERG activation curve together with reduction of inward tail current. All other approaches led to current reduction without changing $V_{1/2}$. In *Xenopus oocytes*, application of adenylate cyclase activators and phosphodiesterase inhibitors is sufficient to produce a shift in $V_{1/2}$.¹⁰ The reason for these different responses to application of PKA-stimulating drugs is unknown, but it may be attributable to cell type-specific factors or to the expression of the regulatory proteins minK or MiRPI¹⁹ modulating the regulation of HERG potassium channel activation.

Immunoprecipitation with an anti-HERG antibody demonstrated direct phosphorylation of the HERG channel protein in *Xenopus oocytes*. We found that both the immature, core-glycosylated form of the protein located in the endoplasmic reticulum, represented by the lower 135 kDa band, and the mature, complexly glycosylated protein located in the plasma membrane are phosphorylated.¹⁶

Our results show that the HERG 4M channel protein is phosphorylated as well, although this mutant does not carry any functional cAMP-dependent phosphorylation sites. This indicates that PKA is not the only protein kinase responsible for HERG channel phosphorylation. The fact that the HERG 4M mutant produces functional channels, together with the biochemical finding that HERG 4M is present at the cell

surface (represented by the 155 kDa band in the Western blot analysis)⁶ suggests that baseline phosphorylation by PKA is not crucial for protein processing. A more detailed pharmacological approach with PMA and forskolin did not reveal additional insights, since there are only 4 PKA-dependent phosphorylation sites in the HERG WT channel protein. In contrast, there are at least 40 phosphorylation sites that are targets for kinases which do not depend on cAMP. In summary, multiple kinases might be responsible for HERG channel phosphorylation under the given experimental conditions. Although the four PKA phosphorylation sites have a dramatic effect on HERG currents, their removal does not significantly alter the level of HERG phosphorylation assessed by ³²P labeling and immunoprecipitation.

The physiological relevance of our findings might be illustrated by the following clinical aspects. Under certain conditions this regulatory pathway may lead to arrhythmogenesis. For example, abnormal β -adrenergic stimulation^{20 21} and pathophysiological situations like sepsis²²⁻²⁴ or ischemia²⁵ can dramatically elevate the cAMP concentration in cardiac myocytes. It is also known that these conditions are associated with a high incidence of cardiac arrhythmias. In addition to this, patients with congenital long QT syndrome develop "torsade de pointes" tachyarrhythmias under emotional or physical stress when cAMP levels are elevated.^{26 27} Furthermore, arrhythmias are a common side effect during therapeutical use of phosphodiesterase inhibitors²⁸ in situations like chronic heart failure²⁹ or cardiogenic shock. Mazur et al demonstrated that systemic application of the PKA inhibitor, H-8, reduced the incidence of induced "torsade de pointes" arrhythmias in rabbits.³⁰ Thus, the link between the PKA system and the HERG potassium channel may be a pathophysiological explanation for arrhythmias, and future therapeutical efforts may act on this arrhythmogenic substrate with the result of new approaches in antiarrhythmic therapy.

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