Missense Mutations in Plakophilin-2 Cause Sodium Current Deficit and Associate With a Brugada Syndrome Phenotype

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Background—Brugada syndrome (BrS) primarily associates with the loss of sodium channel function. Previous studies showed features consistent with sodium current (\(I_{Na}\)) deficit in patients carrying desmosomal mutations, diagnosed with arrhythmogenic cardiomyopathy (or arrhythmogenic right ventricular cardiomyopathy). Experimental models showed correlation between the loss of expression of desmosomal protein plakophilin-2 (PKP2) and reduced \(I_{Na}\). We hypothesized that PKP2 variants that reduce \(I_{Na}\) could yield a BrS phenotype, even without overt structural features characteristic of arrhythmogenic right ventricular cardiomyopathy.

Methods and Results—We searched for PKP2 variants in the genomic DNA of 200 patients with a BrS diagnosis, no signs of arrhythmogenic cardiomyopathy, and no mutations in BrS-related genes SCNSA, CACNA1c, GPD1L, and MEG1. We identified 5 cases of single amino acid substitutions. Mutations were tested in HL-1–derived cells endogenously expressing Na\(_{1.5}\) but made deficient in PKP2 (PKP2-KD). Loss of PKP2 caused decreased \(I_{Na}\) and Na\(_{1.5}\) at the site of cell contact. These deficits were restored by the transfection of wild-type PKP2, but not of BrS-related PKP2 mutants. Human induced pluripotent stem cell cardiomyocytes from a patient with a PKP2 deficit showed drastically reduced \(I_{Na}\). The deficit was restored by transfection of wild type, but not BrS-related PKP2. Super-resolution microscopy in murine PKP2-deficient cardiomyocytes related \(I_{Na}\) deficiency to the reduced number of channels at the intercalated disc and increased separation of microtubules from the cell end.

Conclusions—This is the first systematic retrospective analysis of a patient group to define the coexistence of sodium channelopathy and genetic PKP2 variations. PKP2 mutations may be a molecular substrate leading to the diagnosis of BrS. (Circulation. 2014;129:1092-1103.)

Key Words: arrhythmogenic right ventricular dysplasia-cardiomyopathy ■ Brugada syndrome ■ desmosomes ■ plakophilin 2 ■ sodium channels

Mutations in the PKP2 gene, coding for the desmosomal protein plakophilin-2 (PKP2), cause the most prevalent genetic form of arrhythmogenic cardiomyopathy (AC, also known as arrhythmogenic right ventricular cardiomyopathy). Recent studies have demonstrated that PKP2 not only participates in intercellular coupling, but it also interacts, directly or indirectly, with the voltage-gated sodium channel (VGSC) complex. We have shown that siRNA-mediated loss of PKP2 expression in isolated cells affects the amplitude and kinetics of the sodium current (\(I_{Na}\)), and provided evidence that a mouse model haploinsufficient for PKP2 shows \(I_{Na}\) deficit, leading to flecainide-induced ventricular arrhythmias and sudden death. Moreover, a recent analysis of human heart samples found that the abundance of the immunoreactive signal for the cardiac alpha subunit of the sodium channel, Na\(_{1.5}\), was decreased in 65% of AC patients tested. Overall, the data support the notion that loss and impairment of Na\(_{1.5}\) function at the intercalated disc might be a component of the molecular profile of AC.
associated with mutations in PKP2. Yet, loss of function of the sodium channel has been primarily associated with the phenotype of a different inherited arrhythmia, namely Brugada syndrome (BrS). Here, we speculate that variants of PKP2 that decrease \( I_{Na} \) amplitude could yield a BrS phenotype, even in the absence of the cardiomyopathic features characterizing AC.

We sought to identify PKP2 variants in genomic DNA of patients with a clinical diagnosis of BrS and without mutations in BrS-related genes SCN5A, CACNA1C, GPD1L, and MOGI. We screened the open reading frame of PKP2 samples from 200 patients with a diagnosis of BrS and without clinical signs of AC, and we identified in 5/200 (2.5%) patients the presence of a single-nucleotide replacement leading to an amino acid substitution. We speculated that those variants could be sufficient to affect VGSC function. To characterize the electrophysiological and molecular consequences of these mutants, we developed a new HL-1–derived cardiac cell line that endogenously expresses \( Na_{1.5} \) but is deficient in PKP2 (PKP2-KD). As previously reported in both neonate and adult cardiac myocytes,\(^4\)\(^5\) the loss of PKP2 in these cells caused a decrease in the magnitude of \( I_{Na} \) and decreased abundance of \( Na_{1.5} \) at the site of cell contact. Transient transfection of wild-type (WT) PKP2 restored VGSC function and \( Na_{1.5} \) membrane localization; yet, transfection of PKP2 mutants found in patients with BrS failed to restore function and localization of \( Na_{1.5} \), even if coexpressed with the WT construct. Similarly, human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) from a patient with a PKP2 deficit showed drastically reduced \( I_{Na} \). The deficit was restored by transfection of WT, but not BrS-related PKP2. Further mechanistic insight was gained from the study of PKP2 heterozygous-null (PKP2-Hz) ventricular myocytes. With the use of super-resolution microscopy and scanning patch-clamp methods,\(^3\)\(^9\) we observed that \( I_{Na} \) deficiency was specific to the intercalated disc (ID) and resulted from reduced numbers of functional channels. We also observed increased separation between the microtubule plus end and N-cadherin–containing plaques. Overall, our data show, for the first time, that a clinical phenotype consistent with a diagnosis of BrS can associate in 2% to 3% of patients with missense variants in a desmosomal gene that, in turn, causes \( I_{Na} \) deficit in an experimental system. The possible implications of this finding to our understanding of BrS and AC as separate clinical entities are discussed.

**Methods**

Detailed methods are provided in the online-only Data Supplement.

**Study Population and Genetic Screening**

A total of 200 deidentified patients (179 males) from the Registry of the Molecular Cardiology Laboratories, Magueri Foundation, Pavia, Italy, were included in this study. Patients were selected based on a definite clinical diagnosis of BrS and the absence of mutations on SCN5A, CACNA1C. Genes GPD1L and MOG1 were subsequently screened and no mutation was found. DNA extraction, amplification, and direct sequencing of the entire open reading frame/splice junction of PKP2 followed standard techniques.

**Experiments in HL-1 Cells**

**Cell Culture and Generation of PKP2-Deficient HL-1 Cells**

HL-1 is a cell line derived from the AT-1 mouse atrial cardiomyocyte tumor lineage.\(^6\) Cell culture conditions followed those previously described.\(^10\) To generate stable PKP2-deficient cells (PKP2-KD), a lentiviral shRNA clone (ID TRCN0000123349) was packaged with the use of a TransLenti Viral Packaging System (Open Biosystem). A separate line expressing a nonsilencing Lentivector (PKP2-ϕKD) was used as a control.

**Transient Transfection of PKP2 Constructs**

PKP2-KD cells were transiently transfected with a vector containing cDNA for human PKP2 concatenated to the N-terminal of mCherry for the identification of transfected cells. The following PKP2 variants were generated: D26N, Q62K, S183N, M365V, T526A, and R635Q. A plasmid coding only for mCherry was used as control. Plasmids were introduced by using the Lipofectamine 2000 reagent (Life Technologies).

**Immunohistochemical Analysis of HL-1 Cells**

Samples were imaged by using a Leica SP5 confocal microscope. Colocalization was quantified by the Pearson colocalization coefficient, as described,\(^11\) using the Intensity Correlation Analysis Plugin in the WCIF Image J software (National Institutes of Health).

**Real-Time Polymerase Chain Reaction**

Total RNA was extracted using RNeasy Mini Kit (QIAGEN). The relative quantitation of comparative cycle threshold was used for analysis.

**Whole-Cell Patch Clamp**

Whole-cell \( I_{Na} \) recordings were conducted by using an Axon multiclamp 700B Amplifier and a pClamp system (versions 10.2, Axon Instruments, Foster City, CA); recording solutions and protocols are in the online-only Data Supplement. Human induced pluripotent stem cell cardiomyocytes AC-hiPSC-CMs were obtained from line JK#11, derived from a patient with a clinical diagnosis of AC and a homozygous c.2484C>T mutation in PKP2 causing a cystic splicing with a 7-nucleotide deletion in exon 12. Extensive characterization of the cellular/molecular phenotype in Kim et al\(^12\) and Awad et al.\(^13\) Details are in the online-only Data Supplement. We performed PKP2 rescue experiments with lentiviral constructs containing WT-PKP2 or PKP2 with a c.1904 G>A mutation (p.R635Q), tagged with green fluorescent proteins for verification, according to methods described previously.\(^12\)

**Experiments in PKP2-Hz Mice**

The PKP2-Hz murine model has been described before.\(^6\) All procedures conducted to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 58-23, revised 1996). Dissociation of single adult ventricular myocytes, recording by macropatch, and immunolocalization of relevant proteins followed standard procedures as outlined in the online-only Data Supplement.

**Super-Resolution Scanning Patch Clamp**

This method combines scanning ion conductance microscopy with cell-attached patch-clamp technology for recording of ion channels at a particular subcellular location. Detailed methods are described in Bhargava et al.\(^5\) A brief description is provided in the online-only Data Supplement.

**Super-Resolution Fluorescence Microscopy: Direct Stochastic Optical Reconstruction Microscopy**

To colocalize clusters of microtubule-plus end–binding protein (EB-1) and N-cadherin in single isolated ventricular myocytes, we used 2-color direct stochastic optical reconstruction microscopy (dSTORM). Our spatial resolution in the X–Y plane was estimated at \( \approx 20 \) nm. Detailed methods are given in Agullo-Pascual et al.\(^5\) A brief description is provided in the online-only Data Supplement.
Statistical Analysis
Each individual comparison was limited to 2 data sets (eg, WT versus mutant). Results were statistically analyzed without assuming a defined structure in the data set (nonparametric statistics). Significance was calculated by a Mann-Whitney-Wilcoxon test to assess the null hypothesis that 2 populations were the same, against the alternative hypothesis that a particular population tended to have larger values than the other. No adjustments were made for multiple comparisons because of the nature of the study. Significance was defined by \( P < 0.05 \). For all data plots involving statistics, a dot-plot format was used: open circles represent individual data points, the black circle is the median value, and lower and upper horizontal lines indicate first and third quartiles, respectively.

Results

Genetic Screening
We found 5 single amino acid substitutions on PKP2 in 5 unrelated individuals. Variant Q62K (c.185 C>A, on exon 1), was reported in patients with a diagnosis of AC, and in 3/12 602 (minor allele frequency 0.02%) alleles screened in the National Heart, Lung, and Blood Institute Exome Sequencing Project database (EVS, http://evs.gs.washington.edu/EVS/). It is defined as variant of unknown significance, because of contrasting data on potential deleterious effect: absence of cosegregation with clinical phenotype in some cohorts,\(^{16}\) concomitant presence of additional desmosomal mutations in some affected individuals,\(^{17}\) and detection in individuals without an overt clinical phenotype.\(^{18}\) Four other variants were novel. Three, S183N (c.548G>A), T526A (c.1576A>G), and R635Q (c.1904G>A) are unreported variants, absent in 200 healthy controls screened in our laboratory and 650 healthy controls reported in EVS. The fifth variant, M365V (c.1093A>G) is novel, not present in our controls and reported in 2/13 004 alleles of the EVS (minor allele frequency 0.01%).\(^{14}\) Amino acid substitution D26N (rs143004808) was found in 7 unrelated patients. This variant is present in healthy individuals and not thought to cause disease.\(^{15}\) It was therefore used as additional control in the cell expression systems.

Clinical Characteristics
All patients were evaluated at the Molecular Cardiology Program, Fondazione Maugeri, Pavia, Italy. They were diagnosed with BrS based on clinical history, diagnostic ECG pattern either spontaneous or after flecainide challenge, and the absence of structural cardiomyopathy at echocardiogram or cardiac MRI. Detailed clinical data are presented in Table I in the online-only Data Supplement. In brief, 3 patients had a

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**Figure 1.** A, Top, ECG showing coved-type ST elevation in leads V1 to V2 in patient carriers of PKP2 variants. Q62K, M365V, and T526A carriers showed flecainide-induced ECG; S183N carrier showed spontaneous ECG pattern during febrile episode. B, Left, Spontaneous coved-type ECG in carrier of variant R635Q and corresponding DNA sequence. B, Right, pedestal showing cosegregation between genotype and clinical phenotype in this family. Symbols are as follows: square, male; circle, female; solid, gene carrier with clinical symptoms; empty, asymptomatic, negative genotype; half-full left, gene carrier; and half-full right, clinical symptoms. Hx indicates history; PKP2, plakophilin-2; and SD, sudden death.
saddle-back ECG in baseline with conversion into coved-type diagnostic ECG pattern after flecainide infusion,\textsuperscript{19} whereas 2 showed an abnormal coved-type pattern at the surface ECG (Figure I and Figure I in the online-only Data Supplement). Three patients had a history of syncope at rest or after a large meal, a feature characteristic of the disease.\textsuperscript{19} One patient (mutation T526A) received an implantable cardioverter defibrillator after experiencing 2 syncopal episodes at rest; 2 months later, he experienced a cardiac arrest at rest, with evidence of ventricular fibrillation, treated by the implantable cardioverter defibrillator. His family members agreed to undergo cascade genetic screening. The asymptomatic mother did not carry the R635Q variant. His father, who had a history of syncopal episodes at rest, and his brother, asymptomatic but with suspect ECG, were found to be gene carriers. The brother underwent flecainide challenge that had positive results, confirming the genetic diagnosis. The paternal grandfather had history of syncope and died suddenly during his sleep, but DNA was not available for testing. Altogether, these data show cosegregation between clinical phenotype and the presence of variant R635Q, supporting the hypothesis of a deleterious, disease-causing effect.

The Sodium Channel Complex and PKP2 in HL-1 Cells

Assessment of the relation between PKP2 primary sequence and \( I_{\text{Na}} \) required a system to test sequence variations in PKP2 in the setting of stable, endogenous \( \text{Na}_V_{1.5} \) expression. As a line of cardiac origin,\textsuperscript{10} HL-1s present characteristics of differentiated myocytes, including the expression of \( \text{Na}_V_{1.5} \).\textsuperscript{20} As shown in Figure 2A (black symbols), voltage-clamp steps...
elicited a voltage-dependent, fast inactivating inward current, as previously reported (see Figure IIA and IIB in the online-only Data Supplement). Real-time polymerase chain reaction confirmed transcription of SCN5A. The abundance of SCN5A was 3 orders of magnitude higher than that of SCN1A, SCN3A, and SCN8A (Figure IIC in the online-only Data Supplement). Immunolocalization and Western blot experiments showed expression of Na1.5 (Figure IID and IIE in the online-only Data Supplement). Similarly, we detected the expression of PKP2, with undetectable levels of PKP1 or PKP3 by Western blot (Figure IIE and IIF in the online-only Data Supplement). Next, we established the relation between PKP2 expression and $I_{Na}$.

Generation of PKP2-Deficient HL-1s: Relation Between PKP2 Expression and $I_{Na}$

HL-1s were infected with lentivirus coding for PKP2-shRNA, or for a nonsilencing oligonucleotide. In both cases, the puromycin-resistant gene was used for selection. The corresponding cell lines were dubbed PKP2-KD and PKP2-ϕKD. Western blots showed the expected loss of PKP2 in PKP2-KD but not in PKP2-ϕKD cells (Figure IIIA in the online-only Data Supplement). Interestingly, the loss of PKP2 expression led to significant loss of average peak $I_{Na}$ density (Figure 2A), similar to that reported in neonatal and adult ventricular myocytes. No PKP2-dependent changes in the voltage dependence of steady-state inactivation or time course of recovery from inactivation were detected (see Figure IIB and IIC in the online-only Data Supplement). We observed no change in Na1.5 abundance (Figure IIA in the online-only Data Supplement), but decreased colocalization of Na1.5 with N-cadherin at the site of cell contact in PKP2-deficient cells (Figure 2B).

To confirm the relation between PKP2 expression and $I_{Na}$, PKP2-KD cells were transiently transfected with WT PKP2 (PKP2-KD+PKP2-WT). To identify transfected cells, PKP2 was concatenated in its C end with mCherry. Controls were transiently transfected with mCherry alone (PKP2-KD+mCherry). As shown in Figure 2C, peak $I_{Na}$ density in PKP2-KD+PKP2-WT cells was significantly larger than in PKP2-KD or in

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**Figure 3.** A, Peak $I_{Na}$ as function of voltage in PKP2-KD cells (blue), PKP2-KD cells transfected with wild type PKP2 (black; PKP2-KD+PKP2-WT), and cells transfected with different PKP2 variants (red). Additional $I_{Na}$ properties are displayed in Figures IV and V in the online-only Data Supplement. Data are presented as mean±standard error of the mean for display purposes only. B, Dot plots of peak $I_{Na}$ density at −30 mV measured for each mutant. Additional $I_{Na}$ properties are displayed in Figures IV and V in the online-only Data Supplement. Data are presented as mean±standard error of the mean for display purposes only. C, Pearson coefficient values showing loss of Na1.5 and N-cadherin colocalization for cells transfected with 5 PKP2 mutants, and maintained colocalization in cells transfected with WT or D26N. For B and C, *$P<0.05$; **$P<0.01$; ***$P<0.001$; @$P=0.28$; and #$P=0.48$. Each variant was compared separately against the WT group. n values are in parentheses under each column. PKP2 indicates plakophilin-2; PKP2-KD, HL-1–derived cells endogenously expressing Na1.5 but made deficient in PKP2; and PKP2-WT, PKP2 wild type.
PKP2-KD+mCherry cells (see also Figure III D and III E in the online-only Data Supplement). Thus, the exogenous expression of PKP2-WT rescued $I_{Na}$ deficiency consequent to the loss of expression of endogenous PKP2. Consistently with this observation, the extent of colocalization of Na$_{v}$1.5 and N-cadherin was rescued by transfection of the WT construct, but not by the transfection of mCherry cDNA (Figure 2D).

**BrS-Related PKP2 Variants and the Sodium Channel**

The cellular system described above allowed us to determine whether mutations in PKP2 would alter the relation between PKP2 expression and $I_{Na}$. We generated 6 constructs, all as mCherry concatenants: PKP2-D26N, PKP2-Q62K, PKP2-S183N, PKP2-M365V, PKP2-T526A, and PKP2-R635Q. $I_{Na}$ in cells transfected with PKP2 variants was compared with that in PKP2-KD cells transfected with PKP2-WT or with mCherry. For each experiment, PKP2-KD cells were split from the same flask and divided for treatment with PKP2-WT, mCherry, or the particular PKP2 mutant. $I_{Na}$ was measured from all 3 conditions on each test day to ensure consistency. For each set, data were normalized to the average value of peak $I_{Na}$ density of the PKP2-KD+PKP2-WT group. In contrast with results from PKP2-WT–transfected cells, mutants PKP2-Q62K, PKP2-S183N, PKP2-M365V, PKP2-T526A, and PKP2-R635Q failed to rescue the $I_{Na}$ deficit observed in PKP2-KD cells (Figure 3A); steady-state inactivation and recovery from inactivation were not affected either by loss of PKP2 or by expression of mutants (see Figures IV and V in the online-only Data Supplement). Yet, variant PKP2-D26N, not thought to be causative of disease, behaved as PKP2-WT (Figure 3A, bottom right; cumulative data in Figure 3B). Interestingly, a similar trend was observed for N-cadherin/Na$_{v}$1.5 colocalization. Transient transfection of PKP2-WT, or PKP2-D26N, enhanced colocalization to levels significantly higher than those for any of the 5 mutant PKP2 constructs (Figure VI in the online-only Data Supplement). Pearson coefficient values are displayed in Figure 3C. Western blot showing equivalent levels of PKP2 expression is shown in Figure VII in the online-only Data Supplement.

![Figure 4](image-url)

**Figure 4.** A, Peak $I_{Na}$ as function of voltage in PKP2-KD cells (blue; cells transfected with mCherry), PKP2-KD cells transfected with wild-type PKP2 (black), and cells transfected with PKP2-WT and a PKP2 variant (red). For coexpression, 1:1 plasmid ratio was used. For WT controls, WT plasmid was equal to the sum of WT+variant in test set. Data are presented as mean±standard error of the mean for display purposes only. B, For statistical analysis (MWW test), each variant was compared separately against group PKP2-KD+WT (n=14). P values were as follows (n values in parentheses): PKP2-KD+WT+Q62K (n=9): $P<0.01$; PKP2-KD+WT+S183N (n=7): $P<0.01$; PKP2-KD+WT+M365V (n=9): $P<0.001$; PKP2-KD+WT+T526A (n=8): $P<0.001$; PKP2-KD+WT+R635Q (n=8): $P<0.005$. PKP2-KD+WT+D26N (n=7): $P=0.12$ (NS). MWW indicates Mann-Whitney-Wilcoxon; NS, not significant; PKP2, plakophilin-2; PKP2-KD, HL-1–derived cells endogenously expressing Na$_{v}$1.5 but made deficient in PKP2; and PKP2-WT, PKP2 wild type.
Our results show $I_{Na}$ deficit when PKP2 mutations were expressed in PKP2-null background. BrS patients, on the other hand, were heterozygous for the PKP2 variant. Additional experiments evaluated $I_{Na}$ properties in HL-1s when both WT and PKP2-variant constructs were coexpressed (transfection at 1:1 ratio). As shown in Figure 4 (and Figures VIII, IX, and X in the online-only Data Supplement), $I_{Na}$ in cells coexpressing PKP2-WT and a BrS-related PKP2 variant was less than that recorded in cells expressing only the WT protein, or WT plus variant PKP2-D26N.

**AC-hiPSC-CMs Showed Reduced $I_{Na}$ Rescued by PKP2-WT but not by a PKP2-BrS Variant**

Our data show that the loss of expression, and mutations, as well, in PKP2 leads to decreased $I_{Na}$. Yet, HL-1s derive from a murine atrial myxoma and, as such, their behavior may differ from that of human cardiomyocytes. Therefore, we performed an additional $I_{Na}$ rescue experiment by using a previously characterized hiPSC-CM line from an AC patient with a homozygous loss-of-function PKP2 mutation. As shown in Figure 5, $I_{Na}$ was significantly less in cells from the AC patient (AC-hiPSC-CMs) than in cells from control (H9 human embryonic stem cell-derived cardiomyocytes). More importantly, lentiviral transfection of PKP2-WT but not of PKP2-R635Q significantly increased $I_{Na}$ density. This confirms that $I_{Na}$ depends on the expression/structural integrity of PKP2.

**Analysis of Sodium Channel Functional Expression in PKP2-Hz Cardiomyocytes**

Our data so far were obtained in cells in culture, which differ from adult cardiomyocytes in terms of structural organization/function of junctional complexes. To explore the mechanisms of PKP2-dependent $I_{Na}$ deficiency, we used adult ventricular myocytes from PKP2-Hz mice.

Based on our results, we speculated that PKP2 regulates cell surface expression of functional sodium channels at the ID. As an initial approach, we quantified the amplitude of local $I_{Na}$ by using the macropatch technique. The terms ID and M refer to recordings from the region previously occupied by the ID and from the cell midsection, respectively (diagram to the left of Figure 6A). Figure 6A shows no difference in average peak $I_{Na}$ amplitude recorded from the midsection of cells obtained from PKP2-Hz mice (red) or their control littermates (black). However, the amplitude of $I_{Na}$ measured at the ID (Figure 6B) was significantly reduced in PKP2-Hz in comparison with control (additional data in Figures XI and XII in the online-only Data Supplement). Overall, we show that PKP2 deficit causes selective reduction of $I_{Na}$ at the ID.

To discard the possibility that reduced $I_{Na}$ was consequent to decreased single-channel unitary conductance, we recorded sodium channels from a small, highly localized area at the cell end with the use of super-resolution scanning patch clamp. A topology map of the cell end is shown in Figure 6C, and the unitary measurements are shown in Figure 6D. The data show that reduced $I_{Na}$ in PKP2-deficient cells is consequent to decreased unitary conductance (in fact, a small increase was detected), but to reduced numbers of available $NaV_{1.5}$ channels, specifically at the ID.

The reduced number of available channels could be consequent to decreased open probability of membrane-inserted channels, or to reduced presence of sodium channel–forming proteins. Following on our results in HL-1s (Figure VI in the online-only Data Supplement), we assessed the extent of $NaV_{1.5}$/N-cadherin colocalization at the ID. As shown in Figure 7, $NaV_{1.5}$/N-cadherin colocalization decreased in PKP2-Hz cells in comparison with control, although the amount of total $NaV_{1.5}$ in the cell lysate was unaffected by PKP2 deficit. Altogether, we propose that decreased $I_{Na}$ amplitude in PKP2-deficient cells is consequent to decreased cell surface expression of $NaV_{1.5}$ at the ID.

**PKP2 Deficit Causes Separation of the Microtubule Plus End From N-Cadherin Plaques**

The results were consistent with a role for PKP2 in forward trafficking of $NaV_{1.5}$ (among other not mutually exclusive possibilities). Previous studies indicated that sodium channels are delivered to the cell membrane via the microtubule network, and that microtubules at the ID anchor at N-cadherin–rich sites. We therefore explored whether microtubule arrival to the ID was impaired in PKP2-Hz myocytes. Specifically, we used 2-color dSTORM to determine the distance between the plus end of microtubules (marked by immunoreactive EB-1; see, eg,) and the cell end (defined by the midline of N-cadherin clusters; see the online-only Data Supplement for methods). Figure 8 shows an image of EB-1 and N-cadherin signals acquired by conventional total internal reflectance fluorescence (Figure 8A, left) and dSTORM (Figure 8A, right). The improved resolution of dSTORM (±20 nm versus ±300 nm in total internal reflection fluorescence; see Figure 8A, bottom) allowed us to accurately measure separation distances (B). As shown in C, PKP2 deficiency led to increased average distance between the leading edge of EB-1 clusters and the N-cadherin plaque midline. These results indicate that PKP2 deficiency, directly or indirectly, affects the ability of microtubules to reach the ID, likely impairing delivery of proteins relevant for sodium channel function.

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Dot plot of $I_{Na}$ density for voltage-clamp pulse to −20 mV from a holding potential of −120 mV in WT-hESC-CMs (hESC; n=7), AC-hiPSC-CMs (AC; n=8), AC-hiPSC-CMs+PKP2-WT (AC+WT; n=8), and hiPSC-CMs+PKP2-R635Q (AC+R635Q; n=10). AC versus AC-WT, $P<0.05$; AC vs AC-R635Q, $P=0.85$ (NS). Recordings limited to 1 voltage amplitude, as acceptable recordings (tight and stable gigohm seals and reproducible current traces) were short lived. AC indicates arrhythmogenic cardiomyopathy; hESC, human embryonic stem cells; hiPSC-CMs, human induced pluripotent stem cell cardiomyocytes; PKP2, protein plakophilin-2; and WT, wild type.
Discussion

Previous studies demonstrated reduced Na\textsubscript{V}1.5 at the ID in samples from patients with desmosomal mutations.\textsuperscript{7} Experimental models have shown a correlation between the loss of PKP2 expression and reduced I\textsubscript{Na}.\textsuperscript{4–6} This article represents the first retrospective analysis of a patient group to define the coexistence of clinical sodium channelopathy (BrS) and genetic variation in PKP2. This is also the first study demonstrating that not only loss of PKP2, but also single amino acid mutations, can interfere with I\textsubscript{Na}. Our results support the notion that, in some cases, mutations in PKP2 can be part of the BrS molecular substrate.

Limitations

We focused on the detection of variants in 1 desmosomal gene (PKP2). As such, we did not examine the possibility of other desmosomal mutations. We based this choice on (1) the body of experimental evidence associating PKP2 with sodium current function,\textsuperscript{4–7} (2) the high frequency of PKP2 mutations in comparison with other desmosomal genes,\textsuperscript{1} and (c) the need for an in vitro cell system to test the structure–function relation between a specific desmosomal protein and the sodium channel. Thus, although additional studies have shown that overexpression of a desmoglein-2 mutation also leads to I\textsubscript{Na} dysfunction,\textsuperscript{26} we did not expand our study in that direction because of the likely failure to maintain and successfully transfect healthy HL-1 cells in the absence of desmosomal cadherins.

Our patients did not have mutations in either SCN5A or CACNA1C, the 2 genes most commonly associated with BrS and the subject of a standard test for clinical diagnosis.\textsuperscript{27} We also confirmed the absence of mutations on 2 additional—although rare—BrS genes coding for sodium channel interacting proteins, MOG1 and GPD1L. Although variants in other genes have been found in BrS patients, their occurrence is rare.\textsuperscript{27} As in those cases, we do not know if the PKP2 mutations reported here occur in the context of additional genetic differences. Indeed, there is a large number of known proteins (and likely, a large number of unknown ones) that can affect, directly or indirectly, I\textsubscript{Na}. Rather than extending our search to the entire genome of each patient, we focused on 1 gene while acknowledging that the PKP2 variants found represent part of the BrS molecular substrate.

Exogenous systems are often used to study ion channel structure–function. In most cases, noncardiac cells are used. Here, we chose a cardiac cell line, so that components of the VGSC complex would follow their native transcription/translation process. A great advantage of this system is that, for the first time, we were able to experimentally assess the relation between single amino acid PKP2 mutations, and VGSC function and localization in a cardiac cell. The system

Figure 6. A and B, Peak average I\textsubscript{Na} in macropatches from cell midsection (A; green circle in left inset; n=10 for each group) or from the region previously occupied by the ID (B; yellow circle in left inset; n=7 for WT and 9 for PKP2-Hz). Data are presented as mean±standard error of the mean for display purposes only. Dot plots comparing I\textsubscript{Na} density at −30 mV in are shown in Figure XII in the online-only Data Supplement; P<0.05 for ID recordings, and P=0.97 (NS) for M. C, SICM recording of the end of an adult ventricular myocyte. Notice (from bottom to top) the last striations and then a smooth, T-tubule–free region, closer to cell end. D, Single sodium channel data from either WT or PKP2-Hz cells. Methodological details in Bhargava et al.\textsuperscript{4} ID indicates intercalated disc; NS, not significant; PKP2-Hz, plakophilin-2 heterozygous null; and SICM, scanning ion conductance microscopy.
also has limitations, one of which is that, in its most direct application, it does not model heterozygosity (Figure 3). To circumvent this problem, we performed coexpression experiments (Figure 4). The similarity between results (Figure 3 versus Figure 4) may suggest a possible dominant negative effect of the mutant. However, our experimental conditions are too artificial to make such a conclusion. What we do show is that, even in the presence of WT-PKP2, an $I_{Na}$ deficit was detected.

Our results in HL-1 cells were confirmed and expanded by using the hiPSC-CM system (Figure 5). Technical complexities forced us to limit the test to 1 mutant. We chose mutation R635Q given that cosegregation data were also available (see Figure 1C). Our data show that the relation between PKP2 expression/primary sequence and $I_{Na}$ is also present in human cardiac cells.

Because of the more defined compartmentalization of ID molecules, we chose PKP2-Hz cells to assess the mechanisms by which PKP2 deficiency leads to $I_{Na}$ deficit. We recognize that the mechanisms described here may be influenced by the expression of a mutant allele. Future experiments will involve generation and characterization of a PKP2-knockin mouse model expressing one of these mutations. Overall, our results show a tight convergence across experimental models, demonstrating the importance of PKP2 in the proper function of the sodium channel complex.

**PKP2 and BrS**

BrS is an inherited channelopathy characterized by ST-segment elevation of coved morphology in right precondral leads, increased risk of ventricular tachycardia and ventricular fibrillation, and the absence of cardiac structural disease. SCN5A mutations account for ≈20% to 25% of genotype-positive subjects, and ≈4% of patients carry mutations in the CACNA1c gene. Several other genes have...
been associated with sporadic cases of BrS, but each one accounts for <2% of patients; as such, current guidelines do not advise to screen for them routinely in the general BrS population. Overall, only 25% to 30% of patients with a clinical diagnosis of BrS have a known genotype, implying that additional, still undiscovered genes may be linked to this disease.

When BrS was initially described, some investigators proposed that this condition shared features with AC, thus opening the possibility that they represent 2 poles of a common spectrum ultimately leading to increased risk of sudden death. In fact, on 1 side, some BrS patients show minor right ventricular structural abnormalities, whereas, on the other side, desmosomal mutation carriers can experience ventricular fibrillation and sudden death without overt structural disease. Our study supports the notion that 1 gene (eg, PKP2) can be an underlying factor in both ends of this spectrum. There are other cases in which the same gene is involved in >1 clinical phenotype (in part, depending on whether the mutation leads to the loss or gain of function): SCN5A mutations can give origin to different conditions ranging from long-QT syndrome, to BrS, to progressive cardiac conduction defect, to dilated cardiomyopathy; mutations on potassium channel genes can cause short- or long-QT syndromes; and several genes for sarcomeric proteins when mutated can cause either hypertrophic or dilated cardiomyopathy. This multiplicity of a clinical phenotype is here proposed for the first time for a desmosomal gene that can associate with a spectrum that includes the BrS phenotype.

**PKP2 and the Sodium Channel Complex**

Our previous data demonstrated that PKP2 ablation decreased $I_{Na}$ and elicited reentrant arrhythmias in monolayers of cardiomyocytes. We also showed that PKP2-Hz mice had reduced $I_{Na}$ that facilitated flecainide-induced arrhythmias and sudden death. These results support the role of impaired $I_{Na}$ as a mechanism affecting arrhythmia susceptibility in the concealed phase of AC. Additional data showed that the intensity of immunoreactive Na,1.5 was reduced in most heart sections obtained from AC patients. This finding, consistent with those of Gomes et al, indicate that a reduction in Na,1.5 abundance may be a component of the phenotype in subjects with AC.

The role of PKP2 in preserving $I_{Na}$ may be independent from its function as a component of the desmosome. Indeed, we speculate that some PKP2 mutations may primarily affect 1 function while not disrupting, or only minimally disrupting, the other. In fact, although AC is associated with fibrofatty replacement of ventricular muscle, our data suggest that cytoskeletal alterations affecting ion channel trafficking may precede larger-scale tissue changes and contribute to or even dominate the phenotype. We propose that specific PKP2 mutations can lead to a decreased depolarization reserve that manifests as BrS. Future experiments, with the use of whole animal models, will be necessary to better define the role of PKP2 and other mechanical junction proteins in establishing the depolarizing reserve of the mammalian heart.

Previous studies have shown that N-cadherin–containing complexes act as anchoring points for microtubules at the intercalated disc. PKP2 and N-cadherin are both components of the area composita, a mixed junction of the adult intercalated disc linked to AC. We show that PKP2 deficiency increases the distance between the microtubule plus end and the N-cadherin plaque (Figure 8), suggesting that the integrity of the area composita as a whole, rather than only N-cadherin, is relevant to microtubule anchoring. The evidence that Na,1.5 is delivered to the cell membrane through the microtubular network, and our results showing that reduced $I_{Na}$ is consequent to decreased functional channel expression, lead us to propose that PKP2 is necessary for microtubule anchoring and safe delivery of Na,1.5 to the ID. Of note, Cx43 is also necessary to preserve $I_{Na}$ amplitude, and we recently demonstrated that PKP2 and Cx43 share a subcellular domain, forming a molecular network (the connexome). Whether this domain (likely at the perinexus) constitutes an actual point of anchoring and delivery for microtubules deserves further investigation.

**Conclusions**

This article represents the first systematic retrospective analysis of a large patient population with diagnosis of BrS to define the coexistence of clinical BrS and genetic variations in PKP2. This is also the first study demonstrating that not only the absence of PKP2, but also single amino acid mutations in its sequence, can alter $I_{Na}$. We propose that PKP2 mutations provide at least part of the molecular substrate of BrS. Whether coexistence of PKP2 mutations and a positive flecainide test have value in assessing sudden death risk or progression toward cardiomyopathy is unclear. The inclusion of PKP2 as part of routine BrS genetic testing remains premature; yet, the possibility that some patients showing signs of disease may harbor PKP2 variants should be considered when the genotype is negative for other genes associated with BrS.

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**Disclosures**

None.

**References**


Clinic al Perspective

Brugada syndrome (BrS) is an inherited arrhythmogenic disease primarily associated with loss of sodium channel function. Arrhythmogenic cardiomyopathy (also called arrhythmogenic right ventricular cardiomyopathy) is mostly consequent to mutations in desmosomal proteins. Although early studies proposed that these 2 diseases shared common features, BrS and arrhythmogenic cardiomyopathy have been defined as 2 separate entities. Recent evidence, however, showed that reduced expression of plakophilin-2 (PKP2), a desmosomal protein, leads to sodium channel dysfunction. We thus proposed that some cases of BrS may associate with PKP2 mutations. Here, we report missense mutations in the gene coding for PKP2 in 5/200 patients with a diagnosis of BrS and no identified mutations in genes coding for sodium or calcium channels. Cellular and molecular analysis involving a cell expression system (HL-1 cells), and induced pluripotent stem cell cardiomyocytes from a patient with arrhythmogenic cardiomyopathy, as well, revealed decreased sodium current amplitude in cells expressing the mutations. Additional studies led us to propose that PKP2 is necessary for the delivery of sodium channels to the intercalated discs by the microtubule network. Overall, this is the first evidence that mutations in PKP2 can associate with 2 different phenotypes, BrS and arrhythmogenic cardiomyopathy, reconciling the hypothesis that they represent 2 poles of a common spectrum of manifestations. Data need confirmation in independent cohorts of patients before routine screening for PKP2 variants in BrS patients is recommended. Yet, in the presence of a negative genotype, it seems reasonable to consider the possibility that clinically affected patients may harbor PKP2 mutations.
Missense Mutations in Plakophilin-2 Cause Sodium Current Deficit and Associate With a Brugada Syndrome Phenotype

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DETAILED METHODS

Study population and genetic screening.
DNA was extracted from peripheral white blood cells through conventional techniques and amplified by PCR. The amplified fragments were analyzed by direct sequencing in both directions on the entire open reading frame/splice junction of PKP2 (NM_004572) by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation) on an Applied Biosystem 3130XL automated sequencer.

Studies in HL1 cells

Cell culture and generation of PKP2-deficient HL1 cells.
HL-1 is a cardiac muscle cell line derived from the AT-1 mouse atrial cardiomyocyte tumor lineage. Cell culture conditions followed those previously described\(^1\). To generate a stable PKP2-deficient cell line (PKP2-KD), a lenti-PKP2-shRNA clone (ID TRCN0000123349) was packaged using a TransLenti Viral Packaging System (Open Biosys). The hairpin sequence targeting the 3'-UTR of the PKP2 gene was: CCGGGCATCATTATCCAGGCTTATACTCGAGTATAAGCCTGACTGAATGATGGCTTTTTG. A separate line was generated that expressed a non-silencing Lenti vector (PKP2-ϕKD); this cell line was used as a control. Both control and PKP2-KD HL-1 cells were selected in Claycomb medium containing puromycin.

Transient transfection of PKP2 constructs.
PKP2-KD cells were transiently transfected with a pm Cherry-N1 (CLONTECH) vector containing cDNA for human PKP2 (kindly provided by Dr. Kathleen Green; Northwestern University), concatenated to the N-terminal of mCherry, for identification of the transfected cells. For the experiments where we co-expressed PKP2-WT together with a PKP2-variant (see Figure 4 of the Ms), PKP2-WT was placed in a bi-cistronic (IRES) plasmid with GFP as the second cistron. The PKP2-variant gene was concatenated with mCherry. These manipulations allowed us to a) distinguish both proteins in Western blot by their difference in mobility (molecular weight) and b) recognize cells expressing both genes as targets for patch clamp. Of note, all patch clamp results for the co-expression studies were compared against those obtained from cells co-transfected with the PKP2-WT bicistronic plasmid (expressing GFP) and a plasmid where PKP2-WT was concatenated with mCherry (as we did for the PKP2-variant genes). Plasmid ratio was always 1:1 and the plasmids expressed equivalent amounts of protein, as detected by Western blot. Mutations in the PKP2 gene were introduced using the QuikChange Site-Directed Mutagenesis strategies with PfuTurbo DNA Polymerase (Agilent Technologies). The following PKP2 variants were generated: D26N, Q62K, S183N, M365V, T526A and R635Q (one-letter code used to identify the native amino acid, its position in the human protein sequence, and the substituting residue). A plasmid coding only for mCherry was used as control. Direct sequencing confirmed the presence of all mutations. Plasmids were introduced using the Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer’s instructions. Functional and immunochemical studies were carried out 24-48 hours after transfection.

Immunochemical analysis of HL1 cells

Expression of PKP2 and its variants, as well as abundance and localization of Na\textsubscript{v}1.5 in HL1 cells was assessed by Western blot analysis as well as by conventional immunolocalization and confocal microscopy. For Western blot, cell lysates were applied
on 4-12% gradient SDS gels and transferred onto nitrocellulose membranes, subsequently probed with PKP2 (BD), Nav1.5 (Alomone Labs) and GAPDH (Fitzgerald) antibodies. Secondary antibodies used were: goat-anti rabbit IRDye 800 CM or goat anti-mouse IRDye 680RD antibodies (LI-COR). For immunolocalization, HL-1 cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min and were permeabilized in 0.1% Triton X-100 (Sigma) for 30 min. Cells were subsequently incubated in blocking buffer: 2% bovine serum albumin (Sigma), 2% glycine (Sigma), 0.2% gelatin (Sigma) and 50 mM NH₄Cl (Fluka) for 20 min, followed by an overnight incubation at 4°C with primary antibodies diluted in blocking buffer (1:100). Secondary antibodies were applied for 1 h at room temperature and samples were mounted with ProLong Gold antifade reagent (Invitrogen). Samples were imaged using a Leica SP5 confocal microscope. Antibodies used were: polyclonal rabbit anti-NaV1.5 (Alomone ASC-005), monoclonal mouse anti-N-Cadherin (BD 610920), Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG (Invitrogen). Co-localization was quantified by a Pearson’s co-localization coefficient². Pearson’s coefficient between N-Cadherin and NaV1.5 immunosignals was determined for each cell-cell contact region (as determined by N-Cadherin pattern) using the Intensity Correlation Analysis Plugin in the WCIF Image J software (NIH). All individual values acquired within one coverslip were averaged; the averaged value contributed one data point to the dot plots, and to the statistical analysis (Mann-Whitney-Wilcoxon; MWW). As such, the n values reported correspond to the number of independent coverslips analyzed, each including several tens of individual measures. A similar method was used to assess the co-localization of Nav1.5 with N-cadherin in samples obtained from PPKP2-Hz mice (a total of 319 and 288 regions of interest were analyzed for WT and PKP2+/- cells, respectively).

Real Time PCR
Total RNA was extracted using RNeasy Mini Kit (QIAGEN). The cDNA was generated by reverse transcription PCR with SuperScript VILO cDNA Synthesis Kit. Real Time PCR analysis was performed with TaqMan Gene Expression Assays (Applied Biosystems) applied on a StepOnePlus Real Time PCR System. Each gene expression was normalized to CT(threshold cycle) of GAPDH. Normalized expression was calculated as 1/[log₁₀ 2 × ΔCT x 10].

Whole-cell patch-clamp.
All whole-cell I_{Na} recordings were conducted at room temperature using an Axon multiclamp 700B Amplifier and a pClamp system (versions 10.2, Axon Instruments, Foster City, CA). Pipette resistance was maintained within the range of 1.5 to 2.5 MΩ. Recording pipettes were filled with a solution containing (in mmol/l): NaCl 5, CsF 135, EGTA 10, MgATP 5 and HEPES 5, pH 7.2 with CsOH. Cells were maintained in a solution containing (in mM): NaCl 140, CsCl 4.5, CaCl₂ 1, MgCl₂ 1, CdCl₂ 0.1, HEPES 20 and Glucose 5.5, pH 7.35 with NaOH. To determine the peak current voltage relation, 200 msec voltage pulses were applied to V_m = -90 mV to +40 mV in 5 mV voltage steps, from a holding potential of V_m = -120 mV. Interval between voltage steps was 3 sec. Current densities were determined by dividing current amplitude by the cell capacitance (C_m), as determined by application of +10 mV depolarizing test pulses. Steady state inactivation was determined by stepping V_m from -130 mV to -20 mV, followed by a 30 msec test pulse to V_m = -20mV to elicit I_{Na}. The steady state voltage-dependent inactivation curves were fitted to Boltzmann’s functions. Recovery from inactivation was studied by applying paired voltage clamp steps. Two 20-msec test pulses (S1,S2) to V_m = -20mV (holding potential = -120 mV) were separated by increasing increments of 1 msec to a maximum S1-S2 interval of 80 msec. The S1-S1 interval was kept constant at
3 sec. The time-dependent recovery from inactivation curves were fit with exponential functions.

**Experiments in human induced pluripotent stem cell-derived cardiomyocytes.**

*Generation and characterization of PKP2-deficient human iPSC-CMs.*

The homozygous PKP2-deficient human iPSC-CMs were established from an AC patient with a homozygous c.2484C>T mutation in PKP2 that causes frame-shifted C-terminals and dysfunctional Pkp2 proteins (mutant PKP2 iPSC line (JK#11)). Human embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) culture methods, lentiviral transduction, and derivation of cardiomyocytes using standard cardiogenic protocols were described previously. Cardiomyocytes derived from human ESCs or mutant PKP2 iPSCs (hESC-CMs or hiPSC-CMs) at ~50 days after differentiation (50D) were used for patch-clamp recordings after WT or BrS PKP2 rescue experiments.

**Lentiviral transfection of PKP2 constructs in human iPSC-CMs.**

Wild-type (WT) PKP2 cDNA was first cloned into pcDNA3.1 plasmid and the Xba1-EcoR1 fragment containing WT PKP2 cDNA was then sub-cloned into pCDH-CMV-MCS-EF1-GFP lentivector [System Biosciences, Inc. (SBI)]. SBI lenti packaging and concentration kits were used for lentiviral production following manufacturer’s instructions. The c.1904G>A (p.R635Q) mutation in PKP2 of pCDH-CMV-WT PKP2-EF1-GFP lentivector was introduced using the QuikChange Site-Directed Mutagenesis strategies and DNA sequencing confirmed the c.1904G>A in PKP2. PKP2-deficient hiPSCs were first differentiated to cardiomyocytes till the age of 40 days when the cell clusters were dissociated into a semi-monolayer format containing small clusters. These semi-monolayer cultures of PKP2-deficient hiPSC-CMs were then transduced with lentiviral vectors containing either WT PKP2-GFP or R635Q PKP2-GFP once per day for two consecutive days. At 7-10 days after lentiviral transduction, large hiPSC-CMs with GFP expression were chosen for I_{Na} recordings.

**Experiments in PKP2 heterozygous-null (PKP2-Hz) mice**

The generation and characterization of PKP2-Hz mice has been detailed before. Hearts for cell dissociation and for studies of tissue sections were harvested from adult (3-6 months old) mice. All procedures were in accordance with New York University guidelines for animal use and care (IACUC Protocol 101101-02 to MD approved on 10/09/2012) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 58-23, revised 1996).

**Cell dissociation**

Adult mouse ventricular myocytes were obtained by enzymatic dissociation following standard procedures. Briefly, mice were injected with 0.1 ml heparin (500 IU/ml intraperitoneally) 20 min before heart excision and anesthetized by carbon dioxide inhalation. Deep anesthesia was confirmed by lack of response to otherwise painful stimuli. Hearts were quickly removed from the chest and placed in a Langendorff column. For cell dissociation, the isolated hearts were then perfused sequentially with low calcium, and an enzyme (collagenase, Worthington) solution. Ventricles were cut into small pieces, and gently minced with a Pasteur pipette. Calcium concentration was then increased gradually to normal values. After isolation, cardiomyocytes were plated on laminin coated coverslips or dishes and left to adhere for at least 30 minutes before the start of experiments. Cardiomyocytes were used on the same day of isolation. Cells were washed once with the external recording solution and mounted on the microscope stage for recordings.
**Super-resolution scanning patch clamp**
This method combines scanning ion conductance microscopy (SICM) with cell-attached patch clamp technology for recording of ion channels at a particular subcellular location. A detailed description of this technique can be found in the original publication \(^1\). A brief description is provided below.

**Scanning ion conductance microscopy (SICM)**
SICM is a non-contact scanning probe microscopy technique based on the principle that the flow of ions through the tip of a nanopipette filled with electrolytes decreases when the pipette approaches the surface of the sample. The result is a three-dimensional topography image of live cells with resolution of up to ≤ 20 nm. All topographical images in this study were recorded using a variant of SICM called hopping probe ion conductance microscopy, implemented on a software platform that controls the ICnano sample scan system (Ionscope Ltd, UK).

**Pipette clipping**
After generating the topographical image of the cardiomyocyte surface, the pipette was moved to an area clear of cells or debris. At that coordinate, a custom-built program was used to clip the tip of the pipette against the bottom of the dish. The pipette resistance was continuously monitored and the clipping maneuver stopped once the current through the pipette reached the desired level. At that point, the pipette was repositioned to spatial coordinates that were selected based on the topography image recorded with the sharp pipette.

**Cell-attached recordings**
The repositioned, clipped pipette was lowered at the chosen subcellular location (in our case, the area of the intercalated disc, away from T-tubules or sarcomere structures) to record sodium channels in the cell-attached configuration. Pipettes were filled with a solution containing (in mmol/L): NaCl 148, NaH\(_2\)PO\(_4\) 0.4, MgCl\(_2\) 1, CdCl\(_2\) 0.2, KCl 5.4, HEPES 15, CaCl\(_2\) 1.0 and Glucose 5.5, pH 7.4 with NaOH. Cells were maintained in a solution containing in (mmol/L): 0.33 NaH\(_2\)PO\(_4\), 5 HEPES, 1.0 CaCl\(_2\) and 140 KCl, pH 7.4 with KOH, thus depolarizing the membrane potential to a value estimated to be near zero. To define the unitary sodium channel current-voltage relation, 500 ms voltage clamp pulses were applied to -80, -70, -60 and -50 mV, from a constant holding potential of -120 mV.

Single channel data were analyzed using Clampex version 10.0. All graphs and statistical analysis were performed using Origin version 8.5.

**Macropatch**
Cell-attached recordings of macroscopic sodium currents were obtained using the conventional macropatch technique \(^8\). Recordings were obtained either from the end of the cell, in the area previously occupied by the intercalated disc (ID), or from the midsection of the cell (M). In this case, the assistance of the SICM system was not necessary, given that the areas under the patch were large enough to be resolved by light microscopy. Recording conditions (hardware and software as well as recording solutions) were the same as those used for the cell-attached recording aspect of super-resolution scanning patch clamp.
Super-resolution microscopy: Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

Super-resolution imaging of isolated ventricular myocytes was done using a custom-built fluorescence microscope (Leica DMI3000) configured for total internal reflection fluorescence (TIRF) and highly inclined excitation (HILO) modes as described before\(^9\). Cells plated in 18 mm circular coverslips were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton in PBS. Blocking was done in PBS containing 2% Normal Goat Serum, 2% Glycine, 2% BSA, 0.2% Gelatin, and 50mM NH\(_4\)Cl for 30min. Primary antibodies Rabbit EB-1 (Sigma) and Mouse N-cadherin (BD) were diluted in blocking solution and incubated overnight at 4°C. Primary antibodies were then washed with PBS and secondary antibodies were incubated for 1h at room temperature. Secondary antibodies used were: Mouse Alexa Fluor 647 and Rabbit Alexa Fluor 568 (Invitrogen). Imaging conditions were achieved by addition of 200 mmol/L mercaptoethylamine and an oxygen scavenging system (0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase and 0.8% (wt/wt) glucose) to the fluorophore-containing solution, combined with appropriate laser excitation. Movies containing a minimum of 2000 frames were used to generate reconstructed super-resolved images. Analysis of protein distances was performed using ImageJ software. Images were processed with a smoothing filter, adjusted for brightness and contrast and filtered to a threshold to obtain a binary image. Only the clusters within a distance of 500 nm from the intercalated disc (detected by N-cadherin staining) were considered for analysis. Cluster detection and parameters were obtained using the ImageJ function “Analyze particles”. A line passing through the middle of N-cadherin clusters was drawn to define the estimated position previously occupied by the intercalated disc (ID). The distance, parallel to the long axis of the cell, between the edge of each EB-1 particle and the ID line was measured. When the particle crossed the ID line the distance was considered as a negative value. All individual measurements acquired within one cell were averaged; the averaged value contributed one data point to the statistical analysis (MWW). As such, the n values reported in Figure 8 correspond to the number of independent cells analyzed, each including several tens of measurements. The total number of clusters analyzed was 214 for WT and 193 for PKP2-Hz. The Origin program v.8.5 was used for data management.

Immunolocalization and confocal microscopy

Immunolocalization in murine tissue was carried out as previously described\(^{10}\). Data acquisition and analysis for assessment of protein co-localization (Pearson coefficient) followed procedures described in\(^2\).
OS-Table 1: Clinical features of patients carriers of PKP2 variants. AVNRT: Atrio-ventricular nodal reentrant tachycardia; RVOT: right ventricular outflow tract; PVC: premature ventricular contraction; ICD: implantable cardioverter-defibrillator; EPS: electrical programmed stimulation.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Age at dx</th>
<th>Gender</th>
<th>Clinical history</th>
<th>Brugada type I ECG pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q62K</td>
<td>45</td>
<td>M</td>
<td>Occasional type 2 ECG discovery after evaluation for atypical chest pain; no syncope.</td>
<td>Induced by flecainide</td>
</tr>
<tr>
<td>S183N</td>
<td>44</td>
<td>M</td>
<td>Hx of AVNRT and RVOT VT; 1 syncope at night after a large meal; Spontaneous type 1 ECG and PVCs induced by fever.</td>
<td>Spontaneous, during fever</td>
</tr>
<tr>
<td>M365V</td>
<td>35</td>
<td>M</td>
<td>Occasional type 2 ECG discovery after evaluation for atypical chest pain; no syncope.</td>
<td>Induced by flecainide</td>
</tr>
<tr>
<td>T526A</td>
<td>41</td>
<td>M</td>
<td>2 syncope at rest leading to ICD implant; 1 cardiac arrest at rest, with VF documented and treated by ICD (2 months after implant). Asymptomatic after starting quinidine treatment (1 year follow up).</td>
<td>Induced by flecainide</td>
</tr>
<tr>
<td>R635Q</td>
<td>31</td>
<td>M</td>
<td>2 syncope at rest; spontaneous type 1 ECG pattern; EPS negative; ICD implant; no shocks at follow up.</td>
<td>Spontaneous</td>
</tr>
</tbody>
</table>
Online Supplemental Figures.

**Online Figure 1:** ECG in baseline and after flecainide challenge in three patients carrier of the PKP2 variants Q62K, M365V, T526A.

**Online Figure 2:** Characterization of HL-1 cells. A: Sodium current properties: voltage dependence of steady-state inactivation. B: Sodium current properties: time course of recovery from inactivation. C: Gene expression levels of SCN5A, SCN1A, SCN3A and SCN8A in HL-1 cells were detected by real time qPCR. Each gene expression level was normalized to GAPDH CT. Scale in the ordinates, in log values. The difference between SCN5A and the other transcripts is of three orders of magnitude. For panels A, B and C, data shown are mean +/- standard error of the mean (SEM). D: Left: Immunofluorescence images showing co-localization of Na\(_{v}\)1.5 (green) and N-cadherin (red) at the site of cell-to-cell contact. Scale bar: 20 µm. E: Western blots for Na\(_{v}\)1.5, PKP1, PKP2 and PKP3 proteins in HL1-cells. F: Western blot of PKP1 in HEK cells stably expressing SCN5A (HEK/SCN5A), as well as in lysates of neonatal rat keratinocytes and rat epidermal tissue. Both keratinocytes and epidermis samples, as expected, showed the presence of PKP1. This image is shown as positive control for the PKP1 antibody used in E.

**Online Figure 3:** A: Western blots showing Na\(_{v}\)1.5 and PKP2 signals in HL-1 cells (WT), cells treated with a silencing construct for PKP2 (PKP2-KD), and treated with a non-silencing construct (PKP2-ϕKD). PKP2 signal was absent only in the PKP2-KD group, while Na\(_{v}\)1.5 was found in all 3 cell groups. B and C: voltage dependence of steady-state inactivation (B) and time course of recovery from inactivation (C) in HL1 cells. Black: wild type HL-1 cells (n=11); Red: HL-1 cells treated with silencing construct for PKP2 (PKP2-KD, n=12). Blue: cells treated with non-silencing construct for PKP2 (PKP2-ϕKD n=12). D and E: Voltage dependence of steady-state inactivation (D) and time course of recovery from inactivation (E) in three populations of HL-1 cells: red, HL-1 cells treated with silencing construct for PKP2 (PKP2-KD, n=12); blue, PKP2-KD cells transfected with cDNA coding for mCherry (PKP2-KD+mCherry n=11); black, PKP2-KD cells transfected with wild type PKP2 construct PKP2-KD+PKP2-WT n=13). Panels B to E display data as mean+/-SEM.

**Online Figure 4:** Voltage dependence of steady-state inactivation of sodium current recorded in PKP2-KD (blue), PKP2-KD+PKP2-WT (black) and PKP2-KD cells transfected with the different constructs for the 6 PKP2 variants tested (red). n values same as in Figure 3A,B of the manuscript. All panels display data as mean+/-SEM.

**Online Figure 5:** Time course of recovery from inactivation of sodium current recorded in PKP2-KD (blue), PKP2-KD+PKP2-WT (black) and PKP2-KD cells transfected with the different constructs for the 6 PKP2 variants tested (red). n values as in Figure 3A,B of the main manuscript. All panels display data as mean+/-SEM.

**Online Figure 6:** Immunofluorescence images showing co-localization of PKP2, Na\(_{v}\)1.5 and N-Cadherin in HL-1 cells. A: Na\(_{v}\)1.5 (green) and N-cadherin (pink) co-localization at site of cell-cell contact. Left: PKP2-KD+PKP2-WT cells. Middle: cells transfected with PKP2-D26N. Right: Cells transfected with PKP2-Q62K, showing loss of co-localization. B: Cells treated with the PKP2 mutants S183N, M365V, T526A and R635Q respectively, showing loss of co-localization. Scale bar is 20 µm.
Online Figure 7: Western blots showing expression of Na$_V$1.5 and PKP2 proteins in HL1 PKP2-KD cells and in PKP2-KD cells transiently transfected with PKP2-WT (PKP2-KD+PKP2-WT), with mCherry (PKP2-KD+mCherry) or with the corresponding PKP2 variants (D26N, Q62K, S183N, M365V, T526A, R635Q). Cell lysates were prepared 48 hours after transfection. PKP2 was absent in PKP2-KD+mCherry cells; cells transfected with different PKP2 constructs, including wild type PKP2, show PKP2 and Nav1.5 signal of similar intensity. GAPDH was used as a loading control.

Online Figure 8: Voltage dependence of steady-state inactivation of sodium current recorded in PKP2-KD (blue), PKP2-KD+PKP2-WT (black) and PKP2-KD cells transfected with the different constructs for the 6 PKP2 mutants tested+PKP2-WT in a 1:1 ratio (red). n values as in Figure 4 of the main manuscript. All panels display data as mean+/−SEM.

Online Figure 9: Time course of recovery from inactivation of sodium current recorded in PKP2-KD (blue), PKP2-KD+PKP2-WT (black) and PKP2-KD cells transfected with the different constructs for the 6 PKP2 mutants tested+PKP2-WT in a 1:1 ratio (red). n values as in Figure 4 of the main manuscript. All panels display data as mean+/−SEM.

Online Figure 10: Western blots showing expression of Na$_V$1.5 and PKP2 proteins in HL1 PKP2-KD cells and in PKP2-KD cells transiently transfected with PKP2-WT (PKP2-KD+PKP2-WT), with mCherry (PKP2-KD+mCherry) or with the corresponding PKP2 variants (D26N, Q62K, S183N, M365V, T526A, R635Q) and PKP2-WT 1:1. Cell lysates were prepared 48 hours after transfection. GAPDH was used as a loading control.

Online Figure 11: Sodium channel properties of channels recorded in 2 different locations of PKP2-Hz mouse cardiomyocytes (red) and WT mouse cardiomyocytes (black). On the left, voltage dependence of steady-state inactivation of sodium current and on the right time course of recovery from inactivation. Upper panel (M): mid-center of the cell. Lower panel (ID): intercalated disc. All panels display data as mean+/−SEM. Statistical analysis of ID data is shown in Online Figure 12.

Online Figure 12: Dot plots corresponding to the data presented in Figure 6 of the main manuscript (panels A and B), and in online Figure 11 (panels C and D). Panels A and B: Dot plots comparing $\lambda_{Na}$ density at -30 mV in macropatches from cell midsection (A; n=10 for each group) or from the region previously occupied by the ID (B; n=7 for WT and 9 for PKP2-Hz). p<0.05 for ID recordings, and p= 0.97 (NS) for M. Panels C and D: Dot plots for measurements of V1/2 inactivation (C) and recovery from inactivation (D) recorded from the ID region. WT vs. PKP2-Hz: p=0.001 for V1/2 inactivation; p=0.01 for recovery from inactivation.
REFERENCES


Figure S3
Figure S4

- **PKP2-KD+WT**
- **PKP2-KD+mCherry**
- **PKP2-KD+Q62K**
- **PKP2-KD+S183N**
- **PKP2-KD+M365V**
- **PKP2-KD+D26N**
- **PKP2-KD+T526A**
- **PKP2-KD+R635Q**
- **PKP2-KD+D26N**
Figure S5

Normalized $I_{Na}$ vs Time (msec) for different PKP2-KD constructs:

1. PKP2-KD+WT
2. PKP2-KD+M365V
3. PKP2-KD+Q62K
4. PKP2-KD+S183N
5. PKP2-KD+M365V
6. PKP2-KD+D26N
7. PKP2-KD+R635Q
8. PKP2-KD+T526A

Each construct is represented by a different color on the graphs.
Figure S6A

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** R635Q
** WT D26N Q62K S183N M365V T526A

A

B

C

**merge Na<sub>v</sub>1.5 N-Cadherin**
Figure S6B

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Figure S7
Figure S8

- WT+WT
- mCherry
- WT+Q62K
- WT+S183N
- WT+M365V
- WT+T526A
- WT+R635Q
- WT+D26N
Figure S9

Normalized $I_{Na}$ vs. Time (msec)

- WT+WT
- mCherry
- WT+Q62K
- WT+S183N
- WT+M365V
- WT+T526A
- WT+R635Q
- WT+D26N
Figure S10

WT PKP2 +
mcPKP2 mutants: D26N  Q62K  S183N  M365V  T526A  R635Q  WT  mcN1

Nav1.5 — 250 kD

mcPKP2 —
PKP2 —

GAPDH — 37 kD
Figure S11