Expression of Distinct ERG Proteins in Rat, Mouse, and Human Heart

RELATION TO FUNCTIONAL $I_{Kr}$ CHANNELS*

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One form of inherited long QT syndrome, LQT2, results from mutations in HERG1, the human ether-a-go-go-related gene, which encodes a voltage-gated K$^+$ channel α subunit. Heterologous expression of HERG1 gives rise to K$^+$ currents that are similar (but not identical) to the rapid component of delayed rectification, $I_{Kr}$, in cardiac myocytes. In addition, N-terminal splice variants of HERG1 and MERG1 (mouse ERG1) referred to as HERG1b and MERG1b have been cloned and suggested to play roles in the generation of functional $I_{Kr}$ channels. In the experiments here, antibodies generated against HERG1 were used to examine ERG1 protein expression in heart and in brain. In Western blots of extracts of QT-6 cells expressing HERG1, MERG1, or RERG1 (rat ERG1) probed with antibodies targeted against the C terminus of HERG1, a single 155-kDa protein is identified, whereas a 95-kDa band is evident in blots of extracts from cells expressing MERG1b or HERG1b. In immunoblots of fractionated rat (and mouse) brain and heart membrane proteins, however, two prominent high molecular mass proteins of 165 and 205 kDa were detected. Following treatment with glycopeptidase F, the 165- and 205-kDa proteins were replaced by two new bands at 175 and 130 kDa, suggesting that ERG1 is differentially glycosylated in rat/mouse brain and heart. In human heart, a single HERG1 protein with an apparent molecular mass of 145 kDa is evident. In rat, ERG1 protein (and HERG1) expression is higher in atria than ventricles, whereas in humans, HERG1 expression is higher in ventricular, than atrial, tissue. Taken together, these results suggest that the N-terminal alternatively spliced variants of ERG1 (i.e. ERG1b) are not expressed at the protein level in rat, mouse, or human heart and that these variants do not, therefore, play roles in the generation of functional cardiac $I_{Kr}$ channels.

Long QT syndrome is an acquired or an inherited disorder that can cause syncope and sudden death resulting from episodic ventricular arrhythmias and ventricular fibrillation (1, 2). The characteristic feature identified in surface electrocardiograms of affected individuals is prolongation of the QT interval, consistent with the underlying cause of long QT syndrome being a defect in ventricular repolarization (3, 4). One form of inherited long QT syndrome, LQT2, was localized to chromosome 7 (5), and shown to result from mutations (6) in the human ether-a-go-go-related gene, originally referred to as HERG (7). With the identification of additional members of the ether-a-go-go-related gene (ERG) family (8), the terminology HERG1 seems more appropriate (9).

HERG1 encodes a polypeptide with a predicted molecular mass of 127 kDa and a predicted sequence and membrane topology similar to that of other voltage-gated K$^+$ channel α subunits (10–12). Heterologous expression of HERG1 in Xenopus oocytes (10–12) and in HEK-293 cells (14–16) reveals voltage-gated K$^+$ currents that are similar to the rapid component of delayed rectification, $I_{Kr}$, in myocardial cells (17–22). Like $I_{Kr}$, for example, the currents produced on heterologous expression of HERG are rapidly activating and rapidly inactivating, are K$^+$-selective, and display marked inward rectification at potentials positive to 0 mV (10–14). The detailed properties of the HERG-induced currents (10–16), however, are variable and are not identical to those of (endogenous) $I_{Kr}$ characterized in myocardial cells from several species (17–22), including human (20–22). Additional members of the ERG family, ERG2 and ERG3, have been cloned (8), these subunits appear to be nervous system-specific and therefore likely do not contribute to cardiac $I_{Kr}$. Alternatively spliced variants of HERG1 and MERG1 (the mouse homologue of HERG 1) with unique N termini (referred to as HERG1b and MERB1b), however, have been cloned (8), these subunits appear to be nervous system-specific and therefore likely do not contribute to cardiac $I_{Kr}$. Currently, it is not known which of the diverse ERG family members contribute to cardiac $I_{Kr}$.

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has been documented in ferret heart (26), neither the expression levels nor the distributions of alternatively spliced and/or differentially processed ERG1 proteins have been examined directly to date in the mammalian myocardium.

The experiments here were undertaken to examine ERG1 protein expression in mouse, rat, and human heart. Using antibodies targeted against HERG1, two ERG1 proteins with molecular masses 165 and 205 kDa are detected in rat and mouse atria, ventricles, and brain, and these appear to reflect differentially (N-linked) glycosylated forms of ERG1. In contrast, a single 145-kDa HERG1 protein is identified in immunoblots of human cardiac membrane proteins. In rat, mouse, and human heart, however, no low molecular mass proteins corresponding to the expression of the ERG1b are detected. In rat, ERG1 protein expression and functional IKr expression are higher in atria than ventricles, whereas in mouse and human, ERG1 expression is higher in the ventricles. The results presented demonstrate that the predominant form of ERG1 expressed in myocardial tissues is the full-length ERG1 protein, suggesting that N-terminal ERG1b splice variants do not play a role in the generation of functional IKr channels in cardiac myocytes.

MATERIALS AND METHODS
Polycystic Antibodies against HERG—Peptides corresponding to unique sequences in HERG: (i) residues 174–188, TARESSVRSG-AGGA, in the N terminus and (ii) residues 1145–1159, LTSQPL-GRAGGA, in the C terminus, were generated by the Protein Chemistry Laboratory (Washington University Medical Center). A cysteine residue was added to the N terminus of each peptide to allow coupling to the keyhole limpet hemocyanin carrier protein, and the coupled peptides were sent to Caltag (San Francisco, CA) for injection into rabbits. Sera were screened using enzyme-linked immunosorbent assays, and antibodies were subsequently affinity purified using the ImmunoPuroAntigen/Antibody Immobilization Kit #2 (Pierce). These antibodies are referred to as N-anti-HERG and C-anti-HERG to denote the location of the amino acid sequence in HERG against which the antibody was generated. Enzyme-linked immunosorbent assays on the affinity purified antibodies revealed that each antibody detected only the peptide against which it was generated; no cross-reactivity was evident.

In addition, a polyclonal antiserum generated against a fusion protein corresponding to the C-terminal 181 amino acids in HERG1 coupled to (histidine-tagged) thioredoxin was obtained from Z. Zhou and C. T. January (University of Wisconsin, Madison, WI). Details of the preparation, purification, and characterization of the antiserum have been published (15). An additional affinity purified anti-HERG antibody was generated. Enzyme-linked immunosorbent assays on the location of the amino acid sequence in HERG against which the antibody corresponded to the C-terminal 181 amino acids in HERG1 and the Kvα subunit cDNAs. Transfection efficiencies (generally 25–35%) were determined either by counting GFP-positive cells, which were visualized directly using an inverted epifluorescence microscope, or by subsequent staining for β-galactosidase activity using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma).

Immunohistochemistry—The usefulness of the anti-HERG antibodies for immunohistochemistry was assessed in experiments on ERG1-transfected cells and on cryostat sections of adult rat ventricles. All manipulations were performed at 22–23 °C. Approximately 48 h after transfections, cells were washed with 0.1 M phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (in PBS). Fixed cells were washed with PBS and incubated for 1 h in blocking buffer I (PBS containing 5% normal goat serum, 0.2% Triton X-100, and 0.1% NaN3). The reaction was monitored under brightfield illumination and stopped by addition of PBS. Cells on coverslips were dehydrated in ethanol, cleared in xylene, and mounted with Krystalon for viewing and photography.

For the preparation of cryostat sections, adult (Harlan Sprague-Dawley) rats (~0.5 kg) were anesthetized with CO2/Hearts were rapidly removed, placed in a tissue bath perfused with Tyrode’s solution at 37 °C containing 121 mM NaCl, 5 mM KCl, 15 mM NaHCO3, 1 mM Na2HPO4, 2.8 mM sodium acetate, 1 mM MgCl2, 2.2 mM CaCl2, and 5.5 mM glucose equilibrated with 5% O2/5% CO2 (pH 7.4), and subsequently frozen in isopentane at −40 °C. Small pieces of the ventricles were removed, trimmed, attached to a tissue holder using Histo Prep (DPI) printer, and placed on the rapid freeze stage of a Microm cryostat (Zeiss). Cryostat sections were cut at 20 μm, collected, and air-dried. Dried sections were incubated in blocking buffer II (PBS containing 0.2% Triton X-100 and 0.5% bovine serum albumin (BSA; Sigma)) for 1 h, followed by 2-h incubation in one of the anti-HERG antibodies in blocking buffer II. After washing with PBS, sections were incubated with a Texas Red-conjugated goat anti-rabbit secondary antibody (Sigma) for 1 h, followed by 2-h incubation with a Texas Red-conjugated mouse anti-HERG antibody (Vector, Burlingame, CA) diluted 1:100 for 1 h each. After washing, cells were exposed to the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Bio-Rad); the reaction was monitored under brightfield illumination and stopped by addition of PBS. Cells on coverslips were dehydrated in ethanol, cleared in xylene, and mounted with Krystalon for viewing and photography.

Membrane Preparations—To harvest tissue for biochemistry, adult Long Evans rats and adult C57BL6 mice were anesthetized (5% halothane under 70% nitrous oxide) and decapitated, hearts were rapidly removed, trimmed, attached to a tissue holder using Histo Prep (Electron Microscopy Sciences, Fort Washington, PA) and placed on the rapid freeze stage of a Microm cryostat (Zeiss). Cryostat sections were cut at 20 μm, collected, and air-dried. Dried sections were incubated in blocking buffer II (PBS containing 0.2% Triton X-100 and 0.5% bovine serum albumin (BSA; Sigma)) for 1 h, followed by 2-h incubation in one of the anti-HERG antibodies in blocking buffer II. After washing with PBS, sections were incubated with a Texas Red-conjugated goat anti-rabbit IgG secondary antibody (Jackson, West Grove, PA) diluted 1:2000 in blocking buffer II. After rinsing with PBS, sections were incubated using ImmunoFluore (ICN, Costa Mesa, CA). For photography, sections were placed on the stage of a Zeiss LSM 410 inverted confocal microscope and viewed using a 63×, 3.0 numerical aperture objective. Photomicrographs were viewed through the entire 20 μm in 1-μm steps. Texas Red was excited with a helium/neon (543 nm) laser and imaged on a photomultiplier; images were printed on a Kodak XLS8300 high resolution (300 DPI) printer.
Bound antibodies were detected using the chemiluminescent substrate 
5 mol/liter diethanolamine with 1 mmol/liter MgCl₂, pH 9.8; Tropix). 

Blocking buffer III for 15 min and then for 2 min in assay buffer (0.1 
in blocking buffer III. After incubation, membranes were washed in 
60 buffer (containing 10 mmol/liter Tris-HCl and 1 mmol/liter EDTA, pH 
1–2 g) from individual donors were processed. For rat and mouse, ventricles and atria 
were triturated again. Insoluble material was pelleted (17,500 
3) and the tissue pieces were mechanically dispersed by gentle trituration. 
The resulting suspensions were filtered through gauze, and cells were 
pelleted by centrifugation at 400 rpm for 1 min. Myocytes were resuspended 
in incubation buffer containing 0.5% BSA and rinsed with 2 ml 
of incubation buffer containing 4% BSA. After settling, myocytes were 
resuspended in incubation buffer with 1 mM Ca²⁺. The flow rate was then increased to 10 ml/min; 
when the back pressure stabilized (~30 min), the heart was removed. 
Atria and ventricles were separated, minced, and transferred to fresh 
collagenase-containing perfusion buffer in a shaking water bath at 
37 °C, aerated with humidified 95% O₂/5% CO₂ for the ventricular 
tissue, the collagenase concentration was increased to 400 units/ml. 
The secondary incubations were 5 min for atria and 10 min for ventricles, 
and the tissue pieces were mechanically dispersed by gentle trituration. 
The resulting suspensions were filtered through gauze, and cells were 
pelleted by centrifugation at 400 rpm for 1 min. Myocytes were resuspended 
in incubation buffer containing 0.5% BSA and rinsed with 2 ml 
of incubation buffer containing 4% BSA. After settling, myocytes were 
resuspended in incubation buffer with 1 mM Ca²⁺ at room temperature 
under 100% O₂. The yields of rod-shaped atrial and ventricular myocytes 
were 60–80%, and cells were used within 6 h.

Electrophysiological Recordings—The nystatin perforated patch, whole cell 
recording technique (32) was employed to record K⁺ currents from 
isolated, Ca²⁺-tolerant, rod-shaped adult rat atrial and ventricular 
myocytes (33). The bath solution contained 135 mM NaCl, 5 mM KCl, 
1 mM CaCl₂, 1 mM MgCl₂, 5 mM NaH₂PO₄, 1.0 mM CaCl₂, 0.68 
mM glutamine, 11 mM dextrose, 25 mM HEPES, 5 mM pyruvate, 
and 1 μM insulin (pH 7.3). Following cannulation and flushing with 2 ml 
of cold incubation buffer, the heart was mounted on a Langendorff 
apparatus and perfused retrogradely through the aorta with 50 ml of 
warmed (37 °C) perfusion buffer containing 118 mM NaCl, 37.5 mM 
NaHCO₃, 4.8 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 
0.68 mM glutamine, 16.5 mM dextrose, and 7.5 mM pyruvate, equilibrated 
with humidified 95% O₂/5% CO₂ (pH 7.2). The heart was then 
cut into two 50-g pieces of a normal size and transferred 
to perfusion buffer (100 ml) containing Type II collagenase (140 units/ 
ml; Worthington, Freehold, NJ) and 0.20 mg/ml BSA (Fraction V; Sig- 
ma). After 16 min, Ca²⁺ was added gradually until the free Ca²⁺ 
concentration was 1 mM. The flow rate was then increased to 10 ml/min;

QRS complexes were filtered at 1kHz prior to digitization and 
storage. For experiments, myocytes in 35-mm culture dishes were 
placed in a thermal stage controller (Biophet ΔT system, Butler, PA), 
maintained at 35 °C, and gassed with 100% O₂. E-4031 was kindly 
donated by Eisai Co., Ltd (Tsukuba, Japan). Analysis of digitized data 
was completed using pClamp and Origin (Microcal, Northampton, 
MA). Averaged and normalized data are presented as means ± S.E. 
Statistical significance was assessed using the Student’s t test; p values 
are presented in the text.

To determine whether MERG1 and/or RERG1 is glycosylated, mouse 
and rat brain membrane proteins were incubated in the presence of 
PnGase F (glycopeptidase F; Sigma), an enzyme that cleaves N-linked sugars. 
Prior to exposure to the enzyme, rat/mouse brain membrane proteins (126 μg), 
prepared as described above, were denatured by boiling for 10 min. After cooling, 6.3 μl (1.26 units) of PnGase F was 
added, and the samples were incubated at 37 °C for 24 h. Following the 
second incubation, membrane proteins were fractionated, and Western 
blots were performed as described above with the C-anti-HERG antibody.

Isolation of Adult Rat Cardiac Myocytes—Adult rat cardiac myocytes 
were isolated using a modified version of a protocol described previously 
(31). For each experiment, an adult male Harlan Sprague-Dawley rat 
(250–275 g) was anesthetized with pentobarbital (0.5 ml, 50 mg/ml). 
Each heart was rapidly excised and placed in cold incubation buffer 
containing 35 mM NaCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 
0.68 mM glutamine, 11 mM dextrose, 25 mM HEPES, 5 mM pyruvate, 
and 1 μM insulin (pH 7.3). Following cannulation and flushing with 2 ml 
of cold incubation buffer, the heart was mounted on a Langendorff 
apparatus and perfused retrogradely through the aorta with 50 ml of 
warmed (37 °C) perfusion buffer containing 118 mM NaCl, 37.5 mM 
NaHCO₃, 4.8 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.0 mM CaCl₂, 
0.68 mM glutamine, 16.5 mM dextrose, and 7.5 mM pyruvate, equilibrated 
with humidified 95% O₂/5% CO₂ (pH 7.2). The heart was then 
cut into two 50-g pieces of a normal size and transferred 
to perfusion buffer (100 ml) containing Type II collagenase (140 units/ 
ml; Worthington, Freehold, NJ) and 0.20 mg/ml BSA (Fraction V; Sig- 
ma). After 16 min, Ca²⁺ was added gradually until the free Ca²⁺ 
concentration was 1 mM. The flow rate was then increased to 10 ml/min;

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In parallel experiments, extracts from QT-6 cells transfected with cDNA constructs encoding HERG1, Kv1.2, Kv2.1, or Kv4.2 and from mock transfected QT-6 cells were fractionated by SDS-PAGE, transferred to PVDF membranes, and blotted with the anti-HERG antibodies. Immunoblots with the C-anti-HERG antibody revealed a single protein band at approximately 155 kDa in extracts of QT-6 cells transfected with HERG cDNA (Fig. 1B, lane a). A protein of the same molecular mass was identified in HERG1-transfected HEK-293 cells with this (and the N-anti-HERG) antibody (14, 16), although in HEK-293 cells an additional lower molecular mass (~135 kDa) protein is also expressed (see “Discussion”). Nothing was detected with the C-anti-HERG antibody in extracts from either mock transfected QT-6 cells (Fig. 1B, lane b) or QT-6 cells transfected with the Kv1.2, Kv2.1, or Kv4.2 cDNA. The 155-kDa protein is also identified in extracts of HERG1-transfected QT-6 cells probed with the N-anti-HERG antibody (Fig. 1B, lane c) and, as with the C-anti-HERG antibody (Fig. 1B, lane a), this band is not detected in extracts from either mock transfected QT-6 cells (Fig. 1B, lane d) or QT-6 cells transfected with the Kv1.2, Kv2.1, or Kv4.2 cDNA. The 155-kDa protein was also not seen in Western blots when the antibodies were preincubated with the peptides against which each was generated (data not shown).

Expression of ERG1 and ERG1b Revealed with C-terminal Anti-HERG Antibodies—Subsequent experiments were aimed at determining whether the anti-HERG antibodies could also be used to examine the expression of the alternatively spliced HERG1 variant, HERG1b (Fig. 2A), which has been postulated to play a role in the generation of functional I_Kr channels (9, 23), as well as to detect ERG1 protein expression in other species. Preliminary immunohistochemical experiments, similar to those described above (Fig. 1A), revealed that expression of HERG1, MERG1, and RERG1 is reliably detected with the C-anti-HERG and N-anti-HERG antibodies, whereas only the C-anti-HERG antibody can be used to document HERG1b or MERG1b expression (data not shown). To identify the molecular masses of proteins produced on expression of these constructs, Western blot experiments, similar to those described above (Fig. 1B), were completed on extracts from QT-6 cells transfected with cDNA constructs encoding HERG1, HERG1b, MERG1, MERG1b, RERG1, or Kv1.4 and from mock transfected QT-6 cells. Immunoblots with the C-anti-HERG antibody revealed a single 155-kDa protein in extracts of QT-6 cells transfected with the full-length HERG1, MERG1, or RERG1 cDNA constructs (Fig. 2B, lanes a under Herg1, Merg1, and Rerg1), whereas nothing is detected in extracts of cells expressing Kv1.4 (Fig. 2B) or in mock transfected cells (not shown) with this antibody. Expression of the full-length 155-kDa HERG1, MERG1, and RERG1 proteins is also identified in Western blots probed with the anti-HERG C-terminal fusion protein antiserum (15) (Fig. 2C) and with the Alomone C-terminal anti-HERG antibody (Fig. 2D). All three C-terminal anti-HERG antibodies, therefore, identify the same full-length 155-kDa (human, mouse, and rat) ERG1 protein (Fig. 2 and Table I).

In cells transfected with constructs encoding the truncated HERG1b or MERG1b proteins, the C-anti-HERG antibody also identifies a single protein band, although in this case the molecular mass of the protein is 95 kDa (Fig. 2B, lanes b under Herg1 and Merg1). Expression of the 95-kDa HERG1b and MERG1b proteins is also identified in Western blots probed with the anti-HERG C-terminal fusion protein antiserum (15) (Fig. 2C, lanes b) and with the Alomone C-terminal anti-HERG antibody (Fig. 2D, lanes b). As with the full-length 155-kDa...
ERG1 proteins, all three C-terminal anti-HERG antibodies identify the same 95-kDa protein (Fig. 2, lanes b), suggesting that all of these antibodies recognize the same ERG1b protein(s) and that they can be used to detect ERG1b expression in tissue. As expected, nothing is detected in Western blots of extracts of QT-6 cells expressing HERG1b or MERG1b probed with the N-anti-HERG antibody (data not shown). No specific bands were detected with the C-terminal anti-HERG antibodies in extracts from either mock transfected QT-6 cells (data not shown) or QT-6 cells transfected with a cDNA construct encoding Kv1.4 (Fig. 2, B–D). In addition, neither the 155-kDa nor the 95-kDa proteins was seen in Western blots when the antibodies were preincubated with the peptides (B, D) or the fusion protein (C) against which each antibody was generated (data not shown).

Two ERG1 Proteins in Rat and Mouse Brain—In Western blots of fractionated rat brain membrane proteins, both the C- and the N-anti-HERG antibodies identify a protein at approximately 165 kDa (Fig. 3, A and C), a molecular mass similar to that of the protein (155 kDa) detected with these antibodies in immunoblots of extracts of ERG1-transfected QT-6 (Fig. 1) and HEK-293 (14–16) cells. The 165-kDa protein was not evident when the antibodies were preincubated with the peptides against which each was generated (Fig. 3, A and C, + lanes). In contrast to the findings in QT-6 (Fig. 1) and HEK-293 (14–16) cells, however, an additional high molecular mass (205 kDa) protein, was also detected in blots of fractionated rat brain membrane proteins probed with either the C- or the N-anti-HERG antibodies (Fig. 3, A and C). The 205-kDa band was also not seen when the antibodies were preincubated with the peptides against which each antibody was generated (Fig. 3, A and C, + lanes). With the N-anti-HERG (Fig. 3C), however, the intensity of the 205-kDa band is low relative to the 165-kDa band. This intensity difference is not seen with the C-anti-HERG antibody (Fig. 3, B–D), however, the two ERG1b proteins (see “Discussion”).

Importantly, no protein bands at ~ 95 kDa, as might be expected to result from expression of the N-terminal alternatively spliced ERG1b variant (Fig. 2), are seen in Western blots of fractionated rat brain membrane proteins probed with the C-anti-HERG antibody (Fig. 3A), suggesting that ERG1b is not expressed (at the protein level) in rat brain (see “Discussion”).

In Western blots of fractionated mouse brain membrane proteins (Fig. 3B), virtually identical results were obtained, i.e. two proteins at 165 and 205 kDa were routinely identified.

### TABLE I

| Cell/tissue | Molecular masses |
|-------------|------------------|
|             | ERG1b | ERG1b |
| HEK-293     | 155 kDa | 95 kDa |
| Rat brain   | 165 and 205 | ND |
| Rat heart   | 165 and 205 | ND |
| Mouse brain | 165 and 205 | ND |
| Mouse heart | 165 and 205 | ND |
| Human heart | 145     | ND |

* Molecular masses of the proteins expressed upon heterologous expression of full length HERG1, MERG1, or RERG1.

* Molecular masses of the proteins produced upon heterologous expression of either HERG1b or MERG1b.

ND, not detected.
ERG1 Expression in Rat, Mouse, and Human Heart

Fig. 3. Western blots reveal the presence of two distinct ERG1 proteins in rat and mouse brain. Adult rat (A and C) and mouse (B) brain membrane proteins (35 μg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the C-anti-HERG (A and B) or the N-anti-HERG (C) antibody (diluted 1:500). The antibodies were applied either directly (−) or after preincubation (+) with 10 μg/ml of the peptide against which the antibody was generated. Two bands at approximately 165 and 205 kDa (arrows) are detected with the C-anti-HERG (A and B) and N-anti-HERG (C) antibodies in brain (see text), and there were no prominent low molecular mass bands in these blots (Fig. 3B), as would be expected if MERG1b were expressed. The ERG1 proteins expressed in mouse and rat brain, therefore, are indistinguishable; results similar to those presented in Fig. 3 were obtained in Western blot analyses of seven rat and three mouse brain membrane protein preparations.

The Two MERG1 and RERG1 Proteins Reflect N-Linked Glycosylation—As noted previously, the predicted molecular mass of full-length HERG is 127 kDa (6, 7) and the molecular mass of the protein recognized in HERG1-, MERG1-, and RERG1-transfected QT-6 (Figs. 1 and 2) and in HERG1-transfected HEK-293 (14–16) cells by the C- and the N-anti-HERG antibodies is 155 kDa. The finding of two proteins and, particularly, the 205-kDa protein in rat and mouse brain membrane protein preparations, therefore, was unexpected. It is theoretically possible that the 165- and 205-kDa proteins reflect the expression of distinct ERG subfamily genes, splice variants of the same ERG gene or, alternatively, post-translational modifications of the same ERG holoprotein. Although two additional members, ERG2 and ERG3, of the ERG subfamily have recently been cloned from brain (8), the predicted amino acid sequences of the ERG2 and ERG3 proteins are distinct from ERG1, and neither protein would be detected by the N- and C-anti-HERG antibodies. Although the MERG1 and HERG1 transcripts are alternatively spliced in mouse and human heart (9, 23–25), and this could certainly also occur in rat and mouse brain, splicing reduces the size of the ERG1 message and results in the production of lower (rather than higher) molecular mass ERG1 proteins (see Fig. 2). It seemed more likely, therefore, that the finding of two high molecular mass ERG1 proteins in rat and mouse brain reflects post-translational processing.

Examination of the sequence of HERG1 (RERG1 or MERG1) reveals multiple potential sites for post-translational modifications, including several sites for serine-threonine kinase phosphorylation, N-myristoylation, and N-linked glycosylation; no O-linked glycosylation sites are found. The large difference between the 205-kDa (rat and mouse brain) ERG1 proteins detected in the Western blots (Fig. 3) and the predicted molecular mass (127 kDa) (6, 7) of the ERG1 proteins suggested that N-linked glycosylation might be involved. In addition, it has been reported that HERG1 expressed in HEK-293 cells is glycosylated (14–16). To determine whether ERG1 is (N-linked) glycosylated in vivo, rat and mouse brain membrane proteins were denatured (by boiling) and incubated at 37 °C in the presence of PNGase F (see “Materials and Methods”), which cleaves N-linked sugars. Western blot analyses of samples incubated with PNGase F for 24 h revealed marked reductions in the intensities of both the 165-kDa and the 205-kDa bands, as well as the appearance of two new bands at ~175 and 130 kDa (Fig. 4). The results obtained on the rat (Fig. 4A) and the mouse (Fig. 4B) brain samples are indistinguishable, consistent with the suggestion above that the rat and mouse ERG1 proteins are the same. Importantly, the 175-kDa and the 130-kDa proteins do not reflect breakdown of the ERG1 protein, as evidenced by the fact that the 165- and 205-kDa bands are readily detected, whereas the 175- and 130-kDa proteins are not, in immunoblots of rat and mouse membrane proteins incubated for 24 h at 37 °C in the absence of PNGase F (Fig. 4). Similar results were obtained in Western blots of four rat brain and two mouse brain membrane protein preparations treated with PNGase F. In addition, the results are similar to previous findings on HERG1-transfected HEK-293 (14, 15) cells and suggest that the 165- and 205-kDa bands detected in rat and mouse brain membrane reflect differences in N-linked glycosylation of the same (full-length) ERG1 protein (see “Discussion”).

RERG1 Expression in Rat Heart—Western blots of fractionated rat ventricular and atrial membrane proteins with the C-anti-HERG antibody also revealed proteins of 165 and 205 kDa (Fig. 5A); both bands were eliminated when the antibody was preincubated with the C-terminal peptide against which it was generated. The 165- and 205-kDa bands are indistinguishable from those detected in rat brain membrane preparations (Fig. 3A), as evidenced by Western blots of mixed brain and heart samples in which (only) the same two (165- and 205-kDa) proteins are detected (data not shown). Immunoblots of rat heart membrane proteins with the N-anti-HERG antibody revealed only a prominent band at 165 kDa; the 205-kDa ERG1 protein was not evident. Similar results were obtained with the N-anti-HERG antiserum, which reveals intense labeling of the 165-kDa (but not the 205-kDa) protein, as well as several other unidentified proteins (Fig. 5A). The difficulties encountered with detecting the 205-kDa ERG1 protein in brain (see above and Fig. 3C) and heart (Fig. 5A) with the N-terminal (but not the C-terminal) antibody suggests that the N-terminal amino acid sequence against which the antibody was generated is either modified or inaccessible in the denatured (205-kDa) protein (see below and “Discussion”). The Western blots with the C-anti-HERG and N-anti-HERG antibodies also revealed that ERG1 expression is substantially higher in rat atrial than in...
was preincubated with the C-anti-HERG antibody in immunoblots of rat atrial and ventricular heart membranes with the N anti-HERG antibody. In contrast to the results in brain (Fig. 3), two ERG1 proteins at 165 and 205 kDa were identified with the N-anti-HERG antiserum (diluted 1:500). Similar to the results in brain blotted with either the C-anti-HERG antibody (diluted 1:100) or the N-anti-HERG antiserum (diluted 1:500). Similar to the results in brain (Fig. 3), the 165-kDa ERG1 is detected in rat atrial membranes prepared from ventricles isolated from three different animals. The difference in the labeling patterns with the N- and C-anti-HERG antibodies is intrigu-
ging, particularly in light of the Western blot data (Fig. 5A), demonstrating a difference between the N- and C-anti-HERG antibodies in the detection of the 205-kDa ERG1 protein (see “Discussion”).

**ERG1 Expression in Rat, Mouse, and Human Heart**

**FIG. 5.** ERG1 protein expression in rat heart. A, rat ventricular (lanes V) and atrial (lanes A) membrane proteins (60 µg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and immuno-
blotted with either the C-anti-HERG antibody (diluted 1:100) or the N-anti-HERG antiserum (diluted 1:500). Similar to the results in brain (Fig. 3), two ERG1 proteins at 165 and 205 kDa were identified with the C-anti-HERG antibody in immunoblots of rat atrial and ventricular membrane proteins; both bands were eliminated when the antibody was preincubated (+) with 10 µg/ml of the peptide against which it was generated. In addition, ERG1 protein expression is higher in rat atrial than in rat ventricular membrane protein preparations. In contrast to the results in brain (Fig. 3), only the 165-kDa ERG1 is detected in rat heart membranes with the N anti-HERG antibody. B, immunofluorescence images of cryostat sections of rat ventricular myocardium exposed to either the C-anti-HERG (panels a–c) or the N-anti-HERG (panels d–f) antibody followed by Texas Red-conjugated goat anti-rabbit IgG. Sections photographed in panels c and f were incubated with the anti-
HERG antibodies and 10 µg/ml of the peptide against which each antibody was generated. With the C-anti-HERG (panels a and b) antibody, labeling is evident in the lateral membranes of ventricular myocytes and in the T-tubules; the arrows (panel b) indicate lateral mem-
brane labeling. With the N-anti-HERG antibody, no lateral membrane labeling is evident (panels d and e); rather, this antibody appears to label T-tubular membranes exclusively; the arrows (panel e) indicate the absence of detectable plasmalemmal membrane labeling (see text). Scale bars are 25 µm in panels a, c, d, and f and 5.0 µm in panels b and e.

**FIG. 6.** E-4031-sensitive K⁺ currents, I_K, in adult rat atrial and ventricular myocytes. A, whole cell K⁺ currents recorded at 35 °C from an adult rat atrial myocyte using the nystatin perforated patch technique; the voltage-clamp protocol is displayed in A, and the interpulse interval was 5 s. The holding potential was −50 mV to inactivate voltage-gated Na⁺ channels, and 2 µM nifedipine was added to the bath to suppress voltage-gated Ca²⁺ currents. The tail currents at −30 mV in B are replotted in C at a higher gain. In the presence of 5 µM E-4031, the slowly decaying (I_{K,E}) tail currents were suppressed completely (D). I_{K,E} tails were recorded on repolarization to −30 mV following 200-ms depolarizations from a holding potential of −50 mV to varying test potentials between −30 and +50 mV. Currents in the presence of E-4031 were subtracted from the controls, and the peak amplitudes of the E-4031-sensitive tails were determined from single exponential fits to the tail current decays fitted from the end of the test pulse until the current reached a steady-state level (0.5–1.0 s). Peak tail currents determined in individual cells were then normalized to whole cell mem-
brane capacitance, and mean (± S.E.) peak (I_{K,E}) tail current densities in adult rat atrial (n = 22) and ventricular (n = 22) myocytes are plotted versus test voltage in E (see text).
“Materials and Methods”) under control conditions and following exposure to the class III antiarrhythmic, E-4031, a specific blocker of \( I_{Kr} \) (17–22). To evoke \( I_{Kr} \), cells were depolarized to +20 mV from a holding potential of −50 mV for times ranging from 25 to 575 ms in 50-ms increments (Fig. 6A). These experiments revealed slowly decaying tail currents in adult rat atrial myocytes (Fig. 6, B and C) that were suppressed in the presence of 5 \( \mu M \) E-4031 (Fig. 6D), consistent with the expression of \( I_{Kr} \) (17–22). Results similar to those presented in Fig. 6 (B–D) were obtained on 21 other adult rat atrial and on 22 adult rat ventricular myocytes.

The time and voltage-dependent properties of \( I_{Kr} \) in adult rat atrial and ventricular cells are indistinguishable. Boltzman fits to the mean normalized peak tail current amplitudes as a function of voltage, for example, yielded \( V_0 \) values of −19.4 mV (k = 6.4) and −18.2 mV (k = 11.1) for \( I_{Kr} \) in rat atrial and ventricular myocytes, respectively. To compare \( I_{Kr} \) densities in adult rat atrial and ventricular myocytes, the “E-4031-sensitive” tail currents were obtained by subtraction of the currents in the presence of E-4031 (Fig. 6D) from the control records (Fig. 6C). Peak tail current amplitudes at −30 mV in individual cells were then determined from single exponential fits to the tail current decays in these subtracted records and normalized to the whole cell capacitance. These analyses confirmed that \( I_{Kr} \) density is significantly higher in adult rat atrial than in adult rat ventricular myocytes (Fig. 6E). Mean (± S.E.) peak tail current densities at −30 mV, for example, were 0.62 ± 0.03 pA/pF (n = 22) and 0.36 ± 0.01 pA/pF (n = 22) in adult rat atrial and ventricular myocytes, respectively. Interestingly, these differences in \( I_{Kr} \) density parallel the observed differences in ERG1 protein expression revealed in Western blots (Fig. 5A).

**A Distinct Isoform of HERG Is Expressed in Human Heart**—In contrast to the findings in rat, Western blot analysis of human atrial and ventricular membrane proteins using the C-anti-HERG antibody revealed a single 145-kDa ERG1 protein (Fig. 7A). The 145-kDa band was eliminated when the C-anti-HERG antibody was preincubated with the peptide against which it was generated (Fig. 7A). In addition, the same band was identified in Western blots of human ventricular and atrial membrane proteins probed with the N-anti-HERG antibody, suggesting that the antibodies are detecting ERG1 protein expression. In Western blots of fractionated mouse heart membrane proteins (Fig. 7B), however, results identical to those in rat heart (Fig. 5A), as well as in mouse (and rat) brain (Fig. 3) were obtained, i.e. two proteins at 165 and 205 kDa were routinely identified. Results similar to those presented in Fig. 7 were obtained in Western blot analyses of five human and three mouse heart membrane protein preparations.

The results presented in Fig. 7A reveal that the molecular mass of the single ERG1 protein (145 kDa) identified in human heart, therefore, is substantially lower than the molecular mass of either of the proteins (165 and 205 kDa) identified with this antibody in rat or mouse heart and brain (compare Figs. 3–5 and 7B), suggesting marked species-specific differences in post-translational processing of ERG1 proteins. In addition, and in contrast to the results in rat (Fig. 5A) heart, the Western data (Fig. 7A) suggest that ERG1 protein expression is higher in human ventricular than in atrial tissue. The expression of the ERG1 proteins is also higher in mouse ventricles than atria (Fig. 7B) (see “Discussion”).

**DISCUSSION**

**Generation and Characterization of Specific Anti-HERG Antibodies**—The C- and N-anti-HERG antibodies developed here were generated against unique peptide sequences in the C (residues 1145–1159) and N (residues 174–188) termini, respectively, of HERG1 (Fig. 2A). Experiments completed on HERG1-transfected QT-6 cells revealed that both antibodies are specific for HERG1 and reliably detect HERG1 protein expression using immunohistochemistry or Western blot analysis. Although the sequences of mouse ERG1 (MERG1) and rat ERG1 (RERG1) were not available at the time the peptides were selected for antibody generation, we assumed that the sequences of MERG1 and RERG1 would be very similar to HERG1. Subsequent sequence alignment has indeed revealed that both RERG1 (27) and MERG1 (9, 23) are highly homologous to HERG1; the sequence identity is >97% at the amino acid level. In addition, the C termini of RERG1, MERG1, and HERG1, targeted by the C-anti-HERG antibody (corresponding to residues 1145–1159 in HERG1), are (100%) identical (Fig. 2A). There are several amino acid substitutions in the N-terminal region (corresponding to residues 174–188 in HERG1) against which the N-anti-HERG antibody was generated (6, 7, 9, 23, 25). Nevertheless, 8 of the 15 amino acids (60%) in this sequence differences (between MERG1/RERG1 and HERG1) are (100%) identical (Fig. 2A). There are several amino acid substitutions in the N-terminal region (corresponding to residues 174–188 in HERG1) against which the N-anti-HERG antibody was generated (6, 7, 9, 23, 25). Nevertheless, 8 of the 15 amino acids (60%) in these sequence differences, the N-anti-HERG antibody can be used to detect RERG1 expression (Figs. 2–5). Because of the sequence differences (between MERG1/RERG1 and HERG1) and the variable labeling intensity of the 205-kDa RERG1 protein (relative to the 165-kDa protein), however, the C-anti-HERG antibody is preferred for studying ERG1 protein expression.
Distinct ERG1 Proteins Expressed in Rat (Mouse) and Human Heart—In Western blots of fractionated rat (and mouse) brain and heart membrane proteins probed with the C-anti-HERG antibody, two ERG1 proteins at 165 and 205 kDa were identified (Table I). Because the predicted molecular mass of HERG1 is 127 kDa (6), and the proteins recognized in HERG1-, MERG1-, and RERG1-transfected QT-6 cells by the C-anti-HERG antibody is 155 kDa, the finding of two proteins and, specifically, the 205-kDa protein, in rat and mouse brains (and hearts) was initially unexpected. Although it was theoretically possible that these could be the products of distinct ERG subfamily genes (8) or splice variants of the same ERG1 gene (9, 23–25), the experiments here reveal that the 165- and 205-kDa proteins reflect differentially (N-linked) glycosylated forms of ERG1. It has been reported that HERG1 expressed in HEK-293 cells is also (N-linked) glycosylated to produce proteins of approximately 135 and 155 kDa (14–16). The extent of N-linked glycosylation of RERG1 and MERG1 in rat and mouse brain (and heart), therefore, is considerably greater than for HERG1 in HEK-293 cells. The fact that the molecular mass (145 kDa) of human heart HERG1 is much lower than that of RERG1 or MERG1 (Table I) suggests that the extent of HERG1 glycosylation in vivo is also substantially less than for RERG1 or MERG1. In HERG1 (MERG1/RERG1), there are multiple (5) potential N-linked glycosylation sites, although only two (2) of these (asparagine 598 and 629) are extracellular (Fig. 2A). Recently, it was demonstrated that substitution of glutamine for asparagine at these residues leads to perinuclear localization of the HERG1 protein stably expressed in HEK-293 cells (16). In addition, no intrinsic currents are recorded from HERG1(N598Q) or HERG1(N629Q)-expressing HEK-293 cells (16), suggesting that N-linked glycosylation is required for cell surface expression of functional $I_{K_r}$ channels (16).

In Western blots of fractionated rat brain membrane proteins with the N-anti-HERG antibody, proteins at 165 and 205 kDa were also detected, although the intensity of the 205-kDa band was lower than the 165-kDa band. In immunoblots of rat heart membrane proteins probed with this antibody, however, the 205-kDa protein was not detected. Importantly, the 165- and 205-kDa bands reflect differentially (N-linked) glycosylated forms of ERG1, and both bands are intensely labeled with the C-anti-HERG antibody. For these reasons, the difficulties encountered with using the N-anti-HERG antibody to detect the 205-kDa protein cannot reflect differences in the amino acid sequences of the 165- and 205-kDa ERG1 proteins. Rather, the experimental observations suggest that there are additional differences in post-translational processing of the 205-kDa, relative to the 165-kDa, ERG1 protein. Although further experiments will be necessary to test this hypothesis directly, it is of interest to note that there are two potential N-myristoylation and one protein kinase C phosphorylation sites in RERG1 between amino acids 174–188. Post-translational modifications at nearby sites that limit access to the sequence against which the antibody is directed could also be involved.

Subcellular Localization of ERG1 in Rat Myocardial Cells—Confocal images of cryostat sections of adult rat heart revealed that both the C- and N-anti-HERG antibodies label the membranes of ventricular myocytes; little or no cytosolic labeling was evident. The observed subcellular staining patterns, however, are distinct. Specifically, the C-anti-HERG antibody labels both the T-tubules and the plasma membranes of adult rat ventricular myocytes, and the intensity of the staining in the plasmalemmal and T-tubular membranes is similar. With the N-anti-HERG antibody, in contrast, only the T-tubules are labeled. Taken together with the Western blot data, these results suggest that the 165-kDa ERG1 protein is localized in the T-tubules of adult rat ventricular myocytes. It is tempting to speculate further that the 205-kDa ERG1 protein might be specifically localized in the plasma membranes of rat ventricular myocytes. Further experiments will be necessary to test this hypothesis directly.

The results of the immunohistochemical experiments also suggest that ERG1 is distributed uniformly (or nearly so) in the sarcolemmal and T-tubular membranes of rat ventricular myocytes, i.e. no regions of high staining density or “hot spots” were evident. These results are in marked contrast to previous reports demonstrating that other voltage-gated $K^+$ channel $\alpha$ subunits are concentrated in the regions of the intercalated discs (28, 39). In human atria, for example, Kv1.5 is highly localized at intercalated disc regions, colocalized with connexin 43 and N-cadherin (39). Other Kv $\alpha$ subunits, including Kv4.2, are also nonuniformly distributed in the plasma membranes of rat ventricular myocytes, with the highest densities again being in the regions of the intercalated discs (28). Although the number of studies examining voltage-gated $K^+$ channel distribution in cardiac cells to date is admittedly small, the differences in the distributions of ERG1 seen in the present study and of the Kv $\alpha$ subunits reported previously (25, 39) suggest that distinct mechanisms are in place for targeting different $K^+$ channels to the sarcolemmal membranes of myocardial cells.

Relationship between ERG1 and Functional $I_{K_r}$ Channels—The finding of robust expression of ERG1 proteins in the rat heart was initially surprising because $I_{K_r}$ had not previously been reported in rat atrial cells, and although evident in ventricular cells, the densities of the currents are low (34, 35), particularly compared with other voltage-gated $K^+$ channel currents in these cells (36–38). The electrophysiological experiments here have revealed the presence of $I_{K_r}$ defined as the E4031-sensitive tail currents (17, 18), in isolated adult rat atrial (and ventricular) myocytes. The time- and voltage-dependent properties of the $I_{K_r}$ currents in the two cell types are similar, although $I_{K_r}$ density in atrial cells is significantly higher than in ventricular cells, consistent with the biochemical data showing greater expression of ERG1 proteins in rat atrial, than ventricular, membranes.

Although the results of the experiments presented here are all consistent with a role for ERG1 in the generation of $I_{K_r}$, they do not directly address questions regarding the molecular composition of functional $I_{K_r}$ channels. It is possible, for example, that there are additional members of the ERG subfamily of $K^+$ channel genes in the heart and that these coassemble with ERG1 to produce functional $I_{K_r}$ channels. Two additional ERG subfamily members, ERG2 and ERG3, have been cloned from brain (8), although these appear to be nervous system-specific. Nevertheless, there could certainly be additional ERG subfamilies or additional members of the ERG1, ERG2, and ERG3 subfamilies in the heart that contribute to $I_{K_r}$. MERG1 and HERG1 are also reportedly alternatively spliced in mouse and human heart (9, 23–25), and it has been suggested that the N-terminal splice variants play a role in the generation of functional $I_{K_r}$ channels (9, 23). For both HERG1 and MERG1, splicing reduces the sizes of the transcripts resulting in HERG1b and MERG1b proteins of lower molecular mass than full-length MERG1 and HERG1. The Western blot analysis completed here reveals only full-length HERG1 in atria and ventricles; there is no evidence for the expression of HERG1b (Table I). In both rat and mouse heart, two ERG1 proteins are expressed that reflect differences in N-linked glycosylation of full-length ERG1 (rather than coexpression of ERG1b). The results presented here, therefore, suggest that the ERG1b proteins do not contribute to the generation of functional $I_{K_r}$ channels in human, mouse, or rat heart. Because the experiments
here were performed on adult tissues, it certainly remains a possibility that ERG1b proteins are expressed and contribute to the formation of I\textsubscript{Kr} channels at other developmental stages. It has also been suggested that accessory K\textsuperscript{+} channel subunits that are present in heart contribute (with ERG1) to I\textsubscript{Kr} (40–42). In AT-1 cells (an atrial tumor cell line), for example, I\textsubscript{Kr} is attenuated on exposure to antisense oligodeoxynucleotides targeted against the small (130 amino acids), single transmembrane spanning domain accessory K\textsuperscript{+} channel subunit, minK (40). In addition, heterologously expressed minK and HERG1 reportedly coimmunoprecipitate (41). The functional significance of this finding in terms of HERG1 reportedly coimmunoprecipitate (41). The functional

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