Cardiac \( I_{Kr} \) Channels Minimally Comprise hERG 1a and 1b Subunits*

Previous studies suggest native cardiac \( I_{Kr} \) channels are composed of alpha subunits encoded solely by the 1a transcript of the ERG1 gene. Using isoform-specific ERG1 antibodies, we have new evidence that subunits encoded by an alternate transcript, ERG1b, are also expressed in rat, canine, and human heart. The ERG1a and -1b subunits associate in vivo where they localize to the T tubules of ventricular myocytes. These data indicate native ventricular \( I_{Kr} \) channels are heteromers containing two \( \alpha \) subunit types, ERG1a and -1b. The hERG1b-specific exon thus represents a novel target for screens for mutations causing type 2 long QT syndrome. These findings also suggest phenotypic analyses of existing type 2 mutations causing type 2 long QT syndrome. These findings indicate cardiac \( I_{Kr} \) channels are minimally composed of ERG1a and -1b \( \alpha \) subunits.

Long QT syndrome (LQTS)\(^1\) is an inherited or acquired disease associated with episodic ventricular arrhythmias and sudden death. One form of inherited LQTS (LQTS-2) results from mutations in the human Ether-a-go-go-Related Gene 1 (hERG1a or KCNH2) (1). hERG1 encodes a potassium channel with biophysical and pharmacological properties similar to those of cardiac \( I_{Kr} \), thus explaining the underlying cause of LQTS-2 as a defect in this repolarizing current (2, 3). In mammalian heart, two ERG\(^2\) transcripts, \( I_{a} \) and \( I_{b} \), encode proteins differing in their amino-terminal sequence (see Fig. 1A) and gating properties (4, 5). Expressed in Xenopus oocytes, these subunits preferentially form heteromultimers (4). However, despite high levels of ERG1b transcript (4), first generation ERG1 antibodies against a common epitope identified only ERG1a protein in native tissue (6, 7), suggesting that ERG1b subunits do not contribute to cardiac \( I_{Kr} \) channels. Here we provide the first direct evidence for ERG1b protein expression, localization, and co-assembly with ERG1a in cardiac ventricular myocytes. These findings indicate cardiac \( I_{Kr} \) channels are minimally composed of ERG1a and -1b \( \alpha \) subunits.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies**—Human embryonic kidney 293 (HEK-293) cell lines stably expressing wild-type hERG1a have been described previously (8, 9). Cell lines stably expressing hERG1a and -1b were prepared by transfection of HEK-293/hERG1a stable cells with hERG1b containing a Kozak consensus sequence (4) cloned into the BamHI/EcoRI sites of pcDNA3.1z (Invitrogen). Separate cell colonies were selected after plating cells at low density and grown in media containing 100 \( \mu \)g/ml Zeocin, 500 \( \mu \)g/ml neomycin for selection. All HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium at 37 °C. The pan-ERG1 antibody, ERG1-KA, has been described previously (10). ERG1 isoform-specific antibodies were produced by Bethyl Laboratories (Montgomery, TX) in rabbits. Antisera were affinity-purified using the same peptides employed in immunization. The sequence for the ERG1b peptide is amino acids 12–25 (GALRPRAQKGRVVRR), and the sequence for ERG1a is amino acids 140–153 (SPAHTDNHGRFPTST) (Neocell, Madison, WI). In addition, a hERG1a-specific antibody raised in goat (HERG N-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-coupled secondary antibodies were purchased from Pierce and Santa Cruz Biotechnology. Fluorophore-coupled secondary antibodies were purchased from Molecular Probes (Lake Oswego, OR). Myosin binding protein C (MyBP-C) was a gift from Dvs. Richard Moss and Samantha Harris (University of Wisconsin, Madison).

**Cardiac Tissue Preparation**—Human male ventricular lysate was purchased from ProSci, Inc. (Poway, CA). Human male ventricular tissue was a gift from Dr. Timothy Kamp (Dept. of Medicine, University of Wisconsin, Madison). Canine ventricular myocytes, a gift from the laboratory of Dr. Rob Haworth (Dept. of Surgery, University of Wisconsin, Madison), were isolated from mongrel males and enzymatically treated as previously described (11). Sprague-Dawley rat ventricular myocytes were excised from anesthetized adult males after injection of sodium pentobarbital (100 mg/kg body weight intraperitoneal) as described previously (11). Rat ventricular myocytes were prepared using the same procedure as described for the canine tissue. All procedures have been approved by the Research Animal Resources Center at University of Wisconsin, Madison.

**Cell Membrane Protein Preparations**—Membranes were prepared from myocytes or ventricular tissue after suspension in homogenization buffer (25 mM Tris-HCl, pH 7.4, 10 mM NaEGTA, 20 mM NaEDTA). All buffers used in this procedure contained the following protease inhibitor mixture: 5 \( \mu \)g/ml aprotinin, 50 \( \mu \)g/ml 1,10-phenanthroline, 0.7 \( \mu \)g/ml pepstatin A, 1.56 \( \mu \)g/ml benzamidine, and 1 \( \times \) Complete minitab (Roche Applied Science). Suspensions were homogenized using a Polytron homogenizer at setting 6 for two bursts of 15 s each followed by sonication on ice twice at an amplitude of 20 for 20 s each. Suspensions were spun at 2,000 \( \times \) g for 4 °C for 10 min to remove cellular debris. The supernatants were subject to further centrifugation at 40,000 \( \times \) g for 30 min at 4 °C. The resultant pellet was solubilized on a rotary shaker at 4 °C for 2 h in either Triton X-100 (5 mM NaCl, 25 mM Tris-HCl, pH 7.4, 20 mM NaEDTA, 10 mM NaEGTA, 5 mM glucose, and 1% (v/v) Triton X-100, or RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM NaEGTA, and 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (v/v) sodium dodecyl sulfate). Samples were then spun at 10,000 \( \times \) g for 30 min at 4 °C to remove insoluble material. Cell membrane pellets were prepared by washing plates gently with PBS, aspirating, and adding either Triton X-100 or RIPA buffer. Cells were then scraped, collected in a microcentrifuge tube, and sonicated on ice twice at an amplitude of 20 for 20 s each. The suspension was rotated at 4 °C for 2 h and then centrifuged at 10,000 \( \times \) g for 10 min to remove insoluble material. Protein concentrations of all samples were determined using a modified Bradford assay (DC Protein Assay, Bio-Rad).
Biochemical Analysis—Membrane proteins were deglycosylated using PNGase F and endoglycosidase H (Roche Applied Science) as described previously (8, 12). Proteins were denatured at 60 °C to avoid thermal aggregation at higher temperatures. To determine which proteins were expressed on the surface membrane, proteins were surface-biotinylated using sulfo-NHS-LC-Biotin reagent as described previously (10). Briefly, 100 mm tissue culture dishes with growth at 70–80% confluence were rinsed three times with cold PBS and incubated with freshly prepared biotin reagent (5 mg/ml) in PBS for 45 min at 4 °C. Cells were then rinsed once with 25 ml Tris-HCl, pH 7.5, to quench the reaction, followed by three washes with cold PBS. Membrane protein preparations were prepared as indicated above.

Western Blot Analysis—Membrane proteins (cell lines 2–10 μg/lane, heart lysates 30–50 μg/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels along with prestained molecular weight markers (Bio-Rad) and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 1 h at 100 mV. Western blots were blocked, probed, and analyzed as described earlier (10). For peptide experiments 5 μl of antibody was incubated with 10 μg of peptide in 100 μl of TBS (150 mM NaCl, 25 mM Tris-HCl, pH 7.4) for 6 h at 4 °C and then centrifuged at 10,000 × g for 20 min. The supernatant was carefully removed and used to probe Western blots. Western blot controls include probing blots with secondary antibody alone and peptide block of primary antibody. In the case of heart lysates, a lane containing hERG1a/b cell membrane preparation was included as a positive control.

Co-immunoprecipitation—Membrane lysates (cell lines 100–200 μg reaction, heart lysates 500–1000 μg reaction) in 1 ml of TBS were cleared with 50 μl of protein A- or G-Sepharose beads (Amersham Biosciences) depending on the origin of the immunoprecipitating antibody; protein A was used for rabbit and protein G for goat immunoprecipitating antibodies. Cleared lysates were incubated with antibody (ERG1a 1:200 at 1:20) on a rotating platform for 3–16 h at 4 °C. 50 μl of protein A- or G-coupled beads were added and samples were incubated at 4 °C for an additional 1–3 h. Beads were collected by centrifugation at 10,000 × g and washed three times with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM NaEDTA, 1% (v/v) Triton X-100, followed by one wash with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4. Proteins were eluted with 200 ng/ml antibody-specific peptide for 1 h at 4 °C. Samples were centrifuged at 10,000 × g, and the supernatant was collected. 100 μl of LSB (25 mM Tris-HCl, pH 6.8, 2% (v/v) sodium dodecylsulfate, 10% glycerol) was added to the beads to elute any proteins that remained bound. Additional controls included lysates processed without antibody. Eluted proteins were Western blotted as described above.

Immunohistochemistry—Isolated canine myocytes were fixed in 2% paraformaldehyde-PBS, pH 7.4, for 10 min at room temperature and washed three times in PBS, pH 7.4. Myocytes were then either stored at 4 °C (for up to 8 weeks) or processed immediately. Myocytes were washed once in PBS, pH 7.4, + 1% Triton X-100 and permeabilized in PBS, pH 7.4, + 0.5% Triton X-100 for 10 min at room temperature followed by incubation in 0.75% glycine-PBS (pH 7.4) for 10 min at room temperature to quench any free aldehydes and incubation in blocking buffer (PBS, pH 7.4, + 0.1% Tween-20 + 10% donkey serum + 2% bovine serum albumin) for 2 h at 4 °C with rotation. Cells were washed three times with PBS, pH 7.4, + 0.1% Tween-20 and divided into 0.5-ml aliquots. Each myocyte aliquot was incubated overnight at 4 °C in diluted primary antibody. ERG1b antibodies were diluted 1:1000, ERG1a antibodies (N-20) were diluted 1:10, and myosin binding protein C antibodies were diluted 1:500. Myocytes were washed three times for 1 h in PBS, pH 7.4, + 0.1% Tween-20. Secondary antibodies were diluted in PBS, pH 7.4, + 0.5% Tween-20, + 5% bovine serum albumin, and spun to remove excess. Myocytes were incubated in 0.5 ml of diluted secondary antibody and incubated in the dark 2 h at room temperature with rotation. Donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 568 antibodies were diluted 1:1000. Myocytes were washed briefly 3x with PBS, pH 7.4, + 0.1% Tween-20 followed by two 1-h washes with PBS, pH 7.4, and were stored at 4 °C until viewed on a Zeiss Axiosvert 200 microscope with a ×63 objective. Optical sectioning was accomplished using the Apotome, and three-dimensional rendering was conducted within Axiosvision software. Fluorescent excitation-emission filter set for Alexa 488 (excitation 450–490 nm, emission 515–565 nm) and Alexa 568 (excitation 500–639 nm, emission 560–700 nm) do not overlap. Species specificity of secondary antibodies was confirmed by incubating cells probed with one primary with secondary antibody raised against the other species. No signal was detected demonstrating each second-

ary is species specific. Secondary alone controls were also used to ensure the signal was specific.

RESULTS

We initially observed an ERG1b-sized band on a Western blot of rat heart tissue when we probed with a novel, carboxy-terminal ERG1a antibody termed KA, generated as a tool for a related study (10). As shown in Fig. 1B, the ERG1-KA antibody identified three bands at 160, 120, and 95 kDa. The two higher molecular mass bands are consistent in size with maturely glycosylated and unglycosylated rat ERG1a, respectively (6). The 95-kDa band is consistent in size with ERG1b protein produced in heterologous expression systems (see below) but not observed previously in native tissue. The 95-kDa band cannot represent ERG-USO, another ERG1 transcript that produces a protein of approximately the same size (15) because ERG-USO does not contain the carboxy-terminal sequence against which the ERG1-KA antibody was raised.

To test the hypothesis that the 95-kDa band represents ERG1b, we generated antibodies specific to the ERG1a and ERG1b amino termini. Fig. 2 shows the characterization of the two antisera and the pan-ERG1 antibody ERG1-1a/b on Western blots of membrane proteins prepared from HEK-293 cells stably expressing hERG1a and -1b. The ERG1-KA antibody recognized bands at 155 and 135 kDa, consistent with previously published results identifying these bands as mature and immature hERG1 glycoforms, respectively (Fig. 2, Lane 1) (8). In addition, ERG1-KA recognized three lower molecular mass bands at 95, 85, and 80 kDa. The 95- and 85-kDa bands are consistent with bands attributed to hERG1a in tumor cell lines (14) and heterologously expressed in QT-6 cells (6).

As expected, blots probed with the ERG1a-specific antibody recognized the 155- and 135-kDa bands but not the three lower-mass bands (Fig. 2A, Lane 2). The 155- and 135-kDa bands were eliminated upon incubation of the ERG1a antisera with
the antigenic 1a peptide prior to probing the blots (Lane 3). The ERG1b-specific antibody recognized the 95-, 85-, and 80-kDa bands but not the two higher mass hERG1a bands (Lane 4). These bands were similarly eliminated by preincubation of the antisera with the antigenic 1b peptide (Lane 5). These data show that ERG1-KA and ERG1a antibodies and thus represent ERG1a protein, while ERG1b antisera recognize the 95- and 83-kDa bands, which were also recognized by ERG1-KA antibody (Fig. 3B, Lanes 1 and 2). The ERG1b antibody recognized the bands at 95 and 83 kDa, which were also recognized by ERG1-KA antibody, demonstrating that these bands represent ERG1b isoforms (Fig. 3B, Lanes 1 and 3). These data show conclusively that both ERG1a and -1b proteins are expressed within the ventricle across a range of mammalian species.

To determine whether the mature hERG1b glycoform is expressed on the cell surface, where it could contribute to hERG1 currents, surface proteins were bionitlated prior to cell lysis. Bionitlated proteins were affinity purified with streptavidin beads, blotted, and probed with ERG1-KA antisera. Like hERG1a, only the maturely glycosylated hERG1b (95-kDa) protein band was bionitlated (Fig. 2B, Lane 3), showing hERG1b is expressed on the cell surface in HEK-293 cells.

Next we tested the hypothesis that the lower molecular mass signals observed in native cardiac tissue correspond to the ERG1b protein using the ERG1a- and -1b-specific antisera. In Western blots from two separate human ventricular membrane preparations, the ERG1-KA antibody revealed bands at 140, 120, 94, and 83 kDa (Fig. 3A, Lane 1). The 140- and 120-kDa bands are consistent with previous reports from human tissue (6) and represent the maturely glycosylated and unglycosylated hERG1a, respectively. The 1b-specific antibody recognized the 94- and 83-kDa bands (Fig. 3A, Lane 2) demonstrating that ERG1b protein is expressed in human ventricle.

ERG1b was also observed in canine ventricular tissue. There the ERG1-KA antibody consistently recognized proteins at 160–165 and 90–95 kDa and less consistently at 140–145, 115–125, and 80–85 kDa. Fig. 3 shows that high molecular weight bands at 165 and 140 kDa were recognized by both ERG1-KA and ERG1a antibodies and thus represent ERG1a isoforms (Fig. 3B, Lanes 1 and 2). The ERG1b antibody recognized the bands at 95 and 83 kDa, which were also recognized by ERG1-KA, demonstrating that these bands represent ERG1b isoforms (Fig. 3B, Lanes 1 and 3). These data show conclusively that both ERG1a and -1b proteins are expressed within the ventricle across a range of mammalian species.

Consistent with their biochemical association, ERG1a and ERG1b signals localized to the same subcellular compartment using immunocytochemistry. Both the 1a and 1b antibody in fixed, permeabilized canine myocytes revealed a punctate, Z-line-like fluorescence pattern characteristic of a T tubular lo-

FIG. 2. A, ERG1a and ERG1b-specific antisera characterization. Western blots of membrane proteins from HEK-293 cells stably expressing hERG1a and -1b (4 µg/lane, n = 3). Molecular weight markers, in kDa, are indicated. Lane 1, ERG1-KA antibody (1/5000) recognizes 155-, 135-, 95-, 85-, and 80-kDa bands. Lane 2, ERG1a-specific antibody (1/100) confirming the 155- and 135-kDa bands in Lane 1 represent hERG1a. Lane 3, ERG1a antibody preincubated with 1a-peptide shows no bands. Lane 4, ERG1b-specific antibody confirms that the lower 95-, 85-, and 80-kDa bands represent hERG1b. Lane 5, ERG1b-specific antibody preincubated with 1b-peptide shows no bands. B, ERG1b glycosylation states and surface expression (n = 4). Western blots of HEK-293 ERG1a/1b stable cell line membrane protein preparations probed with ERG1-KA antibody. Lane 1, 2 µg of membrane protein shows predominantly 95- and 85-kDa bands and a faint shadow at 80 kDa. Lane 2, 10 µg of membrane preparation following PNGase F treatment shows an 80-kDa band. Lane 3, 10 µg of membrane preparation following endoglycosidase H treatment shows bands at 95 and 80 kDa. Lane 4, 10 µg of streptavidin purified proteins from surface-bionitlated cells shows only the 95-kDa ERG1b band is on the surface.

FIG. 3. ERG1b expression in vivo. Representative Western blots of 25 µg of membrane proteins from human and canine ventricular myocytes were size-separated by SDS-PAGE on a 7.5% gel and probed with either ERG1-KA, ERG1a, or ERG1b antisera. Molecular masses are indicated on the left of each blot. A, human ventricular membrane proteins. Lane 1, probed with ERG1-KA antisera, exhibits bands at 140, 120, 94, and 83 kDa. Lane 2, probed with 1b-specific antisera, exhibits prominent bands at bands at 94 and 83 kDa. In the example shown additional bands at 102 and 98 kDa are present. Although these are not visible on Western blots probed with ERG1-KA, they are blocked by preincubation of antibody with the 1b peptide, suggesting that they are hERG1b isoforms possibly modified posttranslationally (n = 2 adult male individuals). B, canine ventricular membrane proteins. Lane 1, probed with KA antisera, shows bands at 160, 140, and 95 and a weak band at 83 kDa. Lane 2, probed with 1a-specific antisera, shows bands at 160 and 140 kDa. Lane 3, probed with 1b-specific antisera, shows bands at 95 and 83 kDa (n = 11).
To characterize ERG1 localization more precisely, we stained myocytes concurrently with ERG1a and myosin binding protein C (MyBP-C) antisera (15). Three-dimensional images were rendered from a stack of deconvolved two-dimensional immunofluorescent images. MyBP-C signal, in green, appeared as a repeating pattern of doublets separated by regions devoid of fluorescence that span the width of the cell (Fig. 4, D–F). MyBP-C signal localizes to the myosin-containing sarcomere A-band (16); the unstained areas, between doublets, represent M-lines (Fig. 4 E). ERG1a fluorescent signal, shown in red, is seen in I-bands adjacent to A-bands (Fig. 4, D and E). Both Z lines and T tubules are located in the I-band (17). The punctate red ERG1a signal extends in columns from the cell surface toward the interior, as expected of a T tubular-restricted protein, where it borders the green MyBP-C signal (Fig. 4 E). These data indicate ERG1 signal in canine myocytes is consistent with a T tubular distribution.

**DISCUSSION**

In this study, we have shown ERG1b protein is produced in mammalian heart. The absence of ERG1b protein in previous studies is likely attributable to a lower affinity of the antibodies employed and/or differential accessibility to the common epitope in ERG1a and -1b. Studies by Finley et al. (7) in equine and Rasmussen et al. (18) in rat mention the appearance of an ~100-kDa band in some preparations of cardiac membranes. In retrospect, it is reasonable to suggest these signals represented early indications of the ERG1b isoform.

We also show ERG1a and -1b proteins are associated in ventricular myocytes. In heterologous systems, ERG1a and -1b expression produces currents with biophysical properties that cannot be explained by the coexistence of two populations of homomeric channels (4). The properties of these heteromeric channels more closely resemble those of native Ik channels (19). Localization of ERG1a and -1b to T tubular structures in canine ventricular myocytes is consistent with electron microscopy studies in rat myocytes showing ERG1 protein predominantly localized to the T tubules where it could regulate action potential duration at the site of excitation-contraction coupling (20). These data strongly suggest native Ik channels, which are localized to T tubules, comprise both ERG1a and -1b subunits.

Understanding cardiac Ik physiology and the disease mechanisms of hERG-linked congenital and acquired LQTS necessitates approximating the native state in heterologous systems as closely as possible, minimally requiring coexpression of hERG1a and -1b. In addition, our findings have significant implications for amino-terminal mutations causing LQTS. Approximately 20% of LQTS-2 mutations reside in the amino terminus of hERG1a (pc4.fsm.it:81/cardmoc/hergmut.htm), where they can truncate the protein, alter gating properties, and/or cause trafficking deficiencies (21–25). Because ERG1a and -1b are alternate transcripts produced by the ERG1 gene, mutations in exons encoding the ERG1a amino terminus are not likely to affect the production of wild-type ERG1b from this gene. These findings argue for screening LQTS patients for mutations in the hERG1b-specific exon and assessing the disease mechanism of all mutations in heterologous expression systems in which hERG1a and -1b are co-expressed.

**Acknowledgments**—We thank members of the Robertson lab for support and helpful discussions, Dr. Barry London for assistance with antibodies, Dr. Robert Haworth for canine heart tissue, Dr. Tim Kamp
for human heart tissue, Drs. Rick Moss and Samantha Harris for MyBP-C antibodies, Drs. Craig January and Eugene Kaji for tissue culture space, and Drs. Cindy Czajkowski, Craig January, and Jeff Walker and members of their laboratory for helpful discussions.

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