Protein Kinase A Activity at the Endoplasmic Reticulum Surface Is Responsible for Augmentation of Human ether-a-go-go-related Gene Product (HERG)*

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Human ether-a-go-go-related gene product (HERG) is a cardiac potassium channel commonly implicated in the pathogenesis of the long QT syndrome, type 2 (LQT2). LQT2 mutations typically have incomplete penetrance and affect individuals at various stages of their lives; this may mirror variations in intracellular signaling and HERG regulation. Previous work showed that sustained protein kinase A (PKA) activity augments HERG protein abundance by a mechanism that includes enhanced protein translation. To investigate the subcellular site of this regulation, we generated site-specific probes to the cytoplasmic surface of the endoplasmic reticulum (ER), the presumed locale of channel synthesis. Real-time FRET-based indicators demonstrated both cAMP and PKA activity at the ER. A PKA inhibitor targeted to the ER surface (termed p4PKIg) completely abolished PKA-mediated augmentation of HERG in HEK293 cells as well as rat neonatal cardiomyocytes. Immunofluorescence colocalization, targeted FRET-based PKA biosensors, phosphospecific antibodies, and in vivo phosphorylation experiments confirmed that p4PKIg is preferentially active at the ER surface rather than the plasma membrane. Rerouting this inhibitor to the outer mitochondrial membrane diminishes its ability to block cAMP-dependent HERG induction. Our results support a model where PKA-dependent regulation of HERG synthesis occurs at the ER surface. Furthermore, reagents generated for this study provide novel experimental tools to probe compartmentalized cAMP/PKA signaling within cells.

The long QT syndrome (LQTS) is a potentially lethal cardiac disorder characterized by prolonged ventricular action potential duration as measured by the interval between the start of the Q-wave and end of the T-wave on an electrocardiogram in affected individuals (1). LQTS can lead to torsade de pointes (TdP), ventricular fibrillation, and sudden arrhythmic death. There have hitherto been 13 disease-causing loci linked to hereditary LQTS. Although these represent a wide variety of genes, the majority of LQTS patients carry mutations in cardiac potassium channels. The human ether-a-go-go-related gene product (HERG) is of particular interest, not only because it accounts for over 40% of all inherited LQTS (2) but also because it appears to be central to most acquired forms of long QT syndrome (3, 4).

HERG, also known as KCNH2, codes for the potassium channel responsible for the rapidly activating delayed rectified potassium current I_{Kr} (5). Patients with deleterious variants in this gene are said to carry LQT2 mutations, which typically cause a defect in HERG protein trafficking rather than channel function per se (6, 7). LQT2 mutations frequently have a variable penetrance, even within the same family (8), that may not cause symptoms until later in life. It would therefore appear that the gravity of HERG mutations depends on a multitude of hereditary as well as environmental factors, including variations in channel regulation.

The adrenergic cascade is a prominent signal transduction mechanism in cardiomyocytes that may, if perturbed, contribute to both hereditary and acquired LQTS pathophysiology. For instance, in LQT2 patients, TdP is frequently triggered upon being startled or woken up suddenly from sleep (9), suggesting that acute adrenergic signaling may be involved. Moreover, more than 50% of congestive heart failure-related deaths result from ventricular tachyarrhythmias, including TdP (10). Likewise, animal models link cardiac hypertrophy to prolonged QT intervals (11), and hypertrophied hearts have been shown to elicit disturbed ion channel function from a process of electrical remodeling (12).

Our laboratory has previously shown that HERG is regulated by β-adrenergic pathways, both acutely and chronically. Acute exposure to cAMP causes a current-suppressing phosphorylation by PKA at four different sites on HERG, whereas direct cAMP binding augments current amplitudes (13, 14). Prolonged elevation of cAMP, however, causes significant increases in HERG protein levels (15). This is neither due to increased transcription/mRNA stability nor due to increased HERG protein stability; instead, an increased rate of HERG synthesis is responsible for this effect. The detailed mechanism for this augmented channel synthesis is unknown.

In the present work, we sought to explore the subcellular localization of cAMP/PKA regulation of HERG channel augmentation. In the past 2 decades, the notion that cAMP signal-
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ing is a highly compartmentalized phenomenon has become increasingly prevalent (16, 17). Protein kinase A anchoring proteins (A-kinase anchoring proteins; AKAPs) are scaffolding proteins responsible for maintaining localized PKA niches. AKAPs may specifically target to the ER (18) or to the nuclear membrane (19), among other sites; we have also shown that acute cAMP/PKA regulation of HERG is functionally coupled to an AKAP activity (20). We therefore hypothesize that chronic cAMP-dependent HERG protein augmentation occurs due to localized PKA activity at the ER surface (i.e. the likely site of integral membrane protein translation).

EXPERIMENTAL PROCEDURES

DNA Constructs and Adenoviral Vectors—HERG cDNA (a gift from Michael C. Sanguinetti, University of Utah) was inserted into the pCMV-tag3a vector as a Myc-tagged construct. The following oligonucleotides were synthesized (IDT DNA, Coralville, IA) along with their complementary strands, annealed, and introduced into the C1(1–29)-GFP plasmid containing the first 29 codons of CYP450–2c1 fused to the N terminus of monomeric enhanced green fluorescent protein (mEGFP) (21–23) (a kind gift from Erik Snapp, Albert Einstein College of Medicine) via HindIII and BamHI endonuclease sites: 5′-aagctttatccatatgacgtcccagactctgccggcagaaccatcaacata- ttttattctgcaagaaacaggtgaaatgcaatagttcc-3′ (resulting in CYP450–2c1(1–29)-HA- PKI(6–22)-mEGFP, dubbed p4PKIg) and 5′-aagctttatccatatgacgtcccagactctgccagctgtgcgcgtagaaccatcaaca-gataccacatcgccagctttcgcagagcttggtggtttttctctc-3′ (resulting in CYP450–2c1(1–29)-HA-scrambled PKI(6–22)-mEGFP, dubbed p4scrg).

To inhibit the targetatory (and scramble) peptide to the mitochondrial outer membrane, we ligated the annealed oligonucleotides 5′-ctagcaccatgctaccatga-3′ and 5′-ctagcaccagtaccatga-3′ into Nhel/HindIII digests of p4PKIg (and scrambled p4scrg) constructs fixed with 4% paraformaldehyde 24 h later and stained with ER marker antibodies goat anti-calnexin (Santa Cruz Biotechnology, Inc.) and mouse anti-protein-disulfide isomerase (Stressgen) or the plasma membrane marker antibody mouse anti-pan-cadherin (AbCam).

Corrected sensitized emission fluorescent energy transfer ratios (FRETs) were determined in cells co-transfected with p4AKAR3/pm-AKAR3 along with p4PKIg Y66A/p4scrg Y66A/a1PKIg Y66A/a1scrg Y66A (using 1 μg of biosensor DNA and 1 μg of inhibitor DNA per well in a confluent 6-well culture plate of HEK293 cells), using a modification of several methods (30–32) with modifications. Cells with approximately mean YFP protein-disulfide isomerase (Stressgen) or the plasma membrane marker antibody mouse anti-pan-cadherin (AbCam).

Co-localization was quantified using the intensity correlation analysis plug-in for ImageJ (W. S. Rasband, Image), version 1.410, National Institutes of Health, Bethesda, MD) (29), and Pearson’s correlation coefficient was determined. Cells containing only p4AKAR3, pm-AKAR3, PKIg, and a1PKIg were imaged live. MitoTracker deep red FM (Invitrogen) was used as a mitochondrial stain in the case of a1PKIg-transfected cells. For imaging, a Leica AOBS SP5 laser confocal microscope was used.
background-subtracted, and YFP emission was corrected for bleed-through using the empirically determined formula, YFPC = YFP − (0.91 × CFP), where YFPC is the bleed-through-corrected YFP signal, and CFP and YFP are the raw, background-subtracted signals in the respective channels. The YFP signal was further corrected for bleaching using the equation, YFPcc = (1 + A(n − 1)) × YFPc, where YFPcc is the corrected YFP signal, n is the time point, and A is a constant that was determined empirically from the first nine time points (i.e. prior to forskolin treatment) for each region of interest analyzed. Finally, the FRETc ratios (YFPcc/CFP) were normalized to the first time point of each region of interest, averaged across trials, and plotted as functions of time. Each plot is an average of 9–10 trials. FRET data from cells transfected with the ICUE2 and p4ICUE2 constructs were obtained in an analogous way; however, given that the ICUE2 biosensor responds to forskolin in the opposite direction as AKAR3 (i.e. with rising [cAMP], there is a decrease, not an increase, in resonance energy transfer), we used an inverse function of FRETc = CFP/YFPc.

**cAMP Protein Induction Experiments**—HEK293 cells grown on 6-well plates were transfected with 0.25 μg of HERG cDNA along with 1.0 μg of p4PKIg (or p4scrg) plasmid using FuGene 6 (Roche Applied Science). After 24 h, fresh medium supplemented with 50 μM 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate (CPT-cAMP) (Sigma) or vehicle (DMSO) was introduced for another 24 h; NDET (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.4% deoxycholate, 25 mM Tris, pH 7.5, Roche Complete protease inhibitors) lysis, SDS-PAGE, and Western blot analysis followed. Similarly, RNCMs were co-infected with HERG adenovirus (a kind gift from Richard B. Robinson, Columbia University) and p4PKIg (or p4scrg) adenovirus, at a multiplicity of infection of 5 and 20, respectively. After 48 h, the cells were treated with CPT-cAMP (or DMSO) for another 48 h, lysed in NDET, and analyzed similarly to HEK293 cells.

**In Vivo Phosphorylation Assay**—HEK293 cells stably transfected with p4PKIg (or p4scrg) were plated on gelatin-coated 6-well plates in DMEM and transfected with HERG cDNA. After 24 h, the cells were moved to a 31 °C incubator for another ~72 h so as to promote HERG protein maturation and plasma membrane localization (33, 34). Subsequently, the cells were phosphate-starved in FBS- and phosphate-free DMEM for 2–3 h at 37 °C. Cells were then loaded with [32P]orthophosphate (50–80 mCi/well in 750 μl of total medium) for 1 h and immediately treated with 50 μM forskolin (or DMSO) for 10 min. Lysis with ice-cold NDET buffer supplemented with phospho-STOP phosphatase inhibitors (Roche Applied Science) followed. Postnuclear supernatant was then incubated with 20 μl of mouse anti-Myc 9e10 (Santa Cruz Biotechnology, Inc.) antibody for 2 h and incubated overnight with UltraLink Protein G beads (Thermo Scientific), shaking at 4 °C. Three washes with NDET buffer followed, and the protein sample was eventually eluted, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Autoradiography was detected using HyBlot CL film (Denville Scientific). For each trial, data from 2–3 wells (with the same treatment) were averaged; there were three separate trials in total.

**Results**

**Forskolin Induces [cAMP] Increase at the Surface of the ER**—To determine whether cAMP signaling occurs at the surface of the endoplasmic reticulum, we selectively targeted a [cAMP]-sensitive biosensor to this organelle. Szczesna-Skorpula et al. (21, 22) have shown that the first 29 amino acid residues of cytochrome P450 2C1 are sufficient to target a variety of proteins to the ER; this is achieved by an uncertain mechanism but appears to be a bona fide restriction/retention effect as opposed to ER retrieval/retention as in the case of KDEL motif-mediated processes. Furthermore, the fact that this is originally an N-terminal peptide and that it forms a type I transmembrane domain allows for easy targeting of C-terminally fused proteins, such as GFP (23) with a definite topology; thus, the fused protein is predicted to reside at the ER cytoplasmic surface as opposed to the luminal side of the organelle.

We employed this system and fused ICUE2 (35), an Epac1 (exchange protein that directly binds cAMP)-based FRET biosensor, to the C terminus of the targeting CYP450 domain. To confirm that the construct indeed localizes to the ER, we utilized immunofluorescence imaging. The fluorescent protein signal assumes a reticulate pattern distributed throughout the cytosol, as expected for an ER-localized protein (Fig. 1, A and B). Furthermore, the construct co-localized with the ER markers calnexin (Pearson’s correlation coefficient; r = 0.73 ± 0.04) and protein-disulfide isomerase (r = 0.80 ± 0.04) but not with the plasma membrane marker cadherin (r = 0.16 ± 0.07).

Cells transfected with either freely soluble cytosolic ICUE2 or its targeted form (p4ICUE2) responded to forskolin treatment by a rise in FRETc, indicating that stimulation of adenyl cyclase resulted in cAMP elevation detectable in both the cytosol as well as at the ER surface (Fig. 1C). Although the absolute increases in FRETc were different between the two biosensors, possibly due to the presumably greater anisotropy of the membrane-anchored protein, normalization for amplitude rendered the two data sets virtually superimposable in terms of kinetics. Thus, cAMP signaling is detectable at the ER surface.

**Construction of ER Surface-targeted PKI Inhibitors**—In order to selectively disrupt PKA signaling at the surface of the ER, we designed a fusion construct that would selectively target the inhibitory peptide PKI(6–22) (36) to this organelle. Again, we utilized the targeting P450 2C1 domain and fused it to the PKI fragment; an HA tag spacer was placed in between the two peptides. Finally, mEGFP was positioned at the very C terminus of the fusion to ease detection. We also noticed that the addition of GFP to the C terminus imparted stability and/or proper folding capacity onto the entire construct, because the use of a shortened C-terminal cap (FLAG) was poorly expressed (data not shown). The CYP450(1–29)-HA-PKI-GFP construct was named p4PKIg (Fig. 2A). A control construct was also created, wherein the PKI peptide was scrambled (p4scrg).
Confocal microscopy revealed that both p4PKIg and p4scrg closely co-localized with calnexin (r = 0.88 ± 0.01 and 0.80 ± 0.05, respectively) but not with cadherin (r = 0.09 ± 0.04 and 0.10 ± 0.06, respectively) (Fig. 2, B–D).

**p4PKIg Preferentially Inhibits PKA Activity at the ER over That at the Plasma Membrane**—To determine whether there are differences in PKA activity in p4PKIg-transfected cells, we turned to organelle-targeted FRET-based biosensors. To measure the enzymatic activity, we chose AKAR3, a PKA phosphorylation-sensitive construct developed by Allen et al. (24), along with its plasma membrane-specific variant pm-AKAR3. This biosensor consists of enhanced cyan fluorescent protein and Venus (a yellow fluorescent protein derivative) linked together by FHA1 (forkhead-associated domain 1) and a PKA consensus substrate sequence. High PKA activity leads to phosphorylation of the substrate, which is in turn recognized and bound by FHA1; the consequent conformational change in the biosensor moves the two fluorescent proteins toward each other, thus increasing the probability of fluorescent energy transfer. In addition to the non-targeted and plasma membrane-targeted AKAR3, we also developed an ER surface-targeted inhibitor construct p4AKAR3, by fusing the biosensor to the CYP450 localization domain (Fig. 3A). Because GFP fluorescence from p4PKIg (or p4scrg) would interfere with measurements made on the FRET-based biosensors, we introduced the Y66A mutation into the mEGFP portion of the PKI-based constructs to completely...
disrupt its fluorescence (37). Next, we co-expressed AKAR3-pm/p4AKAR3 along with p4PKIg Y66A/p4scrg Y66A. In either case, anti-GFP immunoblots (detecting p4PKIg Y66A or p4scrg Y66A) indicated approximately equal expression of the inhibitory constructs (Fig. 3, B and C).

FRETc data were then collected from HEK293 cells that were transfected with AKAR3/p4AKAR3 along with p4PKIg Y66A or p4scrg Y66A. The expression of p4PKIg caused a 73.4 ± 4.9% FRETc suppression at the ER (p4AKAR3 readout) after 5 min of 125 μM forskolin compared with the expression of the control construct p4scrg. However, the inhibitor p4PKIg caused significantly less (42.6 ± 8.4%) inhibition at the plasma membrane (pm-AKAR3 read-out) (Fig. 3D).

To further confirm that p4PKIg-expressing cells have a PKA activity preferentially inhibited at the ER, we sought to determine whether the construct differentially affects PKA-dependent phosphorylation of HERG at the two compartments. HERG exists in two glycosylation maturation stages: an immature form (~135 kDa) based primarily in the ER and a mature form (~155 kDa) thought to be quickly exported to the plasma membrane (38). The two forms can be readily separated by SDS-PAGE based on their different gel migration. We performed 32P labeling of intact live cells expressing HERG followed by in vivo phosphorylation after stimulation of CAMP/PKA. We observed that a 10-min treatment with 50 μM forskolin of cells stably expressing p4scrg resulted in a 30.6 ± 8.9% increase in cAMP-dependent phosphorylation of the mature HERG form (transiently transfected) and a 23.6 ± 8.2% increase in phosphorylation of the immature form (Fig. 4).

FIGURE 3. Direct measurement of ER-targeted PKA inhibition. HEK293 cells were transfected with PKA-biosensors AKAR3 targeted to the ER (p4AKAR3) or to the plasma membrane (pm-AKAR3); immunofluorescence images confirm the intended localization (A). Biosensor expressing cells show approximately equal expression of p4PKIg Y66A and p4scrg Y66A when co-transfected with these constructs (B and C). FRETc from cells co-transfected with p4AKAR3 along with p4PKIg Y66A or p4scrg Y66A were recorded and normalized to the maximal response of the p4AKAR3/p4scrg-expressing cells; likewise, FRETc signals from cells co-transfected with pm-AKAR3 along with p4PKIg Y66A or p4scrg Y66A were recorded and normalized to the maximal response of the pm-AKAR3/p4scrg Y66A-expressing subpopulation. A summary plot (D) shows that differential response to the addition of 125 μM forskolin at 180 s is apparent in both cases with preferential inhibition at the ER surface (n = 9–10; *, p < 0.05). PM, plasma membrane; IB, immunoblot. Error bars, S.E.

FIGURE 4. Targeted PKA inhibition effects on in vivo phosphorylation of HERG. HEK293 cells expressing HERG + p4PKIg (or p4scrg) were loaded with 32P radioisotope and then treated with DMSO or 50 μM forskolin for 10 min. Immunoprecipitated HERG was then analyzed using autoradiography (A). After normalization to immunoblot data, the ratio of autoradiography signal from forskolin versus DMSO-treated samples was plotted, showing preferential inhibition of the PKA-dependent phosphorylation of the immature form of HERG (B) (n = 3; *, p < 0.05); IB, immunoblot; Error bars, S.E.
Although the mature HERG form in cells stably expressing p4PKIg still experiences significant cAMP-dependent phosphorylation (32.9 ± 2.5%), the PKA-dependent phosphorylation of the immature form is nearly abolished (2.3 ± 10.0%, p < 0.05). These results indicate that p4PKIg preferentially inhibits PKA-dependent phosphorylation of HERG at the ER compared with that near the plasma membrane.

We further hypothesized that the p4PKIg construct should completely inhibit PKA-dependent phosphorylation of ER-resident membrane proteins. We chose to study this phenomenon on phospholamban (PLN), a small transmembrane protein and a well established PKA target (39) amply expressed in RNCMs (40). Cardiomyocytes infected with p4scrg adenovirus showed strong CPT-cAMP-induced PLN phosphorylation at Ser16 as measured by phospho-specific immunoblots; in addition, there was a slight decrease in total PLN levels, an effect previously attributed to chronic adrenergic tone (41) (Fig. 5A). When normalized to total PLN levels, PLN PKA-dependent phosphorylation was significantly inhibited by p4PKIg compared with p4scrg with no decrease in total PLN abundance (Fig. 5B). Finally, GFP expression (surrogate for p4PKIg/p4scrg levels) was comparable in all samples tested (Fig. 5, A and C). Thus, the p4PKIg construct inhibits a prototypical ER protein phosphorylation in a native system.

PKA Up-regulates HERG at ER Surface

ER-targeted PKI Peptide Inhibits cAMP-dependent Induction of HERG in both HEK293 Cells and Rat Neonatal Cardiomyocytes—To determine if the targeted PKI constructs perturbed cAMP-dependent augmentation of HERG protein, we transiently co-transfected p4PKIg (or p4scrg) and HERG into HEK293 cells. A 24-h treatment with 50 μM CPT-cAMP resulted in a 5-fold induction of HERG levels in p4scrg cells (Fig. 6, A and C). In cells expressing p4PKIg, however, the cAMP-dependent augmentation of HERG was abolished. GFP expression was comparable in all cells tested (Fig. 6B).

We also tested if the same effect is reproducible in a native cell system. For such a purpose, we infected RNCMs with HERG-expressing adenovirus, along with either p4PKIg or p4scrg viruses. A 48-h treatment with 50 μM CPT-cAMP resulted in a >10-fold HERG protein induction in control cells (infected with p4scrg-encoding adenovirus; Fig. 6, A and D). As observed in HEK293 cells there was no significant PKA-dependent induction of HERG in p4PKIg-infected cells.

Cytosolic PKI Peptide Causes Complete Inhibition of the cAMP-dependent Induction of HERG, whereas PKI Peptide Targeted to the Mitochondrial Outer Membrane Does Not—We further hypothesized that if the PKA activity causing the cAMP-dependent up-regulation of HERG protein levels indeed occurs on the surface of the ER, freely diffusible cytosolic PKI constructs should also display inhibitory activity; conversely, PKI constructs targeted to another organelle should not. To this end, we fused HA-PKI(6–22) and HA-scramble PKI to mEGFP in the absence of any targeting domains; we named these constructs PKIg and scrg, respectively. For targeting to another intracellular organelle, we utilized amino acid residues 34–63 from D-AKAP1a; these comprise a signal for targeting to the outer mitochondrial membrane both in their native context (42) and in chimeras (24). We substituted these residues for the ER-targeting motif in both p4PKIg as well as p4scrg, obtaining the mitochondria-targeted constructs a1PKIg and a1scrg, respectively.

As expected, the untargeted PKIg protein distributed freely within the cell, whereas a1PKIg co-localized with mitochondria (Fig. 7A). When co-expressed with HERG, PKIg completely inhibited the cAMP-dependent induction of the channel protein (1.05 ± 0.03-fold increase in HERG with PKIg compared
with a 1.84 ± 0.12-fold increase seen in p4scrg-expressing cells). This confirms that the responsible population of PKA is indeed accessible to the cytosol (Fig. 7B). In contrast, a1PKIg inhibited this effect but to a significantly lesser degree; there was a 1.38 ± 0.15- and 2.00 ± 0.20-fold increase in HERG in a1PKIg- and a1scrg-expressing cells, respectively. The fact that a1PKIg had any effect on HERG abundance could either mean that this construct partially localizes to the ER surface or that cAMP-dependent up-regulation of HERG is, in part, regulated by PKA subpopulation residing in or close to the mitochondria. To examine the latter possibility, we co-expressed the ER PKA sensor, p4AKAR3, with fluorescence-disrupted a1PKIg Y66A or a1scrg Y66A and observed the biosensor’s response to a 5-min 125 μM forskolin treatment. Co-expression of the mitochondrially targeted a1PKIg Y66A caused an intermediate (58.9 ± 11.6%) degree of FRETc suppression at the ER (none occurred with co-expression of a1scrg Y66A). Thus suggests that the a1PKIg construct also affects PKA signaling at the ER surface. Finally, to verify that the effects seen are not merely due to higher expression of the inhibitor constructs compared with the scramble controls, GFP levels were compared across the above experiments (Fig. 7, B and E). Under each condition, the expression of the inhibitor was slightly lower than that of the control peptide. Taken together, these data suggest that the HERG-inducing subpool of PKA is in fact compartmentalized and interacts preferentially at the ER surface.

DISCUSSION

In this paper, we have described the construction of a novel, ER surface-targeted PKI fusion protein that we named p4PKIg. This construct shuttles to the desired organelle, as verified by its co-localization with ER markers. FRET studies using targeted biosensors show that p4PKIg inhibits PKA activity preferentially at the ER rather than at the plasma membrane. Further studies designed to gauge the degree of PKA-dependent phosphorylation of ER-resident (PLN) or ER- and plasma membrane-localized (HERG) proteins confirmed that, indeed, our construct shelters ER surface proteins from the kinase activity but leaves plasma membrane proteins relatively untouched. Not only did this construct function as a valuable tool in inves-
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tigating HERG regulation; it also served to further underscore the concept of compartmentalized PKA activity.

The chief finding of the present study is the fact that p4PKIg completely negates cAMP-dependent HERG protein augmentation. This strongly supports the location of PKA-mediated HERG regulation at the ER membrane surface, as hypothesized in our earlier study (15). There we have shown that the kinase effects on the channel are ablated by geldanamycin-based inhibition of the Hsp90 chaperone. This implies that PKA regulates events upstream of the Hsp90-HERG interaction, which presumably include other chaperone interventions, quality control checkpoints (ER-associated degradation), channel subunit assembly, polypeptide chain folding, and translation itself. There is also evidence that much of the cAMP-dependent regulation is direct (i.e. that PKA phosphorylates the HERG channel directly rather than another effector protein). Such “peri-translational” control by a kinase makes the existence of a PKA-dependent enhancer of transcription unlikely. Therefore, it appears that peritranslational phosphorylation (i.e. during or immediately following translation) of the nascent HERG peptide promotes its folding and/or discourages ER-associated degradation.

HERG channel appears to be a curiously “difficult” protein for a cell to synthesize because typically the majority of the channels reside in the ER of the expressing cell rather than at its plasma membrane. This is in contrast to other similar topology K⁺ channel proteins, such as KCNQ1. Furthermore, the over 300 disease-associated LQTS2 mutations identified to date are dispersed throughout the HERG protein (43). Most of these mutations are deleterious by virtue of interfering with the channel’s biogenesis and trafficking rather than biophysical function (6). It would appear that synthesis and export of HERG is therefore an inefficient process, easily perturbed by even relatively minor mutations. Introduction of negative charge to certain segments of the nascent HERG polypeptide as happens with phosphorylation can be expected to affect folding more dramatically than it would in the case of a more efficiently synthesized protein. Although not a widely observed process, co-translational phosphorylation by PKA has been reported for other proteins, most notably actin (44, 45), where cAMP induces phosphorylation of target residues that become buried immediately following translation (46) of the nascent HERG peptide. Although our FRET studies do suggest that in p4PKIg-expressing cells there is some bleed-through of the PKI inhibitory population is not substantial enough to cause complete inhibitory to this mitochondrially targeted inhibitor. Still, this subpopulation is not enough to cause complete inhibition, as seen with ER-retained p4PKIg or untargeted PKIg. It is also noteworthy that our results show a greater sensitiv-

ity of the AKAR3 biosensors to targeted PKA inhibition than HERG. Specifically, AKAR3-pm is more sensitive to p4PKIg inhibition than is the phosphorylation of the mature form of HERG. Likewise, p4AKAR3 is more sensitive to a1PKIg inhibi-

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tion than is the presumably ER-based CPT-cAMP-dependent change in HERG abundance. There are at least two possible mechanisms for these findings. First, it is possible that only low levels of PKA activity (close to the limits of the dynamic range of AKAR3) are necessary for HERG phosphorylation/abundance regulation. Such a situation may arise when a kinase is physically targeted close to its substrate, as can happen with AKAP targeting of PKA. In support of this mechanism is our previous report of AKAP-like activity associating with HERG (20). Second, a subpopulation of AKAR3-pm may be present at the ER (due to trafficking) and will thus be sensitive to p4PKIg-dependent inhibition. The mature form of HERG is expected to be more restricted to Golgi/plasma membrane regions and thus insensitive to p4PKIg-dependent inhibition. Together, these mechanisms and our data suggest a model of cytoplasmic compartmentalization that is less purely discrete and more a continuum of PKA activity. In this model, there will naturally be some overlap in PKA signaling between the membranes exposed to the cytoplasm (plasma membrane, ER, and Golgi) by virtue of their close proximity.

Finally, it is also important to realize that our study does not take into account compartmentalization of cAMP. Similar to AKAP-mediated localization of PKA subpools, there is now significant evidence that substantial cAMP gradients exist in stimulated cells due to the non-uniform distribution of phosphodiesterases (54). For example, FRET-based studies in RNCMs showed that there is an uneven distribution of cAMP in stimulated myocytes that becomes uniform upon treatment with phosphodiesterase inhibitors (55). It is possible that cAMP and PKA gradients synergistically enhance the compartmentalization of overall adrenergic signaling. Our experiments involving the use of the freely diffusible, non-hydrolyzable cAMP analog CPT-cAMP do not rely on phosphodiesterase activity, yet even these experiments show localized, presumably AKAP-mediated PKA effects.

Our attempts to target PKIg to the plasma membrane (i.e. another organelle where it should only minimally affect the ER surface) have been unsuccessful. Fusions of PKIg and scrg with the CAAAX plasma membrane-targeting motif from K-ras4 (30) in both cases nonspecifically abolished all HERG expression in all subcellular compartments. PKIg/scrg chimeras with the C terminus of the KCNE2 protein, known for its relatively fast plasma membrane trafficking properties (56), resulted in diffusely distributed fluorescence, possibly indicating post-translational cleavage of the chimeras.

Future studies will be needed to elucidate the exact mechanism of the regulation of HERG at the ER surface, a task made difficult by the novelty of such a paradigm. As alluded to above, it would appear that PKA directly or indirectly modulates either HERG synthesis or some early phase of its translation, quality control, and/or folding. Although there are known instances of regulation at each of these steps (57, 58), to our knowledge, evidence implicating the involvement of PKA is scarce.

Finally, localized PKI inhibition will need to be explored in a more native system. Although RNCMs are widely accepted as a relatively easily manipulable cardiac cell model, in our experience, their native ERG channel expression varies widely with each lot prepared. Furthermore, there appears to be a rapid decline in ERG expression over time, a confounding factor in experiments that require several days to complete (which is the case in our adenoviral studies). Last, RNCM HERG detection is complicated by lack of reliable high quality antibodies suited for a native system; as a result, we chose to infect the cardiomyocytes with an adenoviral vector carrying the HERG cDNA as the next best option. However, results from large animal adult cardiomyocytes using more specific and sensitive monoclonal antibodies in the future will be needed.

Acute β-adrenergic receptor stimulation was originally described as having little or no effect on I_{Kr}/HERG as opposed to a dramatic augmentation of I_{Kr}/HERG in response to cAMP and PKA stimulation. The results we present here pertain not so much to hereditary LQTS2, where acute stimuli cause TdP development, but rather to clinical scenarios, where chronic adrenergic tone is involved in cardiac pathology and sudden cardiac death syndrome. For example, we may conjecture that one of the reasons why β-blocker therapy improves mortality due to congestive heart failure-related sudden death (62, 63) is that these compounds paradoxically improve cardiac adrenergic signaling (28) and thus boost the repolarization reserve by augmenting HERG expression.

In conclusion, our results further support the hypothesis that cAMP regulation feeds into an early process in the lifetime of HERG; either the rate/volume of protein synthesis or folding/early quality control within the ER membrane are affected. Furthermore, we report the development of ER surface-localized cAMP/PKA biosensors and a PKA inhibitor. It is becoming clear that the single kinase-based β-adrenergic signaling cascade is a system of immense complexity due to its compartmentalized nature; investigations of this system can therefore no longer rely on blunt tools affecting or detecting signaling indiscriminately throughout the entire cell. The newly developed constructs reported here thus constitute a contribution to a new location-specific tool set.

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REFERENCES

1. Moss, A. J., Schwartz, P. J., Crampton, R. S., Locati, E., and Carleen, E. (1985) Circulation 71, 17–21.
2. Modell, S. M., and Lehmann, M. H. (2006) Genet. Med. 8, 143–155.
3. Mitcheson, J. S., Chen, J., Lin, M., Culberson, C., and Sanguinetti, M. C. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 12329–12333.
4. Farkas, A. S., and Nattel, S. (2010) Drugs 70, 573–603.
5. Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995) Cell 81, 299–307.
6. Anderson, C. L., Delisle, B. P., Anson, B. D., Kilby, J. A., Will, M. L., Tester, D. I., Gong, Q., Zhou, Z., Ackerman, M. J., and January, C. T. (2006) Circulation 113, 365–373.
7. Phirtiyal, P., Jones, E. M., and Robertson, G. A. (2007) J. Biol. Chem. 282, 9874–9882.
