A-Kinase Anchoring Protein Targeting of Protein Kinase A and Regulation of HERG Channels

Yan Li · Jakub Sroubek · Yamini Krishnan · Thomas V. McDonald

Abstract Adrenergic stimulation of the heart initiates a signaling cascade in cardiac myocytes that increases the concentration of cAMP. Although cAMP elevation may occur over a large area of a target-organ cell, its effects are often more restricted due to local concentration of its main effector, protein kinase A (PKA), through A-kinase anchoring proteins (AKAPs). The HERG potassium channel, which produces the cardiac rapidly activating delayed rectifying K^+ current (I_{Kr}), is a target for cAMP/PKA regulation. PKA regulation of the current may play a role in the pathogenesis of hereditary and acquired abnormalities of the channel leading to cardiac arrhythmia. We examined the possible role for AKAP-mediated regulation of HERG channels. Here, we report that the PKA-RII-specific AKAP inhibitory peptide AKAP-*IS* perturbs the distribution of PKA-RII and diminishes the PKA-dependent phosphorylation of HERG protein. The functional consequence of AKAP-*IS* is a reversal of cAMP-dependent regulation of HERG channel activity. In further support of AKAP-mediated targeting of kinase to HERG, PKA activity was coprecipitated from HERG expressed in HEK cells. Velocity gradient centrifugation of solubilized porcine cardiac membrane proteins showed that several PKA-RI and PKA-RII binding proteins cosediment with ERG channels. A physical association of HERG with several specific AKAPs with known cardiac expression, however, was not demonstrable in heterologous cotransfection studies. These results suggest that one or more AKAP(s) targets PKA to HERG channels and may contribute to the acute regulation of I_{Kr} by cAMP.

Introduction

Dynamic control of action potentials in cardiac tissue underlies the normal response to varying physiological demands placed on the heart. In large part, this is governed by changes in ion channel activity in response to the autonomic nervous system cholinergic and adrenergic stimuli commensurate to hemodynamic challenges. Dysregulation of this balance plays a central role in pathological conditions such as heart failure. Spatial and substrate specificity of β-adrenergic signaling is maintained by localization of protein kinase A (PKA) and phosphodiesterases to subcellular microdomains. Localization of these proteins may be achieved by scaffolding adapter proteins termed A-kinase anchoring proteins (AKAPs) (Colledge and Scott 1999; Rubin 1994; Ruehr et al. 2004). The AKAPs are a group of proteins that lack primary structure sequence homology but share function: to localize PKA to subcellular structures, substrates, and oftentimes with other members of the signaling pathway (Smith et al. 2006). The regulatory subunits (RI and RII) of PKA bind and hold the catalytic subunits (C) in an inactive

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Y. Li · T. V. McDonald (✉)
Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: mcdonald@aecom.yu.edu

Y. Li · J. Sroubek · Y. Krishnan · T. V. McDonald
Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

state (R2C2 holoenzyme) until they bind cAMP, whereupon active catalytic portions dissociate toward target proteins (Taylor et al. 1990). The R-subunits bind to their respective RI or RII-specific AKAPs, providing local targeting of the catalytic subunit prior to its activation. The N termini of R-subunits form helix-turn-helix structures that create a binding groove for AKAPs, with specificity determined, in part, by the shallow (RII) or deep (RI) nature of the groove (Banky et al. 2003; Gold et al. 2006).

The human ether-a-go-go related gene (HERG) encodes the voltage-dependent potassium channel HERG and produces the repolarizing current I_{Kr} (Sanguinetti et al. 1995). I_{Kr} is important in controlling the orderly repolarization at the end of each cardiac action potential, particularly near the threshold potential of early afterdepolarizations that can trigger tachyarrhythmias. The importance of HERG/I_{Kr} in adrenergic signaling is highlighted by the fact that disease-causing mutations of HERG often lead to a syndrome in which arrhythmias are triggered by sudden emotional or auditory stress (Schwartz et al. 2001; Wilde et al. 1999). HERG/I_{Kr} responds to acute adrenergic signaling in a complex fashion that includes direct cAMP binding to the channel, PKA-dependent phosphorylation of HERG and phosphorylation-dependent binding to the adaptor protein 14-3-3 (Choe et al. 2006; Kagan et al. 2002; Thomas et al. 2003).

Here, we report the first evidence that acute cAMP/PKA-dependent regulation of HERG is targeted by cellular AKAP(s) to determine the degree of channel phosphorylation and biophysical response to acute cAMP stimulation. Such targeted PKA signaling to HERG may represent potential targets for future therapies in both hereditary and acquired dysregulation of the channel.

Materials and Methods

Cell Culture, Transfection and Expression Constructs

The HEK-293 cell line (from American Type Culture Collection, Manassas, VA) stably expressing C-terminal c-*myc* epitope-tagged HERG cDNA in pCI-Neo vector (Promega, Madison, WI) was cultured in RPMI-1640 (Mediatech, Manassas, VA) supplemented with L-glutamine, 10% fetal calf serum (HyClone, Logan, UT), and penicillin/streptomycin (Mediatech, Manassas, VA). Cultured cells were maintained in 5% CO_2 humidified air at 37°C. Constructs expressing GFP fused AKAP-*IS* and AKAP-*IS*-Scr were expressed in these cells by transient transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM medium (Invitrogen). AKAP-*IS* and AKAP-*IS*-Scramble constructs fused with GFP in pcDNA V5/HIS vectors were provided by Dr. John Scott (Alto et al. 2003) and PKA-R1,

PKA-RII fused with 6xHIS tag in pET14b vector (Novagen, Madison, WI) were from Dr. Charles Rubin (Dong et al. 1998; Li and Rubin 1995).

Immunoprecipitation, SDS-PAGE and Immunoblot Analyses

Cell proteins were harvested for analysis as described previously (McDonald et al. 1997). Briefly, cells were lysed in ice-cold NDET buffer (150 mM NaCl, 25 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1% NP-40, 0.4% deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4 and EDTA-free protease inhibitor cocktail tablets [Roche Pharmaceuticals, Nutley, NJ]) for 1 h with agitation. Lysates were cleared by centrifugation at 13,000 rpm for 10 min. Protein concentration was measured using the BCA method (micro-BCA, Pierce, Rockford, IL).

For immunoprecipitation, cell lysates were precleared with Ultra-Link Protein G-agarose (Pierce) for 30 min at 4°C. Supernatant was then incubated with anti-c-*myc* (A14G; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HERG (C-20) antibody for 1–2 h. A nonspecific IgG was used in parallel experiments to control for nonspecific binding. Antibody–antigen complexes were precipitated by protein-G-agarose for 3 h at room temperature or overnight at 4°C. After thorough washing with PBS, proteins were eluted from the resin with 4× Laemmli sample buffer and subjected to SDS-PAGE and immunoblot analysis.

Nitrocellulose membranes were blocked with 10% fat-free milk in Tris-buffered saline (TBS) and incubated with primary antibody solution for 2 h at room temperature or overnight at 4°C. After washing with TBS-T buffer (1 × TBS 0.5% Tween-20), the membrane was incubated with horseradish peroxidase–conjugated (Pierce) or IRDye-conjugated (Rockland, Gilbertsville, PA) secondary antibody in 5% milk TBS-T buffer for 30 min. Immunoblotting results were visualized with ECL or the Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE). Exposed film (Eastman Kodak, Rochester, NY) was scanned and underwent densitometric analysis. Odyssey software was used to create images and analyze the results.

PKA-Regulatory Subunit Overlay and Far-Western Blotting

PKA-RI and -RII overlay/far-Western was carried out as described by Hausken et al. (1998). Expression of PKA-RI or -RII subunit in pET14b vector (Novagen) was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in *Escherichia coli* BL21(DE3) bacteria. HIS-tagged protein was purified using Ni-NTA resin (Qiagen, Chatsworth, CA). For the overlay assay, cell samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and blocked with 5% nonfat milk and 1% BSA in TBS for 30 min at room

temperature. Purified PKA regulatory subunit was added to a final concentration of 0.5 µg/ml in blocking buffer, for a minimum of 3 h at room temperature. PKA-RI and -RII binding was visualized by subsequent Western blot using PKA-regulatory subunit–specific antibodies (BD Transduction Laboratories, San Diego, CA).

Immunofluorescence Analysis and Confocal Microscopy

HEK-HERG cells were plated in glass-bottomed 35-mm dishes (MatTek, Ashland, MA) at low density. Sample preparation was carried out at room temperature. Samples were rinsed with 1 x PBS three times between steps. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 5 min. After 30-min incubation in blocking buffer (5% BSA, 0.1% Triton X-100, 1 × PBS), samples were incubated with primary antibodies diluted in blocking buffer (1:100 for PKA-RI, -RII, and c-*myc* antibodies from Santa Cruz Biotechnology) for 2 h. Secondary antibodies (Alexa 555 conjugated donkey anti-rabbit, Alexa 647 conjugated donkey anti-mouse antibody; Invitrogen) were reacted with samples at 1:1,000 dilution in blocking buffer for 1 h. After extensive washes, cover glasses were mounted with Gel/Mount (Biomeda, Foster City, CA). Cells were observed using the Leica (Deerfield, IL) AOBS confocal microscope at the Analytical Imaging Facility, Albert Einstein College of Medicine.

In Vitro Phosphorylation

In vitro (or back) phosphorylation was carried out as previously published (Cui et al. 2000). Briefly, HERG protein was isolated from HEK-HERG cells by immunoprecipitation. Antigen–antibody complexes were incubated with purified catalytic subunit of PKA (Sigma, St. Louis, MO) in reaction buffer containing 50 mM Tris–HCl (pH 7.0), 10 mM $MgCl_2$, 0.1 mg/ml BSA and 10 mCi $[\gamma\text{-}^{32}P]$-ATP for 60 min at 30°C. Proteins were washed with ice-cold NDET, separated by SDS-PAGE and transferred to nitrocellulose membrane. Relative specific activity of HERG-^{32}P was determined by taking the ratio of the scintillation count of the ^{32}P signal over the densitometry of the Western blot signal. To assay for coprecipitation of kinase activity with HERG, immunocomplexes were washed three times and resuspended in 110 µl of kinase reaction buffer with or without addition of 500 nM PKI (PKA inhibitor 6–22 NH_2). Next, 5 µCi of $[\gamma\text{-}^{32}P]$ ATP in 10 µl of 880 µM Mg-ATP were added and incubated at 30°C for 30 min. Reactions were analyzed by SDS-PAGE, immunoblot and autoradiography.

Electrophysiology

Whole-cell patch-clamp current recordings were carried out as previously described (Kagan et al. 2002). Polished patch pipettes with tip resistances of 2–3 MΩ were used. All experiments were carried out at 20–22°C. Cells plated onto glass coverslips the night before were placed into a flow chamber mounted onto the stage of an inverted microscope equipped with epifluorescence illumination and micromanipulators to maneuver patch pipettes. Currents were measured with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) controlled by a PC using pClamp9 software for data acquisition and analysis (Axon Instruments). Whole-cell capacitance was recorded (generally 10–30 pF) and compensated by analog circuitry of the amplifier. The calculated junction potential for our experimental solutions was 3–4 mV and was not corrected for analyses. The series resistance of 5–12 MΩ (whole cell) was compensated to 75–90% with amplifier circuitry such that the voltage errors for currents of 2 nA were <5 mV. To study I_{Kr}, currents were elicited from a holding potential of −80 mV to depolarizing steps between –70 and +50 mV for 2 s, followed by sequential repolarizing steps to −40 mV and −120 mV. Signals were analog-filtered at 1,000 Hz and sampled at 5,000 Hz. Voltage-dependent activation data were fitted to the Boltzmann relation: $I = 1/(1 + \exp[(V_{1/2} - V)/k])$. Current densities were calculated as current (pA) divided by cell capacitance (pF). The voltage dependence of activation was obtained by plotting the normalized peak tail current against the test potential. Internal pipette solution was composed of (in mM) KCl 120, $MgCl_2$ 2, $CaCl_2$ 0.5, EGTA 5, ATP-Mg_4 4, HEPES 10 (pH 7.2, osmolality 280 ± 10 mOsm/L). External solution consisted of (in mM) NaCl 150, $CaCl_2$ 1.8, KCl 4, $MgCl_2$ 1, glucose 5, HEPES 10 (pH 7.4, osmolality 320 ± 10 mOsm). Data analysis was performed with Clampfit (Axon Instruments) and Origin 7.5 (Microcal, Northampton, MA) software.

Membrane and Microsome Preparation and Velocity Gradient Centrifugation

Fresh porcine cardiac tissue (∼30 g) was homogenized using Polytron PT-2100 (Kinematica, Bohemia, NY) in ∼100 ml ice-cold lysis buffer (10 mM KCl, 2 mM $MgCl_2$, protease inhibitors) supplemented with 0.25 M sucrose. The crude homogenate was cleared with a slow (1,000 × g) and a subsequent fast (10,000 × g) centrifugation (10 min each); the resulting supernatant was then loaded on top of a sucrose cushion (lysis buffer + 0.5 M sucrose) and centrifuged at 34,000 rpm (∼200,000 × g) for 20 min in SW 41Ti rotor (Beckman Instruments, Palo Alto, CA), yielding a membrane-rich pellet ("microsomal fraction"). Velocity gradient centrifugation was then performed using a

modification of a previously described method (Okamoto et al. 2001; Sargiacomo et al. 1995). Briefly, either confluent 10-cm culture plates of HEK-HERG cells or pig cardiac microsomes (one-sixth of total material) were lysed in 0.5 ml of MBST (1% Triton X-100 + protease inhibitors in 2-(N-morpholino) ethanesulfonic acid buffered saline (MBS), pH ~6.5). The entire lysate was loaded atop a 3.7 ml 5–40% linear sucrose gradient (in MBST) and centrifuged at 50,000 rpm ($\sim 340,000 \times g$) for 16 h in an SW 60 Ti rotor (Beckman Instruments). Liquid fractions ($n = 16$ or 17) of equal volume were collected from the top, while the pellet was extracted with NDET buffer.

Results

Pattern of AKAP Activity in HEK-293 Cells

PKA activation has an inhibitory effect on HERG channels expressed in a variety of heterologous systems (*Xenopus* oocytes, CHO cells and HEK293 cells) (Cui et al. 2000; Thomas et al. 1999). For an initial investigation, we examined the pattern of AKAP activity in HEK-293 cells stably expressing HERG channels (HEK-HERG cells) using a combination of PKA-RI and PKA-RII overlay with far-Western immunoblotting and immunofluorescence microscopy. Overlay assays demonstrated multiple PKA-RI and PKA-RII binding bands (Fig. 1A, C). Confocal micrographs showed that PKA-RI and PKA-RII differed from each other and that both demonstrated some degree of overlap when double-stained for HERG (Fig. 1B, D).

AKAP-*IS* Peptide Affects the Distribution of PKA-RII in HEK-293 Cells

AKAP-*In Silico* (AKAP-*IS*) is a peptide developed in the Scott lab (Alto et al. 2003) that preferentially binds to PKA-RII with an affinity >500 times that for PKA-RI. AKAP-*IS* competes with AKAP for PKA-RII binding, thus disrupting the AKAP-directed PKA-RII targeting. For
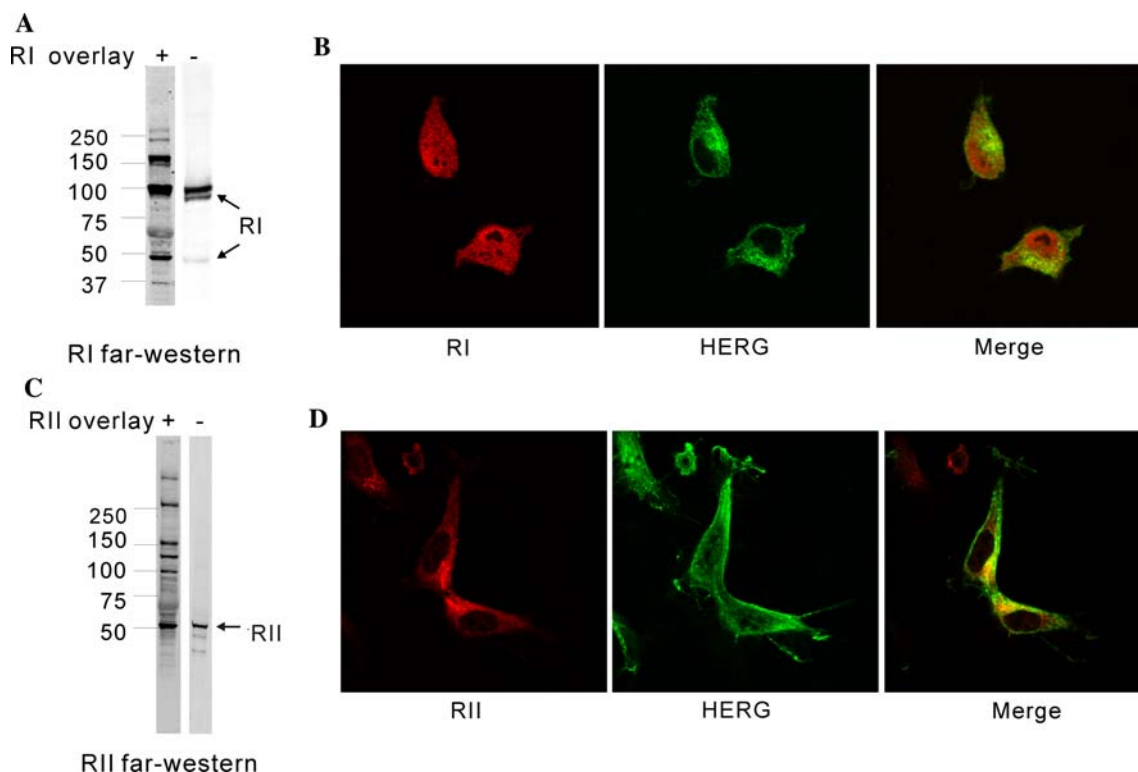

Fig. 1 AKAP activity profile of HEK-HERG cells. (**A**) Representative PKA-RI overlay/far-Western assay results from HEK-HERG cells (HEK-293 cells stably expressing HERG). Several PKA-RI binding bands are seen in the *left lane*. *Right lane* shows results of an identical sample probed with anti-PKA-RI antibody without RI overlay, showing monomer and dimerized RI subunits. The predicted human RIα size is 43 kDa. (**B**) Immunofluorescence confocal images of HEK cells stained for PKA-RI (red) and HERG (green). RI staining pattern is particulate, with both cytoplasmic and nuclear locations. HERG staining is more restricted to peripheral locations, showing some overlap with RI in cytoplasmic areas. (**C**) Representative PKA-RII overlay/far-Western assay results from HEK-HERG cells. PKA-RII binding bands are seen in the *left lane*. *Right lane* shows results of an identical sample probed with anti-PKA-RII antibody without RII overlay showing monomer RII subunits. The predicted human RIIα size is 46 kDa. (**D**) Immunofluorescence confocal images of HEK cells stained for PKA-RII (red) and HERG (green). RI staining pattern is particulate with prominent perinuclear location consistent with Golgi and to a lesser extent more peripheral and cytoplasmic structures. RII and HERG show some degree of overlap in these areas
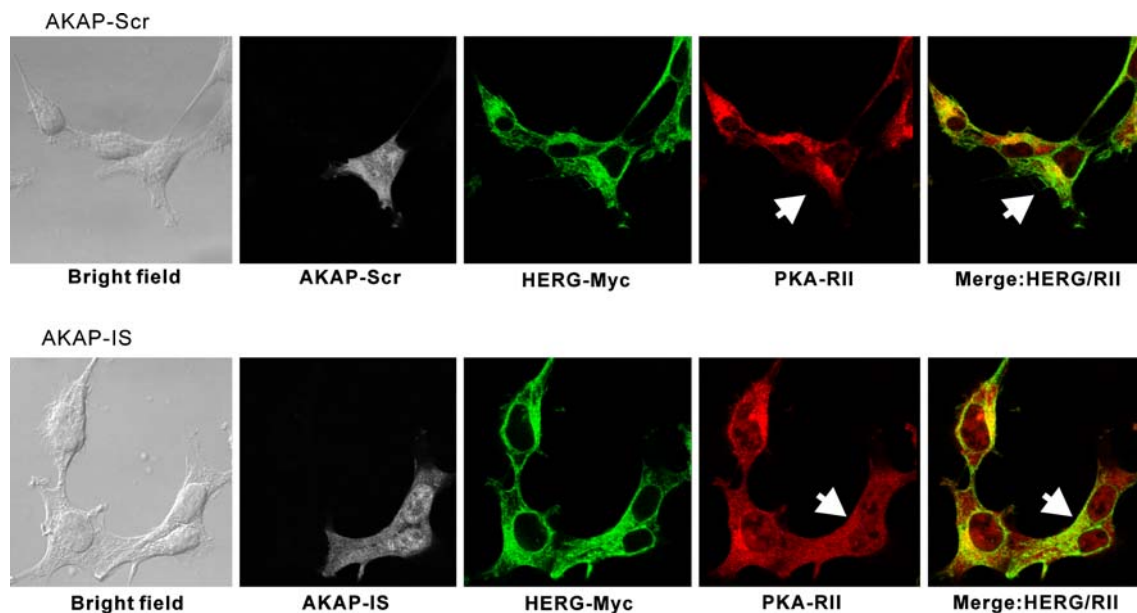
Fig. 2 AKAP-*IS* peptide alters the PKA-RII distribution in HEK-HERG cells. *Upper panel*: Representative confocal immunofluorescence micrographs of HEK-HERG cells transiently transfected with AKAP-Scr-GFP. The AKAP-Scr expressing cell (*arrowhead*) shows RII (red) distribution throughout the cell cytoplasm with enhancement in subcellular structures, membranes and perinuclear locales. The RII distribution pattern of the AKAP-SCR cell is similar to neighboring cells that are not expressing AKAP-Scr. Dual staining for HERG shows considerable overlap (yellow) with RII on the merged image. *Lower panel*: A representative confocal image of HEK-HERG cells transiently transfected with AKAP-*IS*-GFP. The AKAP-*IS* expressing cells (*arrowhead*) show RII (red) staining that is more diffusely distributed than in neighboring cells that are not expressing AKAP-*IS*. Dual staining for HERG shows considerably less overlap (yellow/orange) with RII on the merged image

control purposes, a peptide with the same amino acid composition but in a scrambled order was used (AKAP-Scr). AKAP-Scr or AKAP-*IS* fused with GFP for detection of successful transfection/expression was transiently transfected into HEK-HERG cells. That not all cells were transfected was exploited for determining the effects of AKAP-*IS* within a field of cells subjected to identical fixing/staining conditions. In HEK-HERG cells, AKAP-*IS* altered PKA-RII distribution (Fig. 2). Compared to non-transfected cells or AKAP-Scr expressing cells, AKAP-*IS* expression resulted in more widespread staining of PKA-RII across the subcellular compartments with less intense staining in the perinuclear area (consistent with the Golgi) and less nuclear exclusion. The extent of overlap of PKA-RII with HERG was less apparent in AKAP-*IS* expressing cells. The overall expression and localization of HERG, however, was not affected by AKAP-*IS* expression.

AKAP-*IS* Decreases PKA-Dependent Phosphorylation of HERG Channels

Acute regulation of HERG channel activity by cAMP is the result of PKA phosphorylation of the channel protein at four separate sites (Cui et al. 2000; Thomas et al. 1999). To determine if AKAP targeting of PKA plays a role in this process, we employed in vitro back-phosphorylation as an estimate of the intracellular phosphorylation state of HERG.

In this assay, ^{32}P labeling of HERG in vitro after isolation from cells is an inverse measure of the phosphorylation state of the channel just prior to harvesting cells (Marx et al. 2000). HEK-HERG cells were transfected with either AKAP-*IS* or AKAP-Scr and harvested under control conditions and after administration of CPT-cAMP for 5 min. HERG was immunoprecipitated from these samples with phosphatase inhibitors and subjected to in vitro back-phosphorylation with the catalytic subunit of PKA. Detection of back-phosphorylation by autoradiography and of total HERG by immunoblotting is shown in Fig. 3. PKA activity stimulated by CPT-cAMP markedly diminished the amount of back-phosphorylation, indicating that most of the PKA sites were occupied by nonisotopic phosphate. Transfection with AKAP-*IS*, however, resulted in an increase in the amount of back-phosphorylation after treatment of cells with CPT-cAMP, indicating that disruption of AKAP/PKA-RII targeting reduced intracellular phosphorylation of HERG.

AKAP-*IS* Reverses the Acute cAMP/PKA Regulation of HERG Current

To investigate the functional consequences of disrupted AKAP/PKA-RII targeting for HERG channels, we performed whole-cell patch-clamp recordings of HERG current from HEK-HERG cells transfected with AKAP-*IS* or AKAP-Scr. The current density between the two groups was not
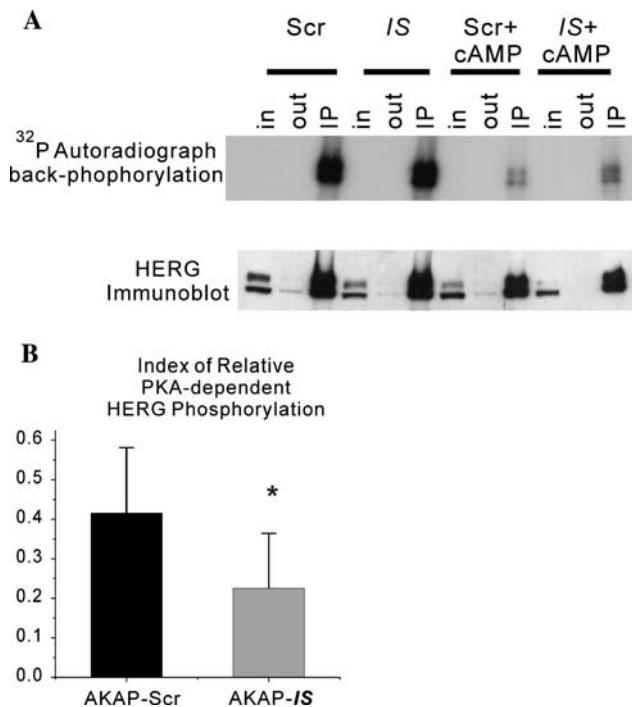
A

B

Index of Relative
PKA-dependent
HERG Phosphorylation

Fig. 3 AKAP-*IS* decreases in situ phosphorylation of HERG protein in HEK cells. (**A**) Back-phosphorylation assay for endogenous PKA-dependent phosphorylation of HERG channels. Nonisotopic phosphorylation of HERG within cells prior to immunoprecipitation prevents in vitro addition of ^{32}P (back-phosphorylation). The amount of in vitro phosphorylation was determined by autoradiography. Subsequent immunoblotting revealed the total HERG protein in the sample. Lanes marked "in" represent the amount of unlabeled protein present in the input lysate. Lanes marked "out" represent protein remaining in the lysate after immunoprecipitation. Lanes labeled "IP" represent the protein that was immunoprecipitated. (**B**) Normalized intracellular PKA phosphorylation index. Endogenous PKA-dependent phosphorylation of HERG was calculated by $1 - (^{32}$P signal/immunoblot signal)$_{cAMP} \div (^{32}$P-signal/immunoblot signal)$_{control}$. In AKAP-*IS* transfected groups, the cAMP-dependent increase of intracellular HERG phosphorylation level was significantly reduced compared to AKAP-SCR transfected cells, suggesting AKAP involvement in PKA phosphorylation of HERG. $p = 0.018$, $n = 5$

significantly different (AKAP-Scr = 26.3 ± 5.3 pA/pF, AKAP-*IS* = 31.714 ± 6.5 pA/pF). Each cell acted as its own control before and after perfusion with CPT-cAMP, as previously reported (Cui et al. 2000). In AKAP-Scr expressing cells, CPT-cAMP perfusion led to inhibition of HERG current by $\sim$40% within 5 min (Fig. 4). Despite reduction in current amplitude, there was little change in $V_{1/2}$ of activation (Fig. 4B, C) consistent with our previous observations (Cui et al. 2000). Perfusion of CPT-cAMP onto AKAP-*IS* transfected cells, however, failed to result in current amplitude reduction; on the contrary, there was a small enhancement of current density (Fig. 4). There was a significant cAMP-dependent shift in the $V_{1/2}$ for the AKAP-IS treated cells, suggesting the effects of direct cAMP activity on the channel or via another unidentified PKA-RI-mediated process.

HERG Coprecipitates PKA Activity from HEK Cells

In experiments where HERG was immunoprecipitated from HEK-HERG cells for in vitro phosphorylation, we also examined ^{32}P labeling in the absence of exogenously added PKA. HERG protein was incubated with γ^{32}P-ATP, with or without the specific PKA inhibitor PKI. Although the extent of labeling was much less than when purified PKA catalytic subunit was added, without exogenous kinase a measurable amount of ^{32}P was detected on HERG channels. A significant fraction of this ^{32}P labeling was prevented by coincubation with PKI (Fig. 5). This result supports the conclusion that PKA coprecipitated with some fraction of HERG expressed in HEK cells.

Pattern of AKAP Activity and ERG Channels from Porcine Ventricular Myocardium

Our results so far have provided evidence that AKAP targeting of PKA-RII is involved in the acute cAMP-dependent regulation of HERG in heterologous expression. The situation in the native tissue (ventricular myocardium) is uncertain at this point. To search for evidence that cardiac AKAPs might target PKA to ERG channels, we combined sucrose gradient sedimentation of membrane proteins from porcine ventricles with PKA-RI/PKA-RII overlay analyses to determine if AKAP activity and ERG channels are localized to similar compartments or complexes. The sucrose density gradient centrifugation technique provides an indication of possible macromolecular complexes based on similar migration within the sucrose gradient (Okamoto et al. 1999; Pasquali et al. 1999; Sugumaran et al. 1998). By introducing Triton X-100, many membrane lipid structures are disrupted but protein–protein complexes and Triton-resistant membrane rafts remain intact (Okamoto et al. 2001; Sargiacomo et al. 1995). The procedure was done in tandem with HEK-HERG cells for comparison (Fig. 6). The first obvious result in these experiments was that both PKA-RI and PKA-RII showed considerable cosedimentation with ERG channels. This overlap was greater in myocardial tissues than in HEK cells. The pattern of PKA-RI/PKA-RII binding furthermore shows that numerous potential AKAPs for both PKA isoforms cosediment with ERG channels. Thus, there is ample potential for AKAP targeting of PKA to ERG channels in the native tissue.

Discussion

Acute β-adrenergic regulation of HERG/I_{Kr} may play an important role in the maintenance of cardiac excitability in situations of both normal and maladaptive physiology. That cAMP and PKA are involved in controlling HERG
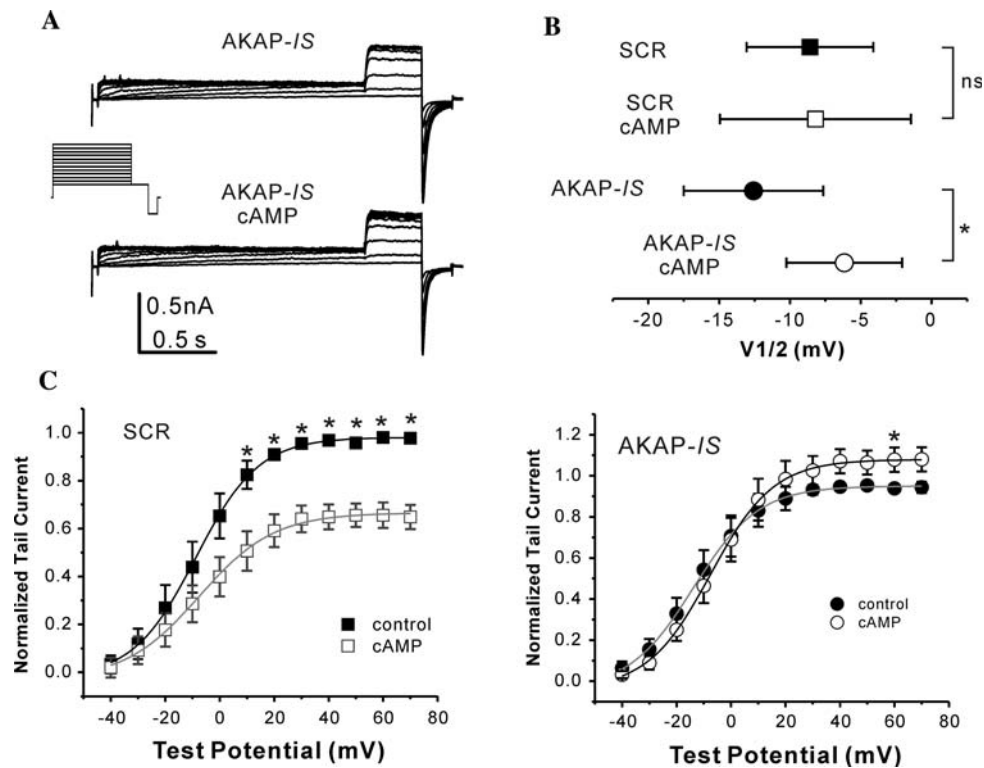
Fig. 4 AKAP-*IS* diminishes the acute cAMP regulation of HERG current. (**A**) Representative HERG K^+ currents from HEK-HERG cells transfected with AKAP-*IS* in response to a series of depolarizing voltage steps (illustrated in the protocol graphic). The two families of currents are from the same cell before and after exposure to CPT-cAMP. (**B**) The $V_{1/2}$ of HERG currents from AKAP-SCR or AKAP-*IS* transfected HEK-HERG cells before and after exposure to CPT-cAMP (in paired control/treated experiments). AKAP-SCR $V_{1/2} =$ -8.6 ± 4.5 mV control, -8.2 ± 6.7 mV cAMP, $n = 8$; AKAP-*IS* $V_{1/2} = -12.6 \pm 4.9$ mV control, -6.2 ± 4.1 mV cAMP, $n = 10$. (**C**) Voltage–activation curves fitted to data from cells expressing AKAP-SCR (*left graph*) or AKAP-IS (*right graph*). Tail current amplitude was normalized to maximum values for each cell before addition of CPT-cAMP. *Difference is statistically significant ($P < 0.05$)
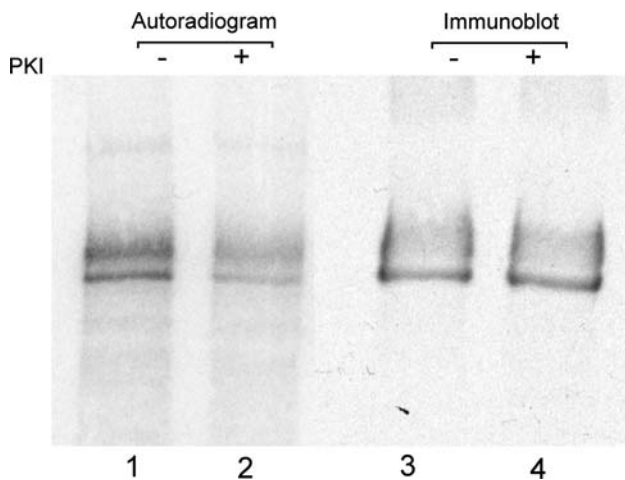

Fig. 5 HERG coprecipitates PKA activity from HEK cells. HERG protein was immunoprecipitated from HEK-HERG cells, washed and incubated with ^{32}Pγ-ATP without exogenous kinase. *Lane 1* shows that HERG protein is phosphorylated under these conditions and that coincubation with the PKA-specific inhibitor PKI diminishes the amount of phosphate added (*lane 2*). *Lanes 3* and *4* show the total amount of HERG protein loaded for each condition

has been shown in vitro (Cui et al. 2000; Thomas et al. 1999) and indirectly from genetic studies (Choe et al. 2006; Schwartz et al. 2001; Wilde et al. 1999). There is evidence, however, that the regulatory system may be more complex in cardiac tissue and may involve additional members of a macromolecular complex (Heath and Terrar 2000; Kagan et al. 2002; Thomas et al. 2003). Here, we provide evidence that that this process may be facilitated by targeting of PKA to the vicinity of the channel by one or more PKA-RII-specific AKAPs. Inhibition of PKA-RII binding to AKAPs in HEK cells with AKAP-*IS* resulted in altered distribution of PKA-RII, reduced PKA-dependent phosphorylation of HERG protein and reversed PKA-dependent functional effects on the channel.

AKAPs are a structurally diverse group of proteins that bind the regulatory subunits (RII and/or RI) of PKA, thereby targeting and concentrating the enzyme to specific locales of action, generally to specific membrane domains (Colledge and Scott 1999; Rubin 1994). AKAPs often coordinate the scaffolding of various other molecules to
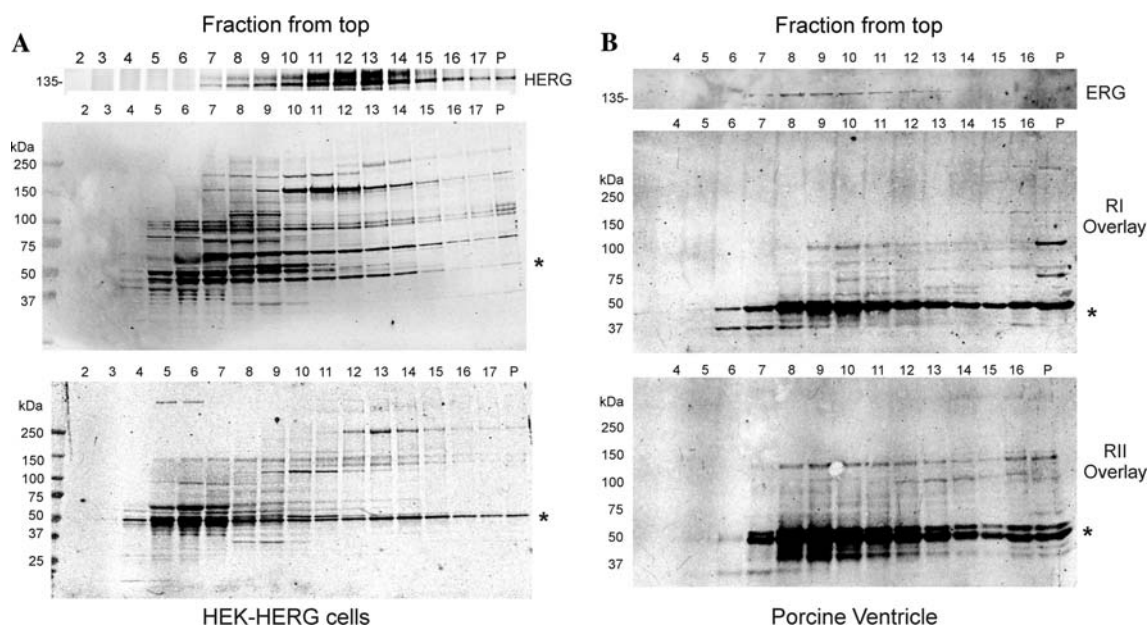
Fig. 6 Cosedimentation of ERG channel PKA and AKAP activity in HEK-HERG cells and porcine heart. Membrane fractions from either HEK-HERG cells (**A**) or porcine ventricular myocardium (**B**) were disbursed in 1% Triton X-100 and subjected to centrifugation through a 5–40% linear sucrose gradient. Equal-volume fractions were analyzed by SDS-PAGE, PKA-RI/ PKA-RII overlay and far-Western blotting. The majority of ERG protein migrated between fractions 8

and 14 from HEK cells and fractions 7 and 13 from porcine myocardium (*top panels*). Endogenous signals from both PKA-RI and PKA-RII (marked by *) show considerable cosedimentation with ERG channels, with greater apparent overlap from cardiac tissue. Several lower–molecular weight (<50 kDa) and larger (>75 kDa) species of RI- and RII-binding proteins also cosediment with ERG proteins

a particular site such as protein phosphatases and phosphodiesterase in addition to the specific substrates, resulting in a macromolecular signaling complex (Appert-Collin et al. 2006; Dodge-Kafka et al. 2006; McConnachie et al. 2006). Since the first report of AKAPs (Sarkar et al. 1984), more continue to be added to the list (Feliciello et al. 2001; Ruehr et al. 2004).

PKA-RII regulatory subunit interactions with AKAPs have been the most thoroughly studied; however, RI subunits are also now recognized as targets for AKAPs (Angelo and Rubin 1998; Huang et al. 1997; Li et al. 2001). The AKAPs that have been best established in the heart include mAKAP (also known as AKAP6), AKAP15/18 (AKAP7), Yotiao (AKAP9), AKAP-Lbc (AKAP13), AKAP79 (AKAP5), gravin (AKAP12), AKAP220 (AKAP11), BIG2, MAP2B, AKAP95 (AKAP8), AKAP149/121 (AKAP1) and D-AKAP-1 (AKAP1) (Ruehr et al. 2004). For the most part, these AKAPs target PKA-RII; however, there is evidence for both PKA-RI and RII targeting in the case of D-AKAP-1 and AKAP220. In some instances, the AKAPs have been shown to enhance cAMP/PKA regulation of ion channels in the heart during periods of sympathetic stimulation such as Yotiao and KCNQ1/I_{Ks} (Marx et al. 2002).

Our results suggest that one or more PKA-RII-specific AKAPs may be involved in mediating adrenergic and cAMP regulation of HERG. The AKAP-*IS* inhibitor is specific to PKA-RII subunits; therefore, we cannot rule out

the potential contribution of PKA-RI-specific AKAP targeting to HERG. That ERG channels comigrated with several RI- and RII-binding proteins raises the possibility that more than one macromolecular signaling complex exists for channel regulation. If more than one AKAP could associate with HERG, adrenergic and PKA-mediated modification of channels may differentially occur at various stages of channel synthesis, assembly, trafficking and regulation of I_{Kr} at the surface.

A pressing question that remains is which AKAP(s) is involved in targeting PKA to the HERG channel. Of the known cardiac AKAPs, we have heterologously coexpressed HERG with Yotiao, AKAP220, Ezrin, MAP2, AKAP15/18 and AKAP75/79 with no evidence of coimmunoprecipitation (supplemental data). These results may indicate that other AKAPs are involved or that some intermediary specific to cardiac myocytes is required for association. Alternatively, AKAPs may colocalize to a particular membrane environment (such as a lipid raft) that brings the channel and PKA into a close approximation without direct protein–protein interaction. In the latter case, standard coimmunoprecipitation assays would fail to detect an association even in heterologous cotransfections. That PKA is targeted via Yotiao to the KCNQ1/KCNE1 complex (Marx et al. 2002) was considered, but velocity gradient centrifugation showed no significant difference in sedimentation of RII when HERG was coexpressed with

either KCNE1 or KCNE2 (supplemental data). Whether associated KCNE in vivo affects AKAP targeting remains an open question.

These results support further investigation into the nature of macromolecular signaling complexes that may regulate HERG/I_{Kr}. Such studies should give attention to the possibility that different complexes may occur at different temporal and spatial sites of HERG biogenesis and activity. Knowledge of specific signal-transduction entities may impact future medical therapies for various cardiovascular disorders.

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