KCNE2 protein is more abundant in ventricles than in atria and can accelerate hERG protein degradation in a phosphorylation-dependent manner

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Zhang M, Wang YH, Jiang M, Zankov DP, Chowdhury S, Kasirajan V, Tseng GN. KCNE2 protein is more abundant in ventricles than in atria and can accelerate hERG protein degradation in a phosphorylation-dependent manner. Am J Physiol Heart Circ Physiol 302: H910–H922, 2012. First published December 16, 2011; doi:10.1152/ajpheart.00691.2011.—KCNE2 functions as an auxiliary subunit in voltage-gated K and HCN channels in the heart. Genetic variations in KCNE2 have been linked to long QT syndrome (LQT6) (1, 25), pointing to its role in maintaining the electrical stability of human ventricles. How- ever, the underlying mechanisms are not clear. Some (2, 14), studies have shown that the mRNA level of KCNE2 is much lower than those of the other KCNE subunits in human hearts, and the KCNE2 mRNA level is much lower in ventricles than in atria. These, and several other studies (19, 20), have created the impression that KCNE2 expression in human heart is limited to pacemaker regions (sinoatrial node and Purkinje fiber) and atrial myocytes. Consequently, it is proposed that KCNE2 expression in human ventricular myocytes is very low and functionally insignificant, making it difficult to explain why genetic variations in KCNE2 are linked to LQT6.

Previously, we used a homemade antibody and a commercial antibody (Alomone catalog No. APC-054) to probe KCNE2 protein expression in the ventricles of human and animal models (rat and dog) (10). We called these antibodies Ab1 and Ab2, respectively. Their epitope sequences are shown in Fig. 1B [based on the human sequence, well conserved in rat, dog, guinea pig, and other species (10)]. We showed that both Ab1 and Ab2 detected a major 25-kDa band in human and rat ventricles, but a 20-kDa band in dog ventricles. In the case of Ab2, the bands can be abolished by preincubating the antibody with excess antigen. We further used Ab2 immunoblot quantification to suggest that KCNE2 expression in the ventricle can be differentially remodeled under different diseased conditions. This implies that aberrant KCNE2 expression may play a role in acquired ventricular arrhythmias.

The aforementioned uncertainty in the literature about KCNE2 protein expression in the ventricle prompted us to revisit this issue. In particular, we want to know whether indeed KCNE2 protein is mainly or preferentially expressed in atria but not or very low in ventricles. We are further motivated by two other concerns. The first one is the variation in the expression of KCNE2 protein expression in the ventricles of human and animal models (rat and dog) (10). We called these antibodies Ab1 and Ab2, respectively. Their epitope sequences are shown in Fig. 1B [based on the human sequence, well conserved in rat, dog, guinea pig, and other species (10)]. We showed that both Ab1 and Ab2 detected a major 25-kDa band in human and rat ventricles, but a 20-kDa band in dog ventricles. In the case of Ab2, the bands can be abolished by preincubating the antibody with excess antigen. We further used Ab2 immunoblot quantification to suggest that KCNE2 expression in the ventricle can be differentially remodeled under different diseased conditions. This implies that aberrant KCNE2 expression may play a role in acquired ventricular arrhythmias.

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Ab2 can detect the same band(s). If the results are positive, we then check whether the native KCNE2 is at a level detectable by Ab1 and Ab2, noting that the native KCNE2 band(s) should be 1 kDa lighter than its HA-tagged counterpart and is expected to be fainter. To help distinguish between native KCNE2 band(s) and unrelated bands, we use adenovirus-mediated expression of small interfering RNA to knock down the expression of native KCNE2 in adult cardiac myocytes. By comparing the immunoblot banding patterns between control myocytes and myocytes with KCNE2 knockdown, we hope to unequivocally validate (or refute) KCNE2 band(s) detected by Ab1 and Ab2.

Our data show that Ab1 can detect native KCNE2 proteins in rat and guinea pig hearts, and in both cases, the KCNE2 protein level is more abundant in ventricles than in atria. Ab1 can also detect native KCNE2 protein in ventricles of nonfailing human hearts. On the other hand, although Ab2 can detect native KCNE2 proteins in the heart, it also detects a major unrelated protein right above the 24 kDa validated KCNE2 band. Despite this problem, Ab2 is useful in another way: it can detect the phosphorylation status of a serine residue (S98) in the epitope region of KCNE2. We show that KCNE2 can suppress the HERG current amplitude by accelerating HERG protein degradation, and S98 phosphorylation seems necessary for this effect of KCNE2.

MATERIALS AND METHODS

Human and animal subjects. Use of human heart specimens and laboratory animals [guinea pigs, spontaneously hypertensive rats (SHR), and dogs] was reviewed annually and approved by the Institutional Review Board and Institutional Animal Care and Use Committee of Virginia Commonwealth University. We use right atrial appendages from patients undergoing elective surgery (removed for lung-heart bypass surgery) and ventricular specimens from end-stage heart failure patients (removed for implantation of left ventricular assist device) or nonfailing donor hearts (not suitable for transplantation).

Cardiac myocyte isolation, culture, adenovirus injection, and canine model of microembolization. Atrial and ventricular myocytes are isolated from guinea pig and SHR hearts using enzymatic digestion followed by gentle trituration as described previously (9). Isolated myocytes are plated on laminin-coated coverslips and maintained in serum-free, Met-free DMEM medium for 30 min at 36°C. 35S-Met COS-7 cells are depleted of Met by incubation in a moist 5% CO2 incubator at 36°C. Cells are plated at a subconfluence level the day before transfection. cDNA transfection is facilitated by lipofectamine 2000 (Invitrogen) based on manufacturer’s instructions. COS-7 cells destined for patch clamp experiments are cotransfected with CD8 cDNA. COS-7 cells are incubated with CD8 mAb-coated beads at room temperature for 10 min, trypsin-digested, plated on poly-L-lysine coated coverslip placed in a cell chamber mounted on the stage of a Nikon inverted microscope, and continuously superfused at room temperature with normal Tyrode’s solution of (in mM) 146 NaCl, 4 KCl, 2 CaCl2, 0.5 MgCl2, 5 HEPES, and 5.5 dextrose (pH 7.3). Cells decorated with beads are used for patch clamp recording using an AxoClamp 200B amplifier. Pipette solution contains (in mM) 105 K-aspartate, 20 KCl, 5 ATP(K), 1 MgCl2, 5 EGTA, and 5 HEPES (pH 7.3). Data acquisition is controlled by Clampex of pClamp10 via a DigiData 1440A interface. Current traces are low-pass (1 kHz) filtered and digitized. Offline data analysis is done using Clampfit in conjunction with Microsoft Excel, SigmaPlot, and SigmaStat.

Oocyte preparation, cRNA injection, and two-electrode voltage clamp experiments. Oocytes are isolated as described before (27) and incubated in an ND96-based medium containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, 5 HEPES, and 2.5 Na-pyruvate (pH 7.5) supplemented with 4% horse serum and penicillin-streptomycin at 16°C. Two to six hours after isolation, oocytes are injected with cRNAs(s) using a Drummond digital microdispenser. Oocytes are incubated in the above medium at 16°C and studied 3–5 days after isolation. Whole oocyte membrane currents are recorded using the 2-cushion pipette voltage clamp method (24). Both current-passing and voltage-recording pipettes have tip resistances of 0.1–0.3 MΩ. During recordings, the oocyte is continuously superfused with a low-Cl ND96 solution to reduce interference from endogenous Cl channels. Voltage clamp is done at room temperature with OC-725B or OC-725C amplifier (Warner Instruments). Data acquisition and analysis are the same as those described for COS-7 patch clamp experiments.

Pulse chase experiments. Twenty-four hours after transfection, COS-7 cells are depleted of methionine (Met) by incubation in serum-free, Met-free DMEM medium for 30 min at 36°C. 35S-Met (200 uCi/ml, [Met] ~0.2 mM) is then added, and cells are incubated for 60 min at 36°C. Afterward, cells are washed and incubated in DMEM containing 2 mM unlabeled Met for different amounts of chase time. At the end of specified chase times, cells are washed, lysed, and solubilized. After lyzing the whole cell lysate (WCL) is subjected to immunoprecipitation as described previously (9). The original WCLs and immunoprecipitates are fractionated by SDS-PAGE (described below). Part of the proteins is blotted to polyvinylidene difluoride (PVDF) membrane and probed by suitable antibodies (9). The original WCLs and immunoprecipitates are fractionated by SDS-PAGE (described below). Part of the proteins is blotted to polyvinylidene difluoride (PVDF) membrane and probed by suitable antibodies. The radioactivity of proteins in the gels is quantified by PhosphorImager.

Biotinylation experiments. Forty-eight hours after cDNA transfection of COS-7 cells, or cRNA injection of oocytes, cells or oocytes are washed with cold PBS twice, followed by incubation in 0.25 mg/ml amine-reactive, disulfide bond containing biotin derivative (EZ-link sulfo-NHS-SS-biotin; Pierce) on ice for 30 min. The biotinylation reaction is quenched by 25 mM Tris-HCl. Cells or oocytes are washed and lysed as described below. The protein concentrations in WCLs are
measured. For each sample, 10% of WCL is saved as direct input for the following immunoblot experiments. To the remaining WCL, a slurry of Neut-Avidin Dynabeads is added at 50 μl beads/200 μg protein, and the mixture is incubated at 4°C overnight. The beads are collected and washed with lysis buffer six times. Biotinylated proteins are eluted by incubating beads in sample buffer containing mercaptoethanol. Biotinylated fractions and direct inputs are fractionated by SDS-PAGE.

**SDS-PAGE, immunoblotting, and related experiments.** Cultured myocytes or COS-7 cells are sonicated, and the suspension is centrifuged at slow speed to remove nuclei and particles. The supernatant is solubilized in a buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mg/ml BSA, and protease inhibitor cocktail. After centrifugation to remove debris, the supernatant is collected as WCL. Oocytes are homogenized with a loose-fit glass homogenizer in a buffer containing protease inhibitors. After low-speed centrifugation to remove nuclei and particles, the supernatant is solubilized by 1% Triton X-100. After centrifugation to remove debris, the supernatant is collected as WCL. Frozen cardiac tissue chunks are pulverized in 10 vol of lysis buffer containing (in mM) 145 NaCl, 0.1 MgCl₂, 15 HEPES, 10 EGTA (pH 7), and 0.5 Triton X-100, and protease inhibitor cocktail, and solubilized for 30 min on ice. The above is homogenized by tip sonicator (2 of 15-s bursts) and then centrifugated to pellet nuclei and particles. The supernatant is collected as whole tissue lysate (WTL) (18). Protein concentrations in WCL and WTL are quantified using BCA kit (Pierce).

In vitro translation is carried out using a rabbit reticulocyte lysate system in the presence of canine pancreatic microsomes (Promega), according to the manufacturer's instructions. Deglycosylation with PNGase F (Sigma–Aldrich) is carried out based on manufacturer's instructions. Dephosphorylation is carried out by adding 20 μl of reaction buffer containing (in mM) 50 Tris-HCl, 10 MgCl₂, 100 NaCl, and 1 DTT to 20 μg of protein (in 5 μl Tris-EDTA buffer). The mix is incubated with 2 units of calf intestinal phosphatase (Sigma P-7640) per milligram of protein at 37°C for 1 h.

Immunoblotting experiments are as described previously (10). Protein samples are boiled for 5 min in sample buffer and loaded onto 4–20% gradient or 15% polyacrylamide gel. After fractionation, the proteins are blotted onto PVDF membranes (Amersham). Residual proteins in the gel are stained with Coomassie blue to serve as loading control or, in the case of radioactively labeled proteins, exposed to phosphomager screen for detection of radioactive bands. The PVDF membranes are blocked in PBS with 5% dried milk-0.1% Tween 20. The membrane is incubated with primary and then secondary antibodies with rinses in reaction buffer containing (in mM) 50 Tris·HCl, 10 MgCl₂, 100 NaCl, and 1 DTT to 20 μg of protein (in 5 μl Tris-EDTA buffer). The mix is incubated with 2 units of calf intestinal phosphatase (Sigma P-7640) per milligram of protein at 37°C for 1 h.

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**Immunofluorescence and confocal microscopy.** Myocytes are fixed in 4% paraformaldehyde, permeabilized by 0.2% Triton X-100, and incubated with primary and then secondary antibodies with rinses in between) / HA mouse mAb/Alexa647-conjugated anti-mouse or 2) β1 rabbit pAb/Alexa488-conjugated anti-rabbit. Nuclei are stained by 4′,6-diamidino-2-phenylindole (DAPI). Myocytes are viewed with a Zeiss 510 Meta confocal laser-scanning microscope.

**Antibodies.** The following antibodies are used: KCNE2 Ab1 (made by ourselves) and Ab2 (Alomone), HA mAb (Covance), actin mAb (Sigma), hERG pAb (Alomone), ERG1a pAb (a kind gift from Dr. Gail A. Robertson, University of Wisconsin at Madison) (11), and Kv4.3 mAb (NeuroMab).

**Statistical analysis.** Statistical tests are carried out using SigmaStat version 2. Two groups are compared by t-test. Multiple groups are compared by one-way ANOVA and, if a significant difference is found, followed by Tukey’s or Dunn’s pair-wise comparisons.

**RESULTS**

Validating Ab1 and Ab2 for detecting native KCNE2 in adult ventricular myocytes. The HA mAb detects two strong bands of 33 and 25 kDa in cultured SHR ventricular myocytes that have been treated with Adv-E2 (HA) overnight, followed by 24 h culture. HA mAb does not detect these bands in cultured SHR myocytes treated with Adv-GFP (Fig. 1C, left, although a faint nonspecific 33-kDa band is seen). Both Ab1 and Ab2 detect the same prominent 33 and 25-kDa HA-KCNE2 bands (Fig. 1, C and D), confirming that they can detect KCNE2 with native-like posttranslational modification(s). However, both Ab1 and Ab2 also detect other bands, some of which are as strong as the HA-KCNE2 bands. Given that the HA epitope adds a mass of 1 kDa, we predict that the native KCNE2 counterparts should be 32 and 24 kDa in size and likely fainter than the highly expressed HA-KCNE2 bands. Do the other prominent bands detected by Ab1 and Ab2 represent highly abundant native KCNE2 protein, or do they represent unrelated proteins recognized by these antibodies? We reason that if they represent highly abundant native KCNE2 protein, they should be suppressed by knocking down KCNE2 expression using RNA interference. We use adenovirus to introduce a short hairpin construct targeting rat KCNE2 nucleotides 319–340 (Adv-E2 si) into cultured SHR ventricular myocytes. The adenovirus also carries a GFP reporter, allowing us to confirm that >40% of the myocytes are infected (GFP⁺) after overnight Adv-E2 si incubation followed by 24 h culture. We are surprised to see that, relative to control culture or culture after incubation with Adv-GFP, Adv-E2 si does not alter the banding pattern detected by either Ab1 or Ab2 (Fig. 1E). We hypothesize that culturing rat ventricular myocytes in the serum-free medium for 3 days may have downregulated the native KCNE2 protein, so that Adv-E2 si does not have any further effect. Therefore, we compare the banding pattern detected by Ab1 between fresh SHR ventricular myocardium and cultured SHR ventricular myocytes (fresh and control culture in Fig. 1E). Indeed, Ab1 detects a clear 24-kDa band in the fresh myocardium (asterisk in Fig. 1E), which is missing in myocytes after culture. This observation supports part of our hypothesis: culturing rat ventricular myocytes under the control conditions downregulates the 24-kDa native KCNE2 protein species. This validates the 24-kDa native KCNE2 band (highlighted by solid black arrows in Figs. 2–5).

The band intensities in the 32 to 33 kDa range (expected for 24-kDa species, so that it is neither downregulated in myocytes after culture. This observation supports part of our hypothesis: culturing rat ventricular myocytes under the control conditions downregulates the 24-kDa native KCNE2 protein species. This validates the 24-kDa native KCNE2 band (highlighted by solid black arrows in Figs. 2–5).

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two N-glycosylation sites (N6 and N29). We showed previously that in vitro translated KCNE2 migrates as 25-, 20-, and 15-kDa bands, and PNGase F treatment (to remove N-glycosylation) collapses the 25- and 20-kDa bands into the 15-kDa band (10). We therefore test whether PNGase F treatment can collapse the 24- and 32-kDa native KCNE2 bands into a 15-kDa band. In view of the uncertainty about the effectiveness of PNGase F treatment (per manufacturer’s manual), WCL from COS-7 cells expressing KCNE2 is treated with PNGase F in a parallel experiment to serve as a positive control (Fig. 2A).

Furthermore, the original WTLs are included in the immunoblot to show that overnight incubation at 37°C without PNGase F does not alter the banding pattern (although the band intensities are reduced). Although the parallel experiment on COS-7-expressed KCNE2 clearly supports the effectiveness of PNGase F deglycosylation, this enzyme does not collapse the 24- or 32-kDa native KCNE2 band detected by Ab1 to the 15-kDa band. Therefore, the shift of the native KCNE2 mass from 15 to 24 and 32 kDa is not due to N-glycosylation. More experimentation is needed to test other possibilities, including O-glycosylation, tight association with other native proteins (not dissociated by the SDS buffer used in immunoblotting), or dimerization (i.e., 2 of the 15-kDa species to form the 32-kDa species).

Open arrows in Fig. 1E highlight the strong bands detected by Ab1 or Ab2 that we suspect to be nonspecific bands. The most troubling one is the prominent band detected by Ab2 right above the 25-kDa HA-KCNE2 band (Fig. 1D, right). Ab2 also detects a similar nonspecific band in the guinea pig ventricle (Fig. 2B, open arrow). Because Ab1 does not have this problem in the relevant molecular weight range (<37-kDa size marker), we use Ab1 to exam-
ine native KCNE2 expression in atria and ventricles of animal models and human hearts.

Using Ab1 to compare native KCNE2 protein expression in atria and ventricles and to examine KCNE2 subcellular distribution. SHR is a good animal model to study cardiac electrical and structural remodeling during aging with chronic hypertension. Based on Ab2 immunoblotting we previously have proposed that KCNE2 upregulation is involved in ventricular electrical remodeling in old SHRs (18–22 mo of age) (10). We now use Ab1 to probe native KCNE2 expression in four old (20 mo) SHR hearts that have experienced different degrees of hypertrophy [Fig. 3A, heart-to-body (H:B) weight ratios listed below the immunoblots]. Ab1 detects 24- and 32-kDa bands in ventricles but mainly the 32-kDa band in atria. Interestingly, in atria from the failing heart (No. 1, H:B weight ratio 7.9), the 32-kDa KCNE2 band is missing despite the confirmation of protein loading by the actin immunoblot of the same membrane. Densitometry analysis shows that on average KCNE2 protein level in atria is only 20% of that in ventricles in old SHRs (Fig. 3B; P < 0.01). Among these four ventricular specimens from old SHRs, the native KCNE2 band intensities are stable, i.e., there is no increase in KCNE2 protein level with a higher H:B weight ratio. This apparent discrepancy with our previous finding using Ab2 (10) will be discussed below (see DISCUSSION).

Guinea pig is a widely used animal model for the rapid and slow delayed rectifier K channels (I_Kr and I_Ks, respectively), two major KCNE2 partner channels in the heart (1, 28). It is not clear whether KCNE2 is expressed in guinea pig hearts. This needs to be considered when using guinea pigs to study native I_Kr and I_Ks. We use Ab1 to probe native KCNE2 protein expression in young adult guinea pig hearts. Ab1 detects 32-...
and 24-kDa, and sometimes very faint 15-kDa, bands in guinea pig atria and ventricles (Fig. 4A). The banding pattern is similar to that seen in SHR ventricles (Fig. 2A). On average, the KCNE2 protein level in guinea pig atria is ~50% of that in guinea pig ventricles (Fig. 4B).

The functional role of KCNE2 does not just depend on the protein expression level. Its subcellular distribution pattern also matters: to be functional, KCNE2 needs to be where its partner channels are, i.e., the cell surface membrane. We use Ab1 to probe KCNE2 protein distribution in atrial and ventricular myocytes of SHR and guinea pig hearts. Despite the caveat that in SHR ventricular myocytes Ab1 detects unrelated proteins in the >37-kDa range (Fig. 1E), the Ab1 immunofluorescence shows preferential cell surface distribution in both ventricular and atrial myocytes of SHR heart (Fig. 3C). This is consistent with the cell surface localization of HA-KCNE2 detected by the HA mAb (Fig. 1F). Ab1 immunofluorescence also exhibits a preferential cell surface localization in both atrial and ventricular myocytes of guinea pig hearts (Fig. 4C, top). There is also a clear striation pattern of Ab1 immunofluorescence in the guinea pig cardiac myocytes. This striation pattern is confirmed by HA immunofluorescence in cultured guinea pig ventricular myocytes expressing HA-KCNE2 (Fig. 4C, bottom). The strong punctate and perinuclear staining in the cytosol of Adv-E2(HA)-treated SHR and guinea pig ventricular myocytes reflects the overexpression of HA-KCNE2. The data presented in Fig. 3 and Fig. 4 show that KCNE2 protein is expressed in ventricular and atrial myocytes of SHR and guinea pig hearts, with preferential cell surface localization and higher abundance in ventricles than in atria.

We use Ab1 to probe native KCNE2 protein in human hearts: atrial specimens from patients in atrial fibrillation or in sinus rhythm, and ventricular specimens from patients in heart failure or nonfailing (NF) (2 each; Fig. 5). Although the actin immunoblot and Coomassie blue stain both confirm even protein loading among the lanes, the KCNE2 band intensities

Fig. 5. Quantifying KCNE2 in 4 human atrial specimens [2 from patients in atrial fibrillation and 2 from patients in sinus rhythm (AF and SR), respectively] and 4 human ventricular specimens [2 from nonfailing and 2 from failing hearts (NF and HF), respectively]. A: membrane is probed for KCNE2 (Ab1), stripped, and reprobed for actin. Coomassie blue (CB) stain confirms even loading (not shown). B: densitometry quantification of KCNE2 protein level. Data analysis is the same as that described for Fig. 3B. Mean values are shown as histogram bars with SE, and data from individual specimens are shown as symbols.
are quite variable. The strongest band is seen in the two nonfailing ventricular specimens (32 kDa; Fig. 5A). A similar but fainter band can be detected in both ventricular specimens from failing hearts. Ab1 detects very faint bands in all four human atrial specimens in the relevant molecular mass range. Although the very limited sample number and large variations in band intensities preclude statistical comparison, densitometry analysis does suggest that the putative KCNE2 protein band in human ventricles is more abundant than that in human atria (Fig. 5B). Therefore, in both human and animal models (old SHR, young guinea pig) KCNE2 protein expression exhibits a gradient of ventricle > atrium.

Ab2 detecting S98-phosphorylated KCNE2. We have made a serendipitous finding that the ability of Ab2 to detect KCNE2 is prevented by pretreating the protein sample with calf intestinal phosphatase (CIP), a nonspecific protein and nucleic acid phosphatase. This suggests that Ab2 detection of KCNE2 requires phosphorylation of its epitope. The confirmation experiment is shown in Fig. 6. Wild-type (WT) KCNE2 is in vitro translated in the presence of γ-32P-ATP, so that the protein can be 32P-labeled via phosphorylation by protein kinases present in the components of the in vitro translation kit. The translation product is divided into two aliquots; one is treated with CIP and the other is similarly processed in the absence of CIP. The two aliquots are fractionated by SDS-PAGE and probed with Ab1 and Ab2. Effective dephosphorylation by CIP is confirmed by the abolition of 32P-radioactivity in the relevant molecular mass range (10–30 kDa; Fig. 6A, bottom). CIP treatment largely abolishes the WT-KCNE2 bands detected by Ab2 (Fig. 6A, middle). On the other hand, Ab1 can detect dephosphorylated WT-KCNE2, although the mobility is slightly faster than without dephosphorylation (Fig. 6A, top).

There are two potential phosphorylation sites in the Ab2 epitope, tyrosine at position 96 (Y96) and serine at position 98 (S98) (Fig. 1B). We remove the phosphorylation site(s) by mutating Y96 to phenylalanine (Y96F) and S98 to alanine (S98A), singly or together. Y96F behaves like WT KCNE2: Ab2 detects the bands before, but not after, dephosphorylation, and Ab1 detects both with slightly higher mobility after dephosphorylation (Fig. 6A). On the other hand, S98A and Y96F/S98A, although clearly detected by Ab1, escape the detection by Ab2 (Fig. 6A). These data indicate that S98 is a phosphorylation site in KCNE2, and S98 phosphorylation is required for Ab2 detection. S98A and Y96F/S98A are 32P-labeled, and the 32P-radioactivity is abolished by CIP (Fig. 6A, bottom), indicating that there is at least one other KCNE2 phosphorylation site in addition to S98.

COS-7 expression confirms the above in a cellular environment: Ab2 can detect WT and Y96F but not S98A or Y96F/S98A, and CIP dephosphorylation greatly reduces the WT and Y96 band intensities detected by Ab2. On the other hand, Ab1 can detect all bands without or with CIP treatment (Fig. 6B). Importantly, the Ab2 immunoreactivity in dog (20-kDa band) and human (32-kDa band) ventricles is also abolished by dephosphorylation with CIP (Fig. 6C), indicating that native KCNE2 in the heart is at least partially S98-phosphorylated.

Phosphorylation status of S98 impacting on KCNE2 modulation of hERG current amplitude and protein level. To explore the consequence(s) of S98 phosphorylation in terms of KCNE2 function, we mutate S98 to aspartate (S98D, mimicking S98 phosphorylation), with S98A mimicking S98 dephosphorylation. The WT and mutant KCNE2 are coexpressed with a partner channel, hERG, in oocytes. To monitor the effects of an auxiliary subunit on its partner channel function (such as KCNE2 modulation of hERG) using voltage clamp of individual cells, oocytes provide an important advantage: by controlling the amounts of cRNAs injected into each oocyte we can control the relative expression levels of KCNE2 and hERG.

![Image](https://www.ajpheart.org/content/31/6/h916/F6.large.jpg)

Fig. 6. Detection of KCNE2 by Ab2 requires phosphorylation of serine at position 98 (S98). A: KCNE2 wild-type (WT) and 3 mutants (Y96F, S98A, and Y96F/S98A) are in vitro translated in the presence of γ-32P-ATP to label proteins phosphorylated during translation. The translation products are divided into 2 aliquots, 1 treated with calf intestinal phosphatase (CIP) and the other processed in the same manner without CIP. The aliquots are fractionated by 2 separate SDS-PAGE; 1 is used to probe with Ab1 (top) and the other with Ab2 (middle). Remaining 32P-radioactivity in the gel (in the 10–30 kDa range) is revealed by phosphoimager (bottom). The farthest left lane is no cRNA negative control. B: WCL from COS-7 cells expressing KCNE2 WT or mutants (listed on top), without or with CIP dephosphorylation (– and +, respectively), probed with Ab1 (top) and Ab2 (bottom). C: WTL from dog and human ventricles are divided into 2 aliquots, 1 treated with CIP and the other processed in the same manner without CIP. The 2 aliquots are fractionated side by side and probed with Ab2. CB stain confirms even loading (not shown). In all 3 panels, size marker positions are marked on the right.
This avoids the issue of heterogeneous KCNE2-to-hERG ratios, which can impact on the degree of channel modulation (28, 31). We use a cRNA molar ratio (hERG:KCNE2) of 1:5 to ensure that hERG is fully associated with, and thus modulated by, KCNE2 even with S98 mutations.

Figure 7A shows that hERG current amplitude is reduced when coexpressed with KCNE2-WT [as reported previously (1)]. S98A attenuates, while S98D accentuates, this hERG current-suppressing effect of KCNE2. There are no changes in the hERG gating kinetics among the four groups of oocytes. These data suggest that S98 phosphorylation is necessary for KCNE2 suppression of hERG current amplitude.

We use biotinylation to quantify oocyte cell surface hERG protein level when the channel is expressed alone or coexpressed with KCNE2 WT, S98A, or S98D. Figure 7B shows that KCNE2-WT reduces the cell surface hERG protein. S98A attenuates, while S98D mimics, this effect of KCNE2. Therefore, the changes in cell surface hERG protein level mirror the changes in hERG current amplitude. Because a similar pattern of changes is seen in whole oocyte hERG protein level, we propose that KCNE2 does not hinder hERG trafficking to the cell membrane. Instead, KCNE2 reduces hERG protein translation and/or accelerates hERG degradation.

**KCNE2 accelerating hERG protein degradation.** We pursue the issue of whether/how KCNE2 affects hERG translation and/or degradation by pulse-chase experiments using the COS-7 expression system. Figure 7C confirms that, similar to the oocyte data, in COS-7 expression the hERG protein levels on cell surface and in WCL are reduced by coexpression with KCNE2 WT; S98A attenuates while S98D accentuates these effects.

For the pulse-chase experiments, 24 h after cDNA transfection we starve COS-7 cells of Met to stop protein translation. We then introduce 35S-Met to synchronize the start of protein translation with all newly translated proteins radioactively labeled with 35S-Met (the pulse). After pulsing for 1 h, the 35S-Met is replaced by high concentration (2 mM) of cold Met, and the newly synthesized cold KCNE2 will dilute the radioactive counterpart in the KCNE2 pool (the chase). After different chase durations, the hERG protein is immunoprecipitated from the WCLs. The immunoprecipitates are fractionated by SDS-PAGE. The proteins are partially blotted to a PVDF membrane, and the membrane is used for immunoblotting with a hERG Ab (IB: hERG, second row). The radioactivity of hERG protein remaining in the gel is measured by phosphoimager (35S hERG, first row). The radioactivity is normalized by the immunoblot band intensity of respective chase time point. Figure 8A shows that at zero chase time, the level of 35S radioactivity is similar between hERG alone and hERG coexpressed with KCNE2. This suggests that the hERG protein...
translation efficiency during the pulse period is similar without versus with KCNE2. The normalized radioactivity in the hERG protein pool decays with longer chase times, and the time course can be used to estimate the time constant of hERG turnover or degradation. KCNE2 coexpression significantly shortens the hERG turnover time ($n = 3, P < 0.05$), indicating that KCNE2 coexpression accelerates hERG protein degradation. Figure 8B shows that, in a parallel pulse-chase experiment, KCNE2 WT accelerates hERG degradation while S98A prevents this effect. This supports the notion that S98 phosphorylation is needed for KCNE2 acceleration of hERG protein degradation.

Figure 9A shows that coexpressing hERG with KCNE2 versus hERG alone in COS-7 cells reduces the hERG protein level in WCL in a time-dependent manner: the degree of decrease is more severe by posttransfection day 2 than day 1. This is consistent with data shown in Fig. 8A: KCNE2 does not hinder hERG protein translation, but accelerates hERG protein degradation. By day 3, hERG alone shows much reduced protein level in WCL, and KCNE2 coexpression reduces the hERG protein to an even lower level. Interestingly, KCNE2 protein level in the same WCLs is well maintained even by day 3. This suggests that although KCNE2 can accelerate hERG protein degradation, KCNE2 itself is not rapidly degraded. Alternatively, the rate of KCNE2 translation may be well maintained to keep up with degradation even on day 3.

**KCNE2 modulating Kv4.3 gating kinetics but not reducing Kv4.3 protein level.** Is the above an overexpression artifact? If so, we expect to see it occur to KCNE2 modulation of other potassium channels. We test the effects of KCNE2 on Kv4.3 expressed in COS-7 cells. KCNE2 coexpression leads to clear changes in the Kv4.3 gating kinetics: slowing the time to peak and inactivation, and shifting the voltage-dependence of activation and inactivation in the positive direction (Fig. 9C) (31). However, KCNE2 does not reduce the Kv4.3 protein level in WCLs even by day 3 (Fig. 9A, top right), and pulse-chase experiment shows that KCNE2 does not accelerate Kv4.3 turnover (Fig. 9B). These observations are clearly different from KCNE2 modulation of hERG (Fig. 9A, bottom).

Testing whether hERG and KCNE2 proteins are degraded by the proteasome or lysosome pathway: It has been shown that in mammalian cell expression systems hERG degradation mainly occurs by the proteasome pathway (4, 6). On the other hand, a previous study showed that KCNE2 uniquely clusters to the lysosomal compartment, where protein degradation occurs (30). Is it possible that hERG, when associated with KCNE2, also traffics to lysosomes at a high rate and becomes degraded there? We test the effects of the proteasome inhibitor [N-acetyl-L-leucyl-L-norleucinal (ALLN), 20 μM] (6) and lysosome inhibitor (leupeptin; 100 μM) on the hERG protein level in COS-7 WCLs when hERG is expressed alone or coexpressed with KCNE2. Treatment with ALLN or leupeptin commences right after transfection and continues for another 48 h before analysis. We confirm that ALLN treatment markedly increases the hERG protein level, whereas leupeptin treatment has very little effect (Fig. 10). Importantly, this pattern is not altered by KCNE2 coexpression, although the hERG protein level is markedly reduced by KCNE2. Figure 10 further shows that KCNE2 degradation also occurs by the proteasome pathway, either expressed alone or coexpressed with hERG.

**DISCUSSION**

Our major findings can be summarized as follows. First, both Ab1 and Ab2 can detect two prominent HA-tagged KCNE2 bands (25 and 33 kDa) expressed in adult ventricular myocytes and validated by HA-mAb. Second, Ab1 detects corresponding native KCNE2 bands of 24 and 32 kDa without interference in the appropriate molecular mass range (<37 kDa), whereas Ab2 recognizes an unrelated band right above the 25-kDa HA-tagged (or the corresponding 24-kDa native) KCNE2 band. Third, Ab1 is used to quantify native KCNE2 protein, and the data show that KCNE2 protein is more abundant in ventricles than in atria of old SHR, young adult guinea pig, and, likely, human hearts. Fourth, Ab1 immunofluorescence suggests that native KCNE2 protein is preferentially expressed in the cell surface membrane of both atrial and ventricular myocytes. Fifth, KCNE2 suppresses hERG current amplitude by accelerating hERG protein degradation, and phosphorylation of serine at position 98 (S98) in the epitope
region for Ab2 is necessary for this KCNE2 effect. Sixth, Ab2 detects S98-phosphorylated KCNE2. Finally, both hERG and KCNE2 proteins are degraded by the proteasome pathway, either when they are expressed separately or together.

Comparison with previous studies. The 24-kDa KCNE2 band size matches the values reported by others in the literature using different KCNE2 antibodies: (1) 24 kDa, KCNE2 in mouse heart, detected by a commercial Ab (Sigma) and by a homemade Ab (22), (2) 25 kDa, KCNE2 in canine Purkinje fiber, by a commercial Ab (Santa Cruz) (19); however, we detect KCNE2 as a 20-kDa band in canine ventricular muscle by both Ab1, Fig. 10, and Ab2 (10). Although the 32-kDa putative KCNE2 band is more consistently seen than the 24-kDa band in SHR hearts (Fig. 3A), this band is not downregulated in SHR ventricular myocytes after culture (as is the case for the 24-kDa band), nor is it suppressed by RNA interference targeting KCNE2 (Fig. 1E).

Despite this uncertainty, we note that excluding the 32-kDa band from our analysis will not alter the conclusion that KCNE2 protein is more abundant in ventricles than in atria. Our conclusion appears to be in conflict with previous reports showing that in human (14) and mouse (5) hearts KCNE2 mRNA level is much lower in ventricles than in atria. Mismatches between mRNA and protein levels have been described for KCNE1 and several other cardiac ion channel genes (15, 16). However, in these cases, the protein levels are disproportionately low relative to the corresponding mRNAs due to microRNA repression of translation, a situation apparently different from that of KCNE2. Furthermore, bioinformatics analysis has suggested that KCNE2 is one of very few genes in the heart that is not subjected to microRNA repression (16). It is possible that the KCNE2 protein is translated with a higher rate and/or degraded at a slower rate in ventricles relative to atria, creating a mismatch between mRNA gradient (atria/ventricles) and protein gradient (atria/ventricles).

A novel mechanism by which KCNE2 modulates the hERG/IKr current density. We have uncovered a novel mechanism by which KCNE2 modulates hERG current amplitude: KCNE2 accelerates hERG protein degradation, reducing whole cell and thus cell surface hERG protein level and suppressing hERG current.

Fig. 9. Differential effects of KCNE2 on hERG and Kv4.3 protein levels in COS-7 expression. A: KCNE2 reduces whole cell protein level of hERG, but not Kv4.3, in a time-dependent manner. A, top: representative immunoblot images of WCLs, with cDNA(s) transfected and number of posttransfection days listed above. A, bottom: ratios of α-subunit protein level when coexpressed with KCNE2 to when expressed alone, (+ KCNE2): (alone), measured on posttransfection day 1. B: pulse-chase experiment in COS-7 cells showing that KCNE2 coexpression does not accelerate Kv4.3 turnover. The format is the same as that of Fig. 8A. As has been reported previously (8), Kv4.3 tends to form high-molecular weight oligomers in addition to the monomer 73-kDa band; the band intensities are combined in quantification. C: Kv4.3 coexpressed with KCNE2 in COS-7 cells is modulated by KCNE2. C, left: KCNE2 slows Kv4.3 activation and inactivation (slowing time to reach peak and decay, currents are recorded at +60 mV). C, middle: KCNE2 shifts the voltage dependence of Kv4.3 activation in the positive direction. C, right: KCNE2 shifts the voltage dependence of Kv4.3 inactivation in the positive direction. Protocols and data analysis have been described previously (31).
current amplitude. This is different from a previous report suggesting that KCNE2 suppresses hERG current amplitude by decreasing the single channel conductance (1). However, these two mechanisms are not mutually exclusive. This mechanism is not shared by KCNE2 modulation of Kv4.3, another KCNE2 partner channel in the heart. Therefore, KCNE2 can modulate partner channels by modulating their gating kinetics (31), single channel conductance (1), and/or the rate of protein degradation.

S98 phosphorylation appears to be necessary for the suppressing effects of KCNE2 on hERG current amplitude and protein level. S98 is in the cytoplasmic domain of KCNE2. Its phosphorylation status may impact on the interactions between vesicles containing KCNE2 as a cargo and other cytosolic proteins involved in cargo recognition and vesicular translocation (7). Because S98 phosphorylation seems critical for KCNE2 modulation of hERG and appears to be altered in diseased hearts (discussed below), identifying the signaling pathway(s), including the participating protein kinase(s) and phosphatase(s), will be of importance.

There is a precedent for KCNE subunit involved in partner Kv channel turnover/degradation: Xu et al. (29) have shown that KCNE1 acts as an endocytic chaperone for KCNQ1, and the trafficking cue may reside in the intracellular COOH-terminal domain of KCNQ1. KCNE1 and KCNE2 are closely related, and each can regulate both hERG (1, 17) and KCNQ1 (23, 28). However, although KCNE2 coexpression reduces the KCNQ1 protein level in oocytes (13) and in COS-7 cells (unpublished results), our preliminary data suggest that this effect does not seem to depend on the phosphorylation of S98.

Implications for the role of KCNE2 in arrhythmias. Our conclusion that KCNE2 protein is relatively abundant in ventricles is consistent with the linkage between genetic variations in KCNE2 and LQT6. Heterologous expression experiments have shown that KCNE2 can engage in quite promiscuous partnerships with voltage-gated K channels [hERG (1), KCNQ1 (26), KCNQ1/KCNE1 (28), and Kv4.x (31), × = 2 and 3] and, unique among KCNE subunits, with HCN channels that mediate the pacemaking currents (20). Experiments conducted in animal models have provided support for KCNE2 modulation of dERG (dog equivalent of hERG) in canine ventricular myocytes (10), KCNQ1/KCNE1 in guinea pig ventricular myocytes (9), and Kv4.2 and Kv1.5 in mouse ventricular myocytes (22). However, direct evidence for such (and potentially other) KCNE2 functions in human heart is still lacking. This important question needs to be addressed before we can fully understand LQT6.
We showed previously that ventricular myocytes from chronically infarcted [myocardial infarction (MI)] canine hearts manifested a higher \( I_{Ks} \) current density than ventricular myocytes isolated from the equivalent region of control canine hearts (10). There was a decrease in Ab2 immunoreactivity in MI versus control hearts (10). However, the Ab1 immunoblot indicates that this was not due to a downregulation of the total KCNE2 protein level during chronic MI (Fig. 11). Furthermore, there was an increase in dERG1a (11) protein level in chronic MI (Fig. 11). In view of the new revelation that Ab2 detection of KCNE2 requires S98 phosphorylation, we suggest that during chronic MI, S98 phosphorylation is reduced despite a modest increase in total KCNE2 protein level. S98 dephosphorylation attenuates the ability of KCNE2 to accelerate dERG1a degradation, leading to an increase in dERG1a protein level and subsequently a rise in \( I_{Ks} \) current density in the ventricular myocytes.

We showed previously that Ab2 detected stronger KCNE2 bands in old SHR ventricles with higher H:B weight ratios (10). However, we do not observe such a correlation between Ab1 immunoblot of old SHR ventricles and the H:B weight ratio (Fig. 3A). It is possible that the increase in Ab2 reactivity reported previously reflected an increase in the degree of S98 phosphorylation in aging SHR ventricles. More experimentation is needed to explore the functional consequences and the partner channels involved.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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