**Functional Effects of KCNE3 Mutation and Its Role in the Development of Brugada Syndrome**

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**Background**—The Brugada syndrome, an inherited syndrome associated with a high incidence of sudden cardiac arrest, has been linked to mutations in 4 different genes, leading to a loss of function in sodium and calcium channel activity. Although the transient outward current (I\textsubscript{to}) is thought to play a prominent role in the expression of the syndrome, mutations in I\textsubscript{to}-related genes have not been identified as yet.

**Methods and Results**—One hundred five probands with the Brugada syndrome were screened for ion channel gene mutations using single-strand conformation polymorphism electrophoresis and direct sequencing. A missense mutation (R99H) in KCNE3 (MiRP2) was detected in 1 proband. The R99H mutation was found 4/4 phenotype-positive and 0/3 phenotype-negative family members. Chinese hamster ovary-K1 cells were cotransfected using wild-type (WT) or mutant KCNE3 and either WT KCND3 or KCNQ1. Whole-cell patch clamp studies were performed after 48 hours. Interactions between Kv4.3 and KCNE3 were analyzed in coimmunoprecipitation experiments in human atrial samples. Cotransfection of R99H-KCNE3 with KCNQ1 produced no alteration in tail current magnitude or kinetics. However, cotransfection of R99H-KCNE3 with KCND3 resulted in a significant increase in the I\textsubscript{to} intensity compared with WT KCNE3+KCND3. Using tissues isolated from the left atrial appendages of human hearts, we also demonstrate that Kv4.3 and KCNE3 can be coimmunoprecipitated.

**Conclusions**—These results provide definitive evidence for a functional role of KCNE3 in the modulation of I\textsubscript{to} in the human heart and suggest that mutations in KCNE3 can underlie the development of the Brugada syndrome. (Circ Arrhythmia Electrophysiol. 2008;1:209-218.)

**Key Words:** genetics ■ sudden cardiac death ■ potassium channels ■ channelopathy ■ electrophysiology

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The Brugada syndrome (BrS) is an inherited syndrome characterized by an ST-segment elevation in the right precordial leads (V\textsubscript{1}–V\textsubscript{3}) and a high incidence of sudden cardiac arrest.\(^1\) This disorder has been linked to mutations in 4 different genes, leading to a loss of function in sodium and calcium channel activity. Mutations in SCN5A, the \(\alpha\)-subunit of the sodium channel, were the first to be associated with BrS.\(^2\) Weiss et al.\(^3\) described a second locus on chromosome 3, close to but distinct from SCN5A, linked to the syndrome in a large pedigree in which the syndrome is associated with progressive conduction disease, a low sensitivity to procainamide, and a relatively good prognosis. The gene was recently identified in a preliminary report as the glycerol-3-phosphate dehydrogenase 1-like gene, and the mutation in glycerol-3-phosphate dehydrogenase 1-like gene was shown to result in a reduction of I\textsubscript{to}.\(^4\) The third and fourth genes associated with BrS were recently identified and shown to encode the \(\alpha\)-subunit (CACNA1C) and \(\beta\)-subunit (CACNB2b) of the L-type cardiac calcium channel.\(^5\) Mutations in the \(\alpha\)- and \(\beta\)-subunits of the calcium channel were also found to lead to a shorter than normal QT interval, in some cases creating a new clinical entity consisting of a combined BrS or short-QT syndrome.\(^5\)

**Clinical Perspective see p 218**

Loss of function mutations of sodium and calcium channels associated with BrS are attributable to 1 of 3 principal mechanisms: (1) truncation of the ion channel protein yielding a nonfunctional channel; (2) alteration in channel gating, such as changes in activation, inactivation, or reactivation kinetics; or (3) altered trafficking of the channels from the
endoplasmic reticulum–Golgi complex to the plasma membrane.5,6

The typical coved-type ST-segment elevation in the ECG is often concealed but can be unmasked by sodium channel blockers and vagal influences.3 The expression of the phenotype and penetrance of the disease appear to be related to factors that alter the balance of outward and inward currents at the end of phase 1 of the epicardial ventricular action potential.8 Experimental studies suggest that the presence of a prominent transient outward current (I\textsubscript{o}) predisposes the myocardium to the development of the BrS by permitting the expression of a prominent phase 1, giving the early phase of the action potential a notched appearance.9-11 Genes that determine or modulate the expression of I\textsubscript{o} have long been considered as candidate genes for the development of BrS.8,12 Augmentation of I\textsubscript{o} via mutations that increase the magnitude or alter the kinetics of I\textsubscript{o}, so as to increase total charge, is expected to lead to the development of the BrS.

A calcium-independent I\textsubscript{o} has been identified in the myocardium of most mammalian species, including humans (for reviews, see Refs. 13 and 14), and it is well established that ventricular epicardial tissue has a more prominent I\textsubscript{o} compared with that of endocardial tissue.15-17 In the human ventricles, Kv4.3 (encoded by the KCND3 gene) is the main pore-forming \( \alpha \)-subunit of I\textsubscript{o}.18 However, currents generated by Kv4.3 channels do not recapitulate all the features of the native I\textsubscript{o}. The electrophysiological properties of Kv4.3 channels are modulated by several \( \beta \)-subunits, including KC1P2 (K\textsuperscript{+}-channel interacting protein), which increases peak current density and accelerates recovery from inactivation,19,20 and the dipeptidyl-aminopeptidase-like protein (DPP9), which has been identified in neuronal and heart tissue and can substantially accelerate inactivation.21 More recently, it has been demonstrated that KCNE3 \( \beta \)-subunits (encoded by the KCNE3 gene) can interact with Kv4.3 channels,22 an interaction that decreases the current density.23 Moreover, it has been demonstrated that the transcription factor Irx5,24 calcinurin, and NfATc325 contribute to the nonuniform distribution of Kv4 expression and, hence, I\textsubscript{o} function in the mouse ventricle.

In this study, we identified a mutation in KCNE3 in the family of a proband diagnosed with BrS. The effects of these changes in KCNE3 were studied by heterologous coexpression of KCNE3 with either Kv7.1 (KvLQT1, KCNQ1) or Kv4.3 channels in Chinese hamster ovary (CHO)-K1 cells. When the mutated KCNE3 was cotransfected with KCNQ1, no alteration in the current magnitude or kinetics consistent with the development of BrS was observed. However, cotransfection of the R99H KCNE3 mutation with KCNND3 resulted in a significant increase in the amplitude of I\textsubscript{o} compared with that of wild-type (WT) KCNE3+KCNDD3. Using tissues isolated from left atrial appendages of human hearts, we further demonstrate that Kv4.3 and KCNE3 can be coimmunoprecipitated. These results provide further evidence for a functional role of KCNE3 (MiR2) in the modulation of I\textsubscript{o} in the human heart and suggest that mutations in KCNE3 can underlie the development of BrS.

Materials and Methods

Mutation Screening

The probands screened included 77 males and 28 females, 52 of whom were symptomatic at the time of presentation. Seventy-one (51 males and 20 females) of the 105 probands displayed a spontaneous type 1 ST-segment elevation in the right precordial leads of the standard ECG. The remaining had a type 1 ST-segment elevation unmasked by raising the position of the right precordial leads by 2 intercostal spaces or with use of sodium channel blockers. Genetic screening was performed as described previously.26 Genomic DNA was extracted using QIAamp DNA Blood mini kit (QIAGEN, Germany). Polymerase chain reaction was used to amplify the DNA fragment corresponding to the single exon of the gene; reactions containing 50 ng of genomic DNA and 10 pmol of the appropriate primers were prepared and underwent polymerase chain reaction through 33 cycles at an annealing temperature of 60°C. The polymerase chain reaction product was cleaved by incubating overnight with BstEI to obtain 2 fragments of an appropriate size. Single-strand conformation polymorphism electrophoresis of the fragments was performed using GeneGel Excel 12.5/24 kits (Amersham Biosciences AB, Sweden). Aberrant conformers were directly sequenced on a 3100-Avant Genetic analyzer from Applied Biosystems (Foster City, CA) using big dye chemistry.

Cell Transfection or Mutagenesis

Human WT-KCNE3 and R99H-KCNE3 were amplified from genomic DNA with primers including the sequences of restriction enzymes: KCNE3f-NheHI-GGAAGAATCTGCTAGCGCCCATGGAG-ACATCAACATGGGACGAC, KCNE3r-BamHI-CCGCTCGAGGATCTTATAGCATAGACACACGGTCTTGT, and KCNE3r-BamHI Xhol-mut CCACCTCGAGGATCTTATAGCATAGACACACGGTCTTGT. Polymerase chain reaction products were then subcloned into pRES2-Ac GFP vector at NheI and BamHI cloning sites. CHO cells were transiently transfected with the complementary DNA, encoding either Kv7.1 (2 \( \mu \)g) and KCNE3 (1 \( \mu \)g) or Kv4.3 (1.5 \( \mu \)g) and KCNE3 (1.5 \( \mu \)g), together with the complementary DNA encoding the CD8 antibody (0.25 \( \mu \)g) by use of FuGENE6 (Roche, Basel Switzerland). Cells were grown on 35-mm culture dishes and placed in a temperature-controlled chamber for electrophysiological study (Medical Systems, Greenvalne, NY) 2 days posttransfection. Before experimental use, cells were incubated with polysyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450; Dynal, Norway). Only beaded cells or green beaded cells in case of KCNE3 were used for electrophysiological recording.

Electrophysiology

Voltage clamp recordings from transfected CHO cells were made using patch pipettes fabricated from borosilicate glass capillaries (1.5 mm OD; Fisher Scientific, Pittsburgh, PA). The pipettes were pulled using a gravity puller (Narashige, Greenvalne, NY) and filled with pipette solution of the following composition (mmol/L): 10 KCl, 125 K-aspartate, 1.0 MgCl\textsubscript{2}, 10 HEPES, 10 NaCl, 5 MgATP, and 10 EGTA, pH 7.2 (KOH). The pipette resistance ranged from 1 to 4 mol/L when filled with the internal solution. The perfusion solution contained (mmol/L): 130 NaCl, 5 KCl, 1.8 CaCl\textsubscript{2}, 1.0 MgCl\textsubscript{2}, 2.8 Na acetate, 10 HEPES, pH 7.3 with NaOH. Current signals were recorded using a MultiClamp 700A amplifier (Axon Instruments, Foster City, CA), and series resistance errors were reduced by about 60% to 70% with electronic compensation. All recordings were made at room temperature. All signals were acquired at 10 to 50 kHz (Digidata 1322, Axon Instruments, Foster City, CA) with a microcomputer running Clampfit 9 software (Axon Instruments, Foster City, CA). Membrane currents were analyzed with Clampfit 9 software.
Coimmunoprecipitation

Left atrial appendage samples were obtained from 5 patients in sinus rhythm undergoing mitral or aortic valve replacement or coronary artery bypass graft surgery. The study was approved by the Ethics Committee of the Hospital Clinico San Carlos, and each patient gave written, informed consent. Samples were homogenized with ice-cold sucrose buffer of the following composition: 0.32 mol/L sucrose, 1 mmol/L EDTA, 5 mM Tris-HCl, pH 7.4, and a mixture of protease inhibitors (10 μg/mL leupeptin, 10 μg/mL pepstatin, 1 mmol/L PMSF). For better preserving the putative interaction between Kv4.3 and KCNE3 proteins, the buffer was supplemented with 0.5 mg/mL freshly prepared 3,3′-dithiobispropionimidate (DTBP, Pierce, Rockford, Ill), a thiol-cleavable crosslinking agent.29 The homogenate was centrifuged at 14 000 revolutions per minute (rpm) for 40 minutes. The crude membrane pellet was incubated (1 hour, 4°C) in TNE (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mg/mL DTBP, and protease inhibitors) and solubilized with 1% Triton X-100. Insoluble material was removed by centrifugation at 14 000 rpm for 40 minutes, and the supernatant was used for immunoprecipitation. The solubilized membrane extract (2 mg of protein/mL) was precleared with protein A-agarose (Sigma, St. Louis, MO) for 2 hours at 4°C. After removing the beads by centrifugation at 14 000 rpm for 10 minutes at 4°C, the extract was incubated (1 hour, 4°C) with Kv4.3 antibodies (4 μg, Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, it was incubated overnight with protein A-agarose at 4°C. The samples were centrifuged at 5000 rpm for 15 minutes at 4°C, and the pellet was resuspended with a buffer of the following composition: 50 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 1 mmol/L EDTA, Triton 1%. After a subsequent centrifugation (5000 rpm, 15 minutes at 4°C), the pellet was finally resuspended in Laemmli buffer and heated at 90°C for 3 minutes. The disulfide linkage in DTBP is cleaved by reduction with 2-mercaptoethanol (10%) present in the loading buffer. Immunoprecipitated proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 12% according to Tutor et al30 with 2-mercaptoethanol (10%) present in the loading buffer. Immunoblots were incubated with the corresponding antibodies to detect each protein. The bound antibodies were detected by chemiluminescence with an ECL detection kit (Amer sham Biosciences AB, Sweden). The antibodies used were anti-KCNE3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), and the corresponding secondary antibody for Kv4.3 and KCNE3 antibodies (antigoat, 1:2500, Santa Cruz Biotechnology). Specificity of the Kv4.3 antibody was validated in rat ventricular samples by coimmunoprecipitation reactions that just include protein A without primary antibody excluded nonspecific adsorption to the beads. The selectivity of the Kv4.3 antibody was tested in experiments developed in rat ventricular myocardium (n=6), because Kv4.3 is also expressed in this tissue, and the Santa Cruz antibody (SC-10647) also identifies this protein (only 3 amino acid difference with the human isofrom). For this purpose, the specific antigenic peptide used was SC-10647-P (Santa Cruz, ratio Ag:Ab=20:1). To exclude a nonspecific crosslinking reaction between KCNE3 and Kv4.3, additional experiments were performed using rat myocardium samples in the absence of crosslinker or in the presence of bis(sulfosuccinimidyl) suberate (Pierce), a nonmembrane permeant crosslinker.

Results

An R99H missense mutation in KCNE3 (MiRP2) was detected in one of our 105 BrS probands. Figure 1A shows the pedigree of the proband’s family illustrating the phenotype-genotype relationships. The proband (II-3, arrow) was asymptomatic until age 36. While resting on a couch, he had a cardiac arrest and was resuscitated. A 12-lead ECG (Figure 1B), recorded a week later, shows a coved-type ST-segment elevation (type I) in leads V1 and V2 and a saddleback ST-segment elevation (type 2) in V3, diagnostic of BrS. Figure 1C illustrates pedigrees of the proband's family. Figure 1D shows a coved-type ST-segment elevation (type I) in leads V1 and V2 and a saddleback ST-segment elevation (type 2) in V3, diagnostic of BrS. Figure 1C illustrates pedigrees of the proband's family.
with the recent report of Bigi et al., who identified this mutation in an alteration of Kv7.1 (SCN5A) has been implicated as a cause of the BrS, we demonstrated that R99H produced a significantly lower current amplitudes recorded on return to −40 mV as a function of the membrane potential of the preceding pulse. Kv7.1 + KCNE3 R99H Current. The R99H mutation produced currents that activated significantly slower (Figure 2E) but with similar deactivation kinetics (Figure 2F). Figure 2B shows Kv7.1 + KCNE3 currents elicited by 2-s pulses from −80 to +60 mV. The current activated rapidly, reached a maximum (τ_{ac} = 29.7 ± 2.2 ms at +60 mV, n = 11; Figure 2E), and did not exhibit further increase or decrease during the application of the depolarizing pulse. Tail currents elicited on return to −40 mV were well fit by a monoeponential function (τ_{act} = 34.4 ± 4.6 ms after pulses to +60 mV, n = 8; Figure 2F).

The Table shows the clinical ECG characteristics for each of the family members. I-1, II-2, and II-4 were diagnosed as BrS, whereas in the case of III-4, the youngest member of the family, the ECG displayed an ST segment elevation in V2 and a very prominent exaggeration of the R wave (J wave) in lead aVR, consistent with the recent report of Bigi et al., who identified this change in aVR as a risk factor for development of life-threatening cardiac events in patients with BrS. Their baseline ECGs also displayed an ST-segment elevation in the right precordial leads in both. In the case of III-1, the ECG after flecainide was typical of BrS, whereas in the case of III-4, the youngest member of the family, the ECG is 0.0090448 (97.5% upper one-side confidence bound), calculated using STATA Version 10.0. (Stata Corporation, TX).

An interaction of SCN5A and KCNE3 has not been reported in the literature; however, previous studies have demonstrated that KCNE3 can interact with Kv7.1 channels to produce a current that exhibits rapid activation and decay kinetics. To determine whether the R99H mutation results in an alteration of Kv7.1 + KCNE3 current, we transiently transfected plasmids encoding the mutant KCNE3 subunit together with Kv7.1 channels. Figure 2A shows Kv7.1 + KCNE3 currents elicited by 2-s pulses from −80 to +60 mV. The current activated rapidly, reached a maximum (τ_{ac} = 29.7 ± 2.2 ms at +60 mV, n = 11; Figure 2E), and did not exhibit further increase or decrease during the application of the depolarizing pulse. Tail currents elicited on return to −40 mV were well fit by a monoeponential function (τ_{act} = 34.4 ± 4.6 ms after pulses to +60 mV, n = 8; Figure 2F). Figure 2B shows Kv7.1 + KCNE3 R99H current. The R99H mutation produced currents that activated significantly slower (Figure 2E) but with similar deactivation kinetics (Figure 2F) compared with KCNE3 WT.

Figure 2C shows the current-voltage relationships and Figure 2D the activation curves constructed by plotting tail current amplitudes recorded on return to −40 mV as a function of the membrane potential of the preceding pulse. Kv7.1 + KCNE3 R99H produced a significantly lower current density (31.4 ± 8.2 pA/pF at +60 mV, n = 8, versus 94.1 ± 19.4 pA/pF, n = 11, P < 0.01) than Kv7.1 + KCNE3 WT. The R99H mutation displayed a smaller tail current amplitude at potentials positive to 20 mV (7.3 ± 1.9 pA/pF after pulses to

<table>
<thead>
<tr>
<th>Mutation carrier</th>
<th>HR, bpm</th>
<th>PQ, ms</th>
<th>QT, ms</th>
<th>QTC, ms</th>
<th>ST elevation (V2), mV</th>
<th>ST elevation +40 (V2), mV</th>
</tr>
</thead>
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<tr>
<td>I-1</td>
<td>88</td>
<td>0.19</td>
<td>0.36</td>
<td>0.44</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>II-2</td>
<td>60</td>
<td>0.17</td>
<td>0.39</td>
<td>0.41</td>
<td>0.41</td>
<td>0.22</td>
</tr>
<tr>
<td>II-3</td>
<td>60</td>
<td>0.16</td>
<td>0.36</td>
<td>0.43</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>III-1*</td>
<td>65</td>
<td>0.22</td>
<td>0.36</td>
<td>0.43</td>
<td>0.07</td>
<td>0.16</td>
</tr>
<tr>
<td>III-2</td>
<td>69</td>
<td>0.17</td>
<td>0.41</td>
<td>0.40</td>
<td>0.03</td>
<td>0.16</td>
</tr>
<tr>
<td>III-3</td>
<td>85</td>
<td>0.13</td>
<td>0.37</td>
<td>0.42</td>
<td>0.15</td>
<td>0.07</td>
</tr>
<tr>
<td>III-4*</td>
<td>90</td>
<td>0.19</td>
<td>0.35</td>
<td>0.44</td>
<td>0.23</td>
<td>0.23</td>
</tr>
</tbody>
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*During flecainide (2 mg/kg maximum 150 mg). HR indicates heart rate; ST elevation +40, ST segment elevation measured 40 ms after termination of the QRS.

Table. Clinical ECG Characteristics

<table>
<thead>
<tr>
<th>Age at time of ECG, y</th>
<th>I-1</th>
<th>II-2</th>
<th>II-3</th>
<th>III-1*</th>
<th>III-2</th>
<th>III-3</th>
<th>III-4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>46</td>
<td>44</td>
<td>23</td>
<td>8</td>
<td>13</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

Following 22-month follow-up period, only 1 additional appropriate ICD shock was delivered.
+60 mV, n=8, versus 16.6±4.2 pA/pF, n=11, P<0.05) and shifted the midpoint of the activation curve (V1/2) to more positive potentials (110.8±24.4 mV versus 61.2±5.9 mV, n=6, P<0.05), without modifying the slope (10.5±1.9 mV). Tail currents elicited with depolarization to +20 mV, the normal plateau potential of the ventricular action potential, were not significantly reduced. It was clear that these effects of R99H could not account for the BrS phenotype of the patients.

Because a more prominent transient outward current (Ito) is thought to underlie the development of the Brugada phenotype,8,10 we considered the hypothesis that the mutated KCNE3 subunit may be interacting with Kv4.3 channels to enhance Ito and, thus, predispose to the development of BrS. We coexpressed Kv4.3 alone or together with WT-KCNE3 (Figure 3). Voltage steps from −50 to +50 mV applied to the Kv4.3 transfected cells elicited a rapidly inactivating component and a small sustained component (Figure 3A). Cotransfection of Kv4.3 and WT KCNE3 resulted in a dramatic reduction in Ito (Figure 3B and 3C) consistent with earlier observations that heterologous expression of KCNE3 can interact with Kv4.3 to reduce the magnitude of the current.23

We next examined the effect of the R99H-KCNE3 on Ito magnitude. Figure 4A and 4B show families of current traces generated by channels formed by Kv4.3+KCNE3 WT and Kv4.3+R99H-KCNE3 channels, respectively. Cotransfection with the R99H mutant resulted in a larger current (Figure 4B). Analysis of the current-voltage relationship of peak Ito+4.3 showed that the current density was significantly greater at potentials positive to −20 mV (52.6±15 pA/pF at +50 mV versus 18.3±4.6 pA/pF, n=9, P<0.01; Figure 4C).

We next assessed whether the difference in current density produced by the coexpression of WT or mutated KCNE3 with Kv4.3 was due to alterations in steady-state gating parameters. Steady-state inactivation of Ito was evaluated using a
standard prepulse-test pulse voltage clamp protocol (top inset of Figure 4). The peak current following a 500-ms prepulse was determined and plotted as a function of the prepulse voltage. The inactivation parameters were obtained by fitting a Boltzmann function to the data (Figure 4D). Coexpression of Kv4.3 with WT KCNE3 or R99H KCNE3 did not significantly modify the voltage dependence of either Kv4.3 subunits as reflected by the marked increase in time constants for 

\[ \tau \text{ (Vh) } \] 

at various potentials. 

**Figure 4.** Representative Kv4.3 currents recorded from Chinese hamster ovary-K1 cells cotransfected with either wild-type (WT) (A) or R99H (B) KCNE3. The voltage protocol used is shown in the inset on top. C, I–V relation for peak \( I_{\text{Kv4.3}} \). Average \( I_{\text{Kv4.3}} \) density is greater in the presence of R99H KCNE3. D, Steady-state Kv4.3 inactivation curves in the absence and presence of WT- and R99H-KCNE3. Continuous lines represent a Boltzmann function fit to the data. Values shown represent mean±SEM.

Because the R99H mutation produced both an increase in peak current and an acceleration of inactivation, we calculated the total charge contributing to the early phases of the action potential. The charge was calculated by integrating the first 40 ms of the current elicited by pulses positive to −10 mV. Figure 5B shows a significant increase in total charge when R99H versus WT-KCNE3 is coexpressed with Kv4.3.

Because \( I_{\text{Kv4.3}} \) magnitude can be influenced by changes in the time course of reactivation, we evaluated the effect of WT- and R99H-KCNE3 on recovery of \( I_{\text{Kv4.3}} \) from inactivation by applying twin pulses of 500-ms duration to +50 mV with a variable interpulse interval (Figure 6). Reactivation of \( I_{\text{Kv4.3}} \) at −80 mV was monoexponential with a \( \tau \) of 198.3±37.2 ms. Coexpression of WT- and R99H-KCNE3 did not modify the recovery process (\( \tau \) of 181.6±12.0 and 181.0±47.4 ms, respectively, Figure 6C).

Next, we assessed whether the combined presence of WT- and R99H-KCNE3 produces the same effects on \( I_{\text{Kv4.3}} \) as R99H-KCNE3 alone. For this purpose, cells were transfected with Kv4.3 encoding gene (1.5 μg), as well as with WT-KCNE3 and R99H-KCNE3 (0.75 μg of each one). Figure 7 summarizes the results obtained in this group of experiments demonstrating that the presence of WT-KCNE3 and R99H-KCNE3 significantly increased the peak current density similarly as R99H-KCNE3 alone did (Figure 7A). Those Kv4.3 channels coexpressed with WT and R99H-KCNE3 subunits also exhibited inactivation kinetics significantly faster than those coexpressed with WT-KCNE3 alone (\( \tau \) at +50 mV 47.9±4.6 ms, \( n=8 \), \( P<0.01 \)). However, despite the acceleration of the inactivation kinetics, the combined presence of WT and R99H-KCNE3 together with Kv4.3 subunits produced a significant increase in total charge crossing the membrane calculated as the current time integral (Figure 7B). Finally, the presence of WT and R99H-KCNE3 (Vh=−40.7±2.2 mV) did not modify the voltage dependence of either Kv4.3+WT-KCNE3 (−39.3±0.7 mV) or
Kv4.3/R99H-KCNE3 (-40.5±0.3 mV; Figure 7C) inactivation.

Although our results provide evidence in support of an important influence of KCNE3 on the kinetics and magnitude of Kv4.3 current expressed in CHO cells, it remains unclear whether the KCNE3 subunits play a functional role in modulation of native I_{Ks} in the human heart. To determine whether KCNE3 proteins associate with Kv4.3 in the human myocardium, we prepared extracts of left human atrial appendages (5 patients) and immunoprecipitated them with human anti-Kv4.3 antibodies and protein A beads, followed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to membranes. Figure 8 pictures Western blots of the immunoprecipitated proteins showing a 80 kDa Kv4.3 band detected using the anti-Kv4.3 antibody. In the same immunoprecipitated proteins, use of an anti-KCNE3 antibody identified a KCNE3 band of 30 kDa. Figure 8 also shows that both anti-Kv4.3 and anti-KCNE3 antibodies failed to detect any band in the supernatant obtained during the immunoprecipitation with Kv4.3 antibody. These results provide evidence in support of an association of subsidiary KCNE3 subunits with Kv4.3 α-subunits in the human heart. We additionally tested the specificity of the Kv4.3 antibody used. These experiments were developed in rat ventricular myocardium (n=6), because Kv4.3 is also expressed in this tissue, and the antibody also identifies this protein (only 3 amino acids of difference with the human isoform). A sample was treated with the antibody following an identical procedure used with the human samples (2 µg per 500 µg of total protein; lane 2). Another sample was exposed to the antibody after incubating it with the antigenic peptide (ratio Ag:Ab=20:1; lane 3). Another rat myocardium sample was treated only with protein A-agarose (lane 4) without adding antibody. Finally, as another negative control, untransfected CHO cells were used (lane 1). As can be observed (Figure 8B), the band was present only in lane 2, demonstrating the specificity of the Kv4.3 antibody. In another set of experiments, the coimmunoprecipitation in rat ventricular myocardium was tested. Rat samples were prepared following the same procedure used for human atrial samples. Lane 4 (Figure 8C) was obtained when using DTBP as crosslinker for better preserving the putative Kv4.3 and KCNE3 interaction (see Materials and Methods). The same results were obtained when a nonpermeant crosslinker was used (bis[sulfosuccinimidyl] suberate, lane 5) and when no crosslinker was added (lanes 2 and 3). In the latter case, the intensity of the KCNE3 bands was somewhat less, even when the total protein charged was doubled. Finally, lane 1 shows that no band was observed with untransfected CHO cells. Therefore, we also observed that association of Kv4.3 and KCNE3 is produced in the rat ventricular myocardium, even in the absence of a crosslinker, which excludes a nonspecific crosslinking reaction between KCNE3 and Kv4.3.

Discussion

To our knowledge, this is the first report of a family of BrS in which the disease phenotype is observed as a result of a mutation in a gene affecting I_{Ks}. Our genetic screen identified
the same missense mutation (R99H) in KCNE3 in all clinically affected members of the BrS family but not in ethnically matched controls. An arginine at codon 99 (R99) of KCNE3 is highly conserved among species. Our results demonstrate that KCNE3/β3-subunits can interact with both Kv7.1 and Kv4.3 channels. WT-KCNE3 subunits interact with Kv7.1 to produce an acceleration of the activation and decay of this current. Cotransfection with R99H-KCNE3 slowed activation kinetics relative to the WT and reduced current amplitude at positive potentials. However, I_{Kv7.1} tail currents were not significantly affected at the normal plateau potentials of the ventricular action potential. These results suggest that interaction of the mutant KCNE3 subunit with Kv7.1 channels is unlikely to contribute to the development of the phenotype in this BrS family. We also demonstrate that the KCNE3 subsidiary subunit interacts with Kv4.3 to produce a reduction in the magnitude and kinetics of I_{Kv4.3}. Interestingly, the gain of function caused by the mutant β-subunit demonstrated a positive dominant effect, because the increase in Kv4.3 current was comparable in the combined presence of WT and R99H KCNE3 as with R99H KCNE3 alone. This gain of function in I_{Kv4.3} is expected to predispose to the development of the BrS phenotype. Finally, using coimmunoprecipitation techniques, we demonstrate that Kv4.3 and KCNE3 coassociate in the human heart, suggesting that this interaction is important to the functional regulation of I_{Kv4.3} by this subsidiary subunit.

Figure 7. A, I-V relation for peak I_{Kv4.3} generated by channels formed by the coexpression of Kv4.3 plus wild-type (WT)-KCNE3, Kv4.3 plus R99H-KCNE3, or Kv4.3 plus WT-KCNE3 and R99H-KCNE3. B, Total charge of the current during the first 40 ms as a function of voltage. C, Steady-state Kv4.3 inactivation curves in the absence and presence of WT-, R99H-, or WT+R99H-KCNE3. Continuous lines represent a Boltzmann function fit to the data. Values shown represent mean±SEM *P<0.05 vs Kv4.3 WT KCNE3.

Figure 8. A, Coimmunoprecipitation of Kv4.3 channel complex with KCNE3 proteins isolated from human atrial tissues. The blots were probed with anti-Kv4.3 (upper) and anti-KCNE3 antibody (lower), respectively. IPP indicates immunoprecipitated proteins; SN, supernatant. B, Western blots showing Kv4.3 expression in untransfected Chinese hamster ovary (CHO) cells (lane 1), in rat ventricular myocardium (lane 2), and in rat ventricular myocardium when the sample was exposed to the antibody after incubating it with the antigenic peptide (lane 3) or when it was treated only with protein A-agarose (lane 4). C, Coimmunoprecipitation of Kv4.3 channel complex with KCNE3 proteins isolated from rat ventricular myocardium. The blots were probed with anti-Kv4.3 (upper) and anti-KCNE3 antibody (lower), respectively. Association of Kv4.3 and KCNE3 is produced in the absence of crosslinker (lanes 2 and 3), in the presence of DTBP (lane 4), or when a nonpermeant crosslinker was used (bis[sulfosuccinimidyl] suberate, lane 5). No bands were observed in the case of untransfected CHO cells (lane 1).
Contribution of R99H KCNE3 Mutation to the Electrocardiographic and Arrhythmic Manifestation of BrS

Patch clamp analysis of WT-KCNE3 interaction with Kv4.3 demonstrates that this ancillary subunit produces a substantial increase in the activation rate, as well as a faster decay of the tail current, consistent with previously published results. However, cotransfection of the R99H-KCNE3 mutation did not alter the magnitude or kinetics of delayed rectifier current ($I_{Ks}$) at potentials consistent with the action potential, indicating that alteration of this current is not responsible for the development of the BrS phenotype.

A recent study showed that coexpression of KCNE3 with Kv4.3 in Xenopus oocytes results in a reduction in $I_{Ks}$ compared with the expression of Kv4.3 alone. Coexpression of KCNE3 with Kv4.3 in our mammalian cell line caused a similar reduction in $I_{Ks}$. The R99H mutation in KCNE3 reversed this effect of the subsidiary subunit, pointing to the ability of the mutation to cause a gain of function in $I_{Ks}$. The electrocardiographic and arrhythmic manifestation of BrS are thought to be due to the amplification of intrinsic heterogeneities in the early phases of the action potential among epicardial, mid-, and endocardial cells, particularly in the right ventricle. In BrS, a decrease in inward currents, such as $I_{Na}$ or $I_{Ca}$, or an increase in one of the repolarizing currents active during phase 1 of the action potential, particularly $I_{Ks}$, can accentuate the spike-and-dome morphology of the epicardial action potential, giving rise to a down-sloping ST-segment elevation with a negative T wave, the typical BrS ECG. A further outward shift in the balance of current can lead to loss of the action potential dome, creating both a transmural and epicardial dispersion of repolarization. The transmural dispersion of repolarization gives rise to an ST-segment elevation, creating a vulnerable window across the ventricular wall, whereas the epicardial dispersion of repolarization gives rise to a phase 2 reentrant extrasystole that captures the vulnerable window to precipitate a rapid polymorphic ventricular tachycardia in the form of reentry.

$I_{Ks}$ levels play a pivotal role in the manifestation of the syndrome, and a lower intensity of the current in females is thought to protect them from the arrhythmic consequence of the inherited genetic defects responsible for BrS. Although genes that modulate $I_{Ks}$ have long been considered candidate genes for the disease, until this report, none have been uncovered. One possible explanation is that gain of function mutations in $I_{Ks}$ is likely to be lethal in utero. A minor slowing of the inactivation kinetics of the current can give rise to a dramatic increase in total charge causing marked abbreviation of the action potential throughout much of the myocardium, thus leading to contractile failure. The R99H mutation in KCNE3 leads to a very significant increase in peak current density, as well as an acceleration of inactivation kinetics, so that total charge increases only modestly.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

The Brugada syndrome (BrS) is an inherited syndrome associated with a high incidence of sudden cardiac arrest. This disorder has previously been linked to mutations in four different genes, leading to a loss of function in sodium and calcium channel activity. Mutations in *SCN5A* encoding the α-subunit of the sodium channel were first identified in 1998. After a hiatus of nearly a decade, three additional genes were identified in 2007. A mutation in glyceral-3-phosphate dehydrogenase-1-like (*GPD1L*) gene was shown to result in a reduction of *I\textsubscript{to}* and to be a rare cause of BrS. The third and fourth genes associated with BrS, *CACNA1C* and *CACNB2b*, encode the α1- and β-subunits of the L-type cardiac calcium channel. Loss of function mutations in *SCN5A* are found in 14.3% of BrS probands in our registry, and loss of function mutations in the calcium channel genes are found in 11.5% of probands. The present study identifies a fifth gene, *KCNE3*, encoding a β-subunit of the Kv4.3 channel responsible for carrying the transient outward current (*I\textsubscript{to}*). Although the presence of a prominent *I\textsubscript{to}* has long been thought to be a critical component of the mechanism responsible for BrS defects, defects in genes associated with *I\textsubscript{to}* have not been described to be associated with BrS. The present study provides definitive evidence for a functional role of *KCNE3* in the modulation of *I\textsubscript{to}* in the human heart and suggests that mutations in *KCNE3* leading to a gain of function of *I\textsubscript{to}* may underlie the development of BrS.
Functional Effects of KCNE3 Mutation and Its Role in the Development of Brugada Syndrome

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