Deletion of Protein Kinase A Phosphorylation Sites in the HERG Potassium Channel Inhibits Activation Shift by Protein Kinase A

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We investigated the role of protein kinase A (PKA) in regulation of the human ether-a-go-go-related gene (HERG) potassium channel activation. HERG clones with single mutations destroying one of four consensus PKA phosphorylation sites (S283A, S890A, T895A, S1137A), as well as one clone carrying all mutations with no PKA phosphorylation sites (HERG 4m) were constructed. These clones were expressed heterologously in Xenopus oocytes, and HERG potassium currents were measured with the two microelectrode voltage clamp technique. Application of the cAMP-specific phosphodiesterase (PDE IV) inhibitor Ro-20–1724 (100 μM), which results in an increased cAMP level and PKA stimulation, induced a reduction of HERG wild type outward currents by 19.1% due to a shift in the activation curve of 12.4 mV. When 100 μM Ro-20–1724 was applied to the HERG 4m channel, missing all PKA sites, there was no significant shift in the activation curve, and the current amplitude was not reduced. Furthermore, the adenylate cyclase activator forskolin that leads to PKA activation (400 μM, 60 min), shifted HERG wild type channel activation by 14.1 mV and reduced currents by 39.9%, whereas HERG 4m channels showed only a small shift of 4.3 mV and a weaker current reduction of 22.3%. We conclude that PKA regulates HERG channel activation, and direct phosphorylation of the HERG channel protein has a functional role that may be important in regulation of cardiac repolarization.

In cardiac myocytes, repolarization of the action potential is produced by different potassium currents (1). Activation of the rapid component of the delayed rectifier potassium current, \( I_{Kr} \), initiates repolarization and terminates the plateau phase of the cardiac action potential. The human ether-a-go-go-related gene (HERG) encodes the voltage-gated potassium channel underlying \( I_{Kr} \). This has been demonstrated in macroscopic current measurements (3, 4) and single channel measurements (5). HERG channels are one primary target for the pharmacological management of arrhythmias with class III antiarrhythmic drugs: \( I_{Kr} \) is blocked and the cardiac action potential is prolonged (5–7). Mutations in HERG produce chromosome 7-linked congenital long QT syndrome (LQT2) (3). These mutations are associated with delayed cardiac repolarization, prolonged electrocardiographic QT intervals (8), and a high risk for the development of ventricular “torsade de pointes” arrhythmias and sudden cardiac death (9).

Cyclic AMP-dependent protein kinase (PKA) is a key enzyme for numerous regulatory processes in almost all types of cells. It has been demonstrated that PKA regulates ion channels in native tissue (10). PKA is a serine/threonine kinase that can be stimulated by extracellular signals that elevate intracellular cAMP concentrations. CAMP binds to the regulatory subunit of the enzyme, which leads to dissociation of regulatory and catalytic subunits. The catalytic subunit phosphorylates the substrate at its specific phosphorylation sites. The substrate may either be the effector protein or another protein that mediates the effect. Increased cAMP concentrations can be obtained experimentally with the adenylate cyclase activator forskolin (11) and with the selective cAMP-specific phosphodiesterase (PDE IV) inhibitor Ro-20–1724 (12). Recently, we have demonstrated that PKA is involved in the regulation of \( I_{Kr} \) in guinea pig cardiac myocytes and HERG channels expressed heterologously in Xenopus oocytes (13). Incubation of the cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) leads to a HERG current reduction due to a shift in the activation curve that is mediated mainly by PKA. To investigate the biochemical pathways of this regulation process in more detail, we generated single mutations of all four PKA-specific phosphorylation sites in HERG, combined all of them, and expressed the resulting channel proteins in Xenopus oocytes. Because all enzymes involved in the PKA cascade are present endogenously in Xenopus oocytes, we could use indirect protein kinase A activators and inhibitors to study the role of PKA in HERG channel regulation.

Our findings show that direct phosphorylation of the channel protein by PKA is involved in the regulation of HERG channel activation, and the PKA-mediated part of the shift in the HERG activation curve can be abolished by mutating all PKA-specific phosphorylation sites in the HERG channel protein.

EXPERIMENTAL PROCEDURES

Solutions and Drug Administration—Voltage clamp measurements of Xenopus oocytes were performed in a low K⁺ solution containing (in mM) 5 KCl, 100 NaCl, 1.5 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.3). Current and voltage electrodes were filled with 3 M KCl solution.

Forskolin (Calbiochem) and Ro-20–1724 (Calbiochem) were dissolved in Me₂SO to a stock solution of 100 mM and stored at –20 °C. Phorbol-12,13-didecanoate (Calbiochem) was dissolved in Me₂SO to a stock solution of 10 mM 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. All measurements were carried out at room temperature (20 °C).
**Electrophysiology and Data Analysis**—The two-microelectrode voltage-clamp configuration was used to record currents from *Xenopus laevis* oocytes. Microelectrodes had tip resistances ranging from 1 to 5 megaohms. Data were low-pass filtered at 1–2 kHz (−3 dB, four-pole Bessel filter) before digitalization at 5–10 kHz. Recordings were performed using a commercially available amplifier (Warner OC-725A, Warner Instruments, Hamden, CT) and Pclamp software (Axon Instruments, Foster City, CA) for data acquisition and analysis. No leak subtraction was done during the experiments. The recording chamber was continually perfused. Activation curves were fitted with a Boltzmann distribution:

\[ G(V) = G_{\text{max}}/(1 - \exp(\frac{V_{1/2} - V}{k})) \]

where \( V \) is the test pulse potential, \( V_{1/2} \) is the half-maximal activation potential, and \( k \) is the slope of the activation curve. Statistical data are expressed as mean ± S.D. \((n = \text{number of experiments performed})\). We used paired and unpaired Student’s \( t \) test to compare the statistical significance of the results: \( p < 0.05 \) was considered to be statistically significant.

**Site-directed Mutagenesis**—The HERG wild type (HERG WT) clone (3) was a gift from M. T. Keating (GenBank™ accession number hs04270), which contains the HERG potassium channel coding region downstream the SP6 polymerase promoter. By use of computer analysis with the program HUSAR PROSITE, we searched for consensus PKA phosphorylation sites with the amino acid sequence Arg-Lys-(2)-Xaa-Ser-Thr, where (2) means Arg-Arg and Lys-Lys are also allowed. The program identified four sites. The serine or threonine residues of the PKA phosphorylation sites (Ser-283, Ser-890, Thr-895, Ser-1137) were replaced with the nonphosphorylatable alanine to eliminate PKA-mediated phosphorylation at this position. This resulted in the mutated channels HERG S283A, HERG S890A, HERG T895A, HERG S1137A. The mutations were generated with polymerase chain reaction by mutagenesis on the double-stranded plasmid using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The polymerase chain reaction products between unique anchor sites were sequenced (MWG-Biotech, Ebersberg, Germany), and fragments between these unique restriction sites were cut out using the following combinations of restriction enzymes: BstEII (1137) and NcoI (668) for HERG S283A; FseI (2779) and XhoI (2107) for HERG S890A and HERG T895A; BamHI (3532) and FseI (2779) for HERG S1137A. The mutated fragments were subcloned into the original HERG plasmid and sequenced again.

The HERG 4M clone (combination of all four mutations) was generated by introducing mutation S890A into the T895A clone as described above, generating HERG S890A,T895A. Then the restriction fragments containing mutations S283A and S1137A were subcloned into HERG

**FIG. 1.** Hypothetical membrane folding model for the HERG potassium channel. The amino and carboxyl termini and the six membrane-spanning domains are indicated. The locations of four putative PKA-specific phosphorylation sites and mutations generated in this study are illustrated. For simplicity, only the mutated amino acids are shown.
Finally, cDNA of HERG 4M was verified by DNA sequencing.

Expression of HERG Channels in Xenopus Oocytes—Complementary RNA was prepared from the corresponding cDNA (HERG WT, HERG S283A, HERG S890A, HERG T895A, HERG S1137A, HERG 4M) in the pSP64 transcription vector (Promega, Madison, WI) with the mMES-SAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX) by use of SP6 polymerase after linearization with EcoRI (Roche Molecular Biochemicals). Injection of RNA (50–500 ng/ml) into stage V and VI defolliculated oocytes was performed by using a Nanoject automatic injector (Drummond, Broomall, PA). The volume of injected cRNA solution was 46 nl/oocyte, and measurements were made 2–10 days after injection.

RESULTS

Expression of HERG Channels with Deleted Consensus Phosphorylation Sites for PKA—We performed site-directed mutagenesis of HERG to generate mutated channels that lack consensus PKA phosphorylation sites (HERG S283A, HERG S890A, HERG T895A, HERG S1137A; and in combination HERG 4M, see Fig. 1) and expressed these mutant channels in Xenopus oocytes. All clones generated in this study resulted in functional potassium channels with current kinetics similar to those of HERG WT.

HERG Wild Type Channel Activation Curve Was Shifted by Increasing cAMP Levels via Inhibition of the PDE IV—To investigate the effects of the PDE IV inhibitor Ro-20–1724 (IC50 = 2.2 μM) on HERG potassium channels, we measured currents using a two-step protocol (see Fig. 2A). A variable first step (test pulse) was applied at different potentials from −80 mV to +80 mV (increment 10 mV) for 0.4 s and a second step at −120 mV 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 preceding test pulse potential, which results in the activation curve. In the control currents, the voltage for half-maximal activation \( V_{1/2} \) was −4.3 mV (Fig. 2, A and C). When 100 μM Ro-20–1724 was applied into the bath for 30 min (Fig. 2B), the activation curve was shifted by 14.0 mV to 9.7 mV (Fig. 2C). The average shift in five experiments was 12.4 ± 3.2 mV. This was statistically significant. We used an additional protocol that evokes outward tail currents as shown in Fig. 2D. The variable test pulse from −280 to 80 mV (increment 10 mV) for 0.4 s was followed by a constant return pulse to 260 mV (0.4 s). The control HERG current had an activation threshold of −40 mV, then the current amplitude reached a maximum at 0 mV because of inward rectification that is characteristic to this channel (3, 5, 14). Typical current traces are shown in Fig. 2D (control measurements) and Fig. 2E (after application of 100 μM Ro-20–1724 into the bath for 30 min). The maximum outward current amplitude during the test pulse was reduced significantly by 19.1 ± 12.8% (n = 5). Fig. 2F displays the current amplitude during the test pulse as a function of the test pulse potential (control measurement and after incubation with 100 μM Ro-20–1724 for 30 min).

Deletion of Phosphorylation Sites Inhibits the Activation Shift Caused by PDE IV Inhibition in HERG 4M—We mutated all four consensus PKA phosphorylation sites in the HERG channel protein (HERG 4M). The current kinetics of the HERG 4M clone was almost identical to the wild type HERG channel
ent experimental approach, we applied forskolin to the 
activation sites is responsible for the activation shift induced by PKA.

To further elucidate the role of PKA phosphorylation of HERG WT channels at its PKA phosphorylation sites regulates the PKA-mediated activation shift in HERG 4M after 30 min of perfusion (Fig. 3, C). The average $V_{1/2}$ of HERG 4M during control experiments was $-16.4 \pm 1.6 \text{mV}$ ($n = 6$) (HERG wild type gave $-4.7 \pm 1.5 \text{mV}$ ($n = 3$)). In contrast to HERG WT, addition of 100 $\mu\text{M}$ Ro-20–1724 caused virtually no change of current properties in HERG 4M after 30 min of perfusion (Fig. 3, D and E). $V_{1/2}$ was only shifted by $3.8 \pm 1.7 \text{mV}$ to $-12.6 \pm 2.4 \text{mV}$ ($n = 6$) (Fig. 3C and Fig. 6A), and there was no significant current reduction ($\Delta V = +2.8 \pm 8.1\%$ ($n = 6$) (Fig. 3F)). Thus, direct phosphorylation of HERG WT channels at its PKA phosphorylation sites is responsible for the activation shift induced by PKA.

Elevation of cAMP Levels by Forskolin Leads to a Shift of the HERG Channel Activation Curve—To further elucidate the role of protein kinase A in the HERG activation process in a different experimental approach, we applied forskolin to the Xenopus oocytes expressing HERG WT channels. Forskolin is an adenylate cyclase activator (EC$_{50}$ = 4.0 $\mu\text{M}$) that increases intracellular cAMP levels and subsequently stimulates PKA.

After having obtained the control measurements (Fig. 4, A and D), forskolin (400 $\mu\text{M}$) was perfused into the bath for 60 min. The resulting activation curve is shifted by 13.8 $\text{mV}$ (panel C). Maximum outward current amplitude (measured at the end of the test pulse in panels D and E) was reduced by 59.0%. Protocols and plots were identical to those shown in Fig. 2. Bath: 5 mM K$^+$.

FIG. 4. Forskolin (400 $\mu\text{M}$), an adenylate cyclase activator that leads to PKA stimulation, shifts the activation curve in HERG WT currents. Original currents obtained from the same oocyte before (panels A and D) and after exposure to 400 $\mu\text{M}$ forskolin (60 min; panels B and E). The resulting activation curve is shifted by 13.8 $\text{mV}$ (panel C). Maximum outward current amplitude (measured at the end of the test pulse in panels D and E) was reduced by 59.0%. Protocols and plots were identical to those shown in Fig. 2. Bath: 5 mM K$^+$.
potentials as well (T895A, S283A, and especially S890A) (Fig. 6B). The values were $V_{1/2} = -5.9 \pm 2.4$ mV for T895A ($n = 9$), $V_{1/2} = -6.0 \pm 3.0$ mV for S283A ($n = 6$), $V_{1/2} = -8.0 \pm 4.8$ mV for S890A ($n = 6$), and $V_{1/2} = -2.6 \pm 1.9$ mV for S1137A ($n = 7$).

**DISCUSSION**

This study demonstrates that HERG potassium channel activation is regulated by direct phosphorylation of the HERG protein. This has been shown by mutating all four consensus
PKA phosphorylation sites in the HERG protein (resulting in the HERG 4m channel) and analyzing the electrophysiological effects of PKA stimulation by adding Ro-20–1724, an inhibitor of the PDE IV, or adding the adenylate cyclase activator forskolin. Our results demonstrated that the HERG wild type activation curve was shifted and the repolarizing HERG current amplitude was reduced by protein kinase A, whereas in the HERG 4m channel these effects were virtually absent. Because all PKA sites are mutated in the HERG 4m channel, we conclude that PKA produces these electrophysiological effects by direct phosphorylation of the channel protein.

Regulation of HERG channels by PKA has been found recently in another expression system as well. Palma et al. (15) could demonstrate that stimulation of PKA reduces HERG currents in HERG-transfected cultural cells. In isolated guinea pig cardiomyocytes, the rapid component of the delayed rectifier potassium current, I_{Kr}, that is produced by the HERG potassium channel was decreased by stimulating protein kinases, presumably PKA (13). Therefore regulation of HERG channels by PKA may be of physiological relevance.

The baseline midpoint of the activation curve was −4.7 mV for HERG WT and −14.8 mV for HERG 4m (Fig. 6A). This difference in the baseline activation curves indicates that the positions of the mutations and its phosphorylation by PKA are crucial for activation. Even more so, because the difference in activation after stimulation of PKA was higher (22 mV) (Fig. 6A).

There are two possible explanations for the difference in the baseline half-maximal activation voltage between HERG WT and HERG 4m channels. Either mutating the serines or threonines to alanines causes this effect, but more likely the naturally occurring higher phosphorylation occupancy of HERG WT compared with HERG 4m induces this baseline shift. All four mutations contribute to the baseline shift, because single mutations shift the activation curve to a smaller extent compared with HERG 4m (Fig. 6B).

In a previous study (13), we demonstrated that the phorbol ester PMA produces a stronger PKA-dependent shift in the activation kinetics of HERG channels (37 mV) compared with the shift by Ro-20–1724 (14.0 mV) or forskolin (14.1 mV). PMA is a very potent but not specific compound. It is known to be an activator of protein kinase C and other protein kinases, presumably PKA (13). Therefore regulation of HERG channels by PKA may be of physiological relevance.

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