14-3-3 Is a Regulator of the Cardiac Voltage-Gated Sodium Channel Nav1.5

Marie Allouis,* Françoise Le Bouffant,* Ronald Wilders, David Péroz, Jean-Jacques Schott, Jacques Noireaud, Hervé Le Marec, Jean Mérot, Denis Escande, Isabelle Baró

Abstract—The voltage-sensitive Na⁺ channel Na₁.5 plays a crucial role in generating and propagating the cardiac action potential and its dysfunction promotes cardiac arrhythmias. The channel takes part into a large molecular complex containing regulatory proteins. Thus, factors that modulate its biosynthesis, localization, activity, and/or degradation are of great interest from both a physiological and pathological standpoint. Using a yeast 2-hybrid screen, we unveiled a novel partner, 14-3-3, interacting with the Na₁.5 cytoplasmic I interdomain. The interaction was confirmed by coimmunoprecipitation of 14-3-3 and full-length Na₁.5 both in COS-7 cells expressing recombinant Na₁.5 and in mouse cardiac myocytes. Using immunocytochemistry, we also found that 14-3-3 and Na₁.5 colocalized at the intercalated discs. We tested the functional link between Na₁.5 and 14-3-3 using the whole-cell patch-clamp configuration. Coexpressing Na₁.5, the β1 subunit and 14-3-3 induced a negative shift in the inactivation curve of the Na⁺ current, a delayed recovery from inactivation, but no changes in the activation curve or in the current density. The negative shift was reversed, and the recovery from inactivation was normalized by overexpressing the Na₁.5 cytoplasmic I interdomain interacting with 14-3-3. Reversal was also obtained with the dominant negative R56,60A 14-3-3 mutant, suggesting that dimerization of 14-3-3 is needed for current regulation. Computer simulations suggest that the absence of 14-3-3 could exert proarrhythmic effects on cardiac electrical restitution properties. Based on these findings, we propose that the 14-3-3 protein is a novel component of the cardiac Na⁺ channel acting as a cofactor for the regulation of the cardiac Na⁺ current. (Circ Res. 2006;98:1538-1546.)

Key Words: Na⁺ channel ■ auxiliary subunit ■ congenital heart disease

Voltage-gated Na⁺ channels are responsible for initiation and propagation of the action potential in most electrically excitable cells. They are transmembrane glycoprotein complexes consisting of a pore-forming α subunit and accessory subunits. The α subunit has 4 homologous domains, each containing 6 transmembrane segments and an outer pore-forming loop. The 3 interdomain linkers (ID 1 to III), and the N- and C-terminal ends of the protein are cytosolic. Ten α-subunit isoforms have been cloned from different mammalian tissues. Among accessory subunits regulating voltage-gated Na⁺ channels, 4 β-subunit isoforms have been identified and shown to modulate the channel-gating properties. In the human heart, SCN5A and SCN1B encode the major voltage-sensitive Na⁺ channel α subunit Na₁.5 (also called hH1) and the β1 subunit (hB1), respectively. Recent studies have shown that Na₁.5 associates with other proteins that regulate its biosynthesis, localization, activity, and/or degradation. Given the major role of Na₁.5 in the cardiac action potential, the expression and activity of these auxiliary proteins are likely important determinants for the electrical excitability of the cardiomyocytes and cardiac conduction.

In the present study, we report and characterize the association of Na₁.5 with 14-3-3 proteins. 14-3-3 family members form a group of highly conserved 30-kDa cytosolic acidic proteins expressed in a wide range of organisms and tissues. In mammals, this family consists of 7 members encoded by separate and differentially expressed genes. 14-3-3 proteins can interact with numerous cellular proteins and are crucial for signaling, cell growth, division, adhesion, differentiation, apoptosis, and ion-channel regulation. We show that 14-3-3 and Na₁.5 colocalize at the intercalated discs in cardiomyocytes. We found that the interaction of the 2 proteins altered the cardiac Na⁺ channel function. Considering that 14-3-3 proteins are expressed in cardiac myocytes, we also show that the Na⁺ current alterations attributable to 14-3-3 absence are expected to result in the alteration of the restitution of action potential duration and conduction velocity facilitating arrhythmia occurrence.

Based on these findings, we propose that the 14-3-3 protein is a novel member of the cardiac Na⁺ channel acting as a cofactor for the regulation of the cardiac Na⁺ current.
Materials and Methods
An expanded Materials and Methods section containing details for plasmids, yeast 2-hybrid screening, coimmunoprecipitation, pull-down assays, Western blotting, immunocytochemistry, electrophysiology, and computer simulations is available in the online data supplement at http://circres.ahajournals.org.

Yeast Two-Hybrid Screening
The first interdomain fragment of human Na\(_{\text{1.5}}\) (hNa\(_{\text{1.5}}\) ID I) was used as bait. The yeast reporter strain L40, which contains the reporter gene HIS3 downstream of the binding sequence for LexA, was sequentially transformed with the pVJL10-ID I plasmid and with a mouse cDNA library, using the lithium acetate method\(^8\) and subsequently treated as previously described.\(^9\)

Coimmunoprecipitation, Pulldown, and Western Blotting
African green monkey kidney-derived COS-7 cells (ATCC), mouse heart, anti–14-3-3 mouse monoclonal antibody (Santa Cruz Biotechnology) and anti-Na\(_{\text{1.5}}\) rabbit polyclonal antibody (ASC-005, Alomone Labs) were used. GST-F11 fusion protein (F11 corresponding to residue 417 to 467 of human Na\(_{\text{1.5}}\)) has been used during pulldown experiments on mouse heart lysate.

Immunocytochemistry
Immunostaining was performed as described by Mohler et al\(^{10}\) on COS-7 cells expressing the human tagged Na\(_{\text{1.5}}\)-GFP and HA-14-3-3\(\eta\) and on freshly isolated rabbit cardiomyocytes\(^{11}\) using also anti-HA rabbit polyclonal antibody (Clontech) and Alexa Fluor 488- or 568-conjugated secondary antibodies (Molecular Probes).

Electrophysiology
Patch-clamp studies were performed on COS-7 cells transiently expressing hNa\(_{\text{1.5}}\) and h\(\beta\)_1 subunits, with or without human wild-type and/or double mutant R56,60A 14-3-3\(\eta\), using the whole-cell configuration at room temperature.

Computer Simulations
The functional role of the 14-3-3 protein in shaping the cardiac action potential was assessed by computer simulations using the human ventricular cell model by ten Tusscher et al.\(^{12}\)

Statistics
Data are presented as mean±SEM. Statistical significance of the observed effects was assessed by means of the t test or 2-way ANOVA followed by a Tukey test for multiple comparisons when needed. A value of \(P<0.05\) was considered significant.

Results

The Intracellular Interdomain ID I of Na\(_{\text{1.5}}\) Interacts With 14-3-3\(\eta\)
The yeast 2-hybrid was used to screen a mouse cDNA library with hNa\(_{\text{1.5}}\) cytoplasmic I interdomain (ID I) as bait (amino acids 417 to 711; Figure 1A). Among the 22 clones able to grow in the absence of histidine, we identified full-length 14-3-3\(\eta\) cDNA by sequencing and database searching. The specificity of the interaction was confirmed by cotransformation of the bait and 14-3-3\(\eta\) or GAL4 AD into the yeast L40 (Figure 1B). To refine the site responsible for the interaction site, we created LexA-fusion protein baits containing various fragments of Na\(_{\text{1.5}}\) ID I (Figure 2A) and tested their ability to interact with 14-3-3\(\eta\) (Figure 2B). 14-3-3\(\eta\) interacted with Na\(_{\text{1.5}}\) ID I fragment 1 (F1) and more precisely with its N terminus, ie, the 417 to 467 amino acid sequence of Na\(_{\text{1.5}}\) (F11). A 99% identity between mouse and human 14-3-3\(\eta\) protein sequences and a 100% identity between residues 417 to 467 of mouse and human Na\(_{\text{1.5}}\) was observed. We challenged the specificity of the interaction with other 14-3-3 isoforms. Using mouse \(\tau\) and \(\zeta\) isoforms of 14-3-3, we observed that these isoforms were able to induce yeast growth (Figure 3). Indeed there are 82% and 86% homology between mouse protein sequences of \(\eta\) and \(\tau\) isoforms and \(\eta\) and \(\zeta\) isoforms, respectively.

14-3-3 Interacts With Na\(_{\text{1.5}}\) in Transfected COS-7 Cells and in the Heart
To provide biochemical evidence for the interaction between full-length Na\(_{\text{1.5}}\) and 14-3-3, we immunoprecipitated the complex from IGEPA extracts of Na\(_{\text{1.5}}\)-GFP–transfected COS-7 cells or mouse heart (Figure 4A and 4B) using antibodies against GFP, 14-3-3, or Na\(_{\text{1.5}}\) proteins. When 14-3-3 immunoprecipitates were blotted and probed with the anti-GFP (COS-7 cells) or anti-Na\(_{\text{1.5}}\) antibody (mouse heart), coprecipitation of the full-length channel was revealed. Inversely, 14-3-3 was detected in the immunoprecipitated Na\(_{\text{1.5}}\) complex from mouse heart extract. The interaction of 14-3-3 and F11 of Na\(_{\text{1.5}}\) was confirmed by pulldown experiments on mouse heart lysate showing the presence of 14-3-3 among the proteins specifically bound to GST-F11 (Figure 4C and 4D).
We determined the subcellular distribution of Na$_{1.5}$ and 14-3-3 using confocal microscopy. Immunostaining revealed colocalization of Na$_{1.5}$ and 14-3-3 in the plasma membrane of transfected COS-7 cells (supplemental Figure 1). In rabbit cardiomyocytes, 14-3-3 localization was assessed by using a monoclonal antibody that detects members of the 14-3-3 protein family. Fluorescent labeling of polymerized actin with phalloidin–Texas Red stained the actin thin filaments of transfected COS-7 cells (supplemental Figure I). In rabbit cardiomyocytes, 14-3-3 localization was assessed by using a monoclonal antibody that detects members of the 14-3-3 protein family. Fluorescent labeling of polymerized actin with phalloidin–Texas Red stained the actin thin filaments of transfected COS-7 cells (supplemental Figure I). In rabbit cardiomyocytes, 14-3-3 localization was assessed by using a monoclonal antibody that detects members of the 14-3-3 protein family. Fluorescent labeling of polymerized actin with phalloidin–Texas Red stained the actin thin filaments of transfected COS-7 cells (supplemental Figure I).

14-3-3 Modulates the Na$^+$ Current by Direct Protein–Protein Interaction

To investigate the functional consequence of 14-3-3$\eta$ on the channel activity, we used the whole-cell configuration of the patch-clamp technique. The presence of overexpressed human 14-3-3$\eta$ (h14-3-3$\eta$) in COS-7 cells expressing hNa$_{1.5}$ and h$\beta$1 subunits, did not modify the Na$^+$ current density ($-96.1\pm6.6$ pA/pF; $n=29$ versus $-99.3\pm5.2$ pA/pF; $n=32$; in the absence and in the presence of exogenous 14-3-3$\eta$, respectively; current measured at $-20$ mV; Figure 6A). The voltage dependence of the Na$^+$ current activation and inactivation were also investigated. No changes were observed in the activation parameters ($V_{1/2}_{\text{act}}: -35.7\pm0.9$ mV, $n=10$, versus $-34.6\pm1.1$ mV, $n=16$; slope: $5.1\pm0.2$ mV versus $5.4\pm0.3$ mV; Figure 6B). On the other hand, the presence of exogenous 14-3-3$\eta$ shifted the inactivation curve toward more negative values ($V_{1/2}_{\text{inact}}: -79.2\pm1.2$ mV, $n=13$, to $-84.5\pm0.9$ mV, $n=21$; $P<0.001$; Figure 6B) without change in the slope ($-6.0\pm0.2$ mV versus $-6.1\pm0.1$ mV). Inactivation was not significantly accelerated nor decelerated at any potential in the presence of exogenous 14-3-3$\eta$ (not shown). Finally, recovery from inactivation was decelerated in the presence of exogenous 14-3-3$\eta$ (2-way ANOVA: $P<0.001$; Figure 6C). The time course of recovery from steady-state inactivation was quantified by the time to reach 50% and 75% recovery ($t_{1/2}$ and $t_{3/4}$, respectively). Recovery from inactivation in the presence of 14-3-3$\eta$ was slower ($t_{1/2}: 18.0\pm2.0$ ms; $t_{3/4}: 52.3\pm6.3$ ms) than in its absence ($t_{1/2}: 9.5\pm1.2$ ms; $t_{3/4}: 22.5\pm5.6$ ms; $P<0.01$). Coexpression of 14-3-3 and F1 prevented this deceleration as demonstrated by $t_{1/2}$ and $t_{3/4}$ values of 9.3±1.4 and 20.1±2.9 ms, respectively ($P<0.01$ versus +14-3-3; non-significantly different (NS) versus absence of 14-3-3).

To further evaluate the implication of protein–protein interaction in these changes, we used the F1 peptide (ie, amino acids 417 to 507 of Na$_{1.5}$) to compete with full-length Na$_{1.5}$ for association with exogenous 14-3-3$\eta$. We first tested the effects of coexpressing Na$_{1.5}$ and F1 (pIRES-F1-Na$_{1.5}$). We observed no change in the current density ($-121.8\pm21.2$ pA/pF, $n=19$, in the presence of F1) nor in the inactivation curve ($V_{1/2}_{\text{inact}}: -80.2\pm2.6$ mV; slope: $-6.2\pm0.3$ mV; $n=6$; in the presence of F1; not illustrated). This suggests that endogenous 14-3-3 does not regulate overexpressed Na$_{1.5}$.

In cells expressing 14-3-3$\eta$ and Na$_{1.5}$ (pIRES-14-3-3$\eta$-Na$_{1.5}$), F1 (pCDNA3-F1) reversed the inactivation shift ($V_{1/2}_{\text{inact}}: -79.8\pm1.2$ mV; slope: $-6.2\pm0.5$ mV; $n=8$; NS in comparison with cells expressing Na$_{1.5}$ alone; Figure 6D), produced no change in current density ($-97.7\pm22.3$ pA/pF; $n=16$; NS in comparison with cells expressing Na$_{1.5}$ alone), and prevented the recovery from inactivation deceleration (2-way ANOVA: NS in comparison with cells expressing Na$_{1.5}$ alone; Figure 6C).
14-3-3 Forms Dimers for Functional Regulation of Na1.5

14-3-3 proteins have a dimeric structure and monomeric proteins may not regulate their target. Double mutant R56,60A 14-3-3/H9257 has been shown to associate with wild-type 14-3-3/H9257 but to impair binding with ligands, resulting in a dominant negative activity (DN-14-3-3/H9257). Here, DN-14-3-3/H9257 prevented the inactivation shift induced by wild-type 14-3-3/H9257 (V1/2inact: 79.5 ± 1.5 mV; slope: -6.2 ± 0.3 mV; n=8; both NS in comparison with cells expressing Na1.5 alone; Figure 6D). This suggests the requirement of 2 intact binding sites on 14-3-3 dimer to regulate the Na+ current.

14-3-3 Affects the Cardiac Action Potential

We performed computer simulations to assess the functional role of the 14-3-3 protein in shaping the cardiac action potential. The effects of the absence of 14-3-3 on INa were implemented into a human ventricular cell model by a

**Figure 4.** Direct interaction of Na1.5 and 14-3-3. A and B, Coimmunoprecipitation of full-length Na1.5 with 14-3-3. A, COS-7 cells were cotransfected with h14-3-3-3γ and either hNa1.5-GFP or GFP coding vectors. Cell extracts were incubated with anti-GFP or anti-14-3-3 antibodies (IP). The lysate and immunoprecipitated protein complex were examined by immunoblotting with anti-GFP antibody. The immunoprecipitation conducted in the absence of antibody (con) shows the absence of nonspecific interactions with protein-G sepharose beads. B, Mouse heart cell extract was incubated with anti-Na1.5 or anti–14-3-3 antibodies (IP). The lysate and immunoprecipitated protein complex were examined by immunoblotting with anti-Na1.5 (top) or anti–14-3-3 (bottom) antibody. Control lane, The immunoprecipitation was effected with the corresponding antibody exhausted with the appropriate blocking peptide. C and D, Pulldown assay of 14-3-3 from mouse heart with GST-F11 baits. C, Immunoblotting of mouse heart lysate. A specific 27-kDa band corresponding to 14-3-3 was detected with anti–14-3-3 and anti-mouse secondary antibodies together with a nonspecific 24-kDa band detected with anti-mouse secondary antibody alone (left). D, 14-3-3 was specifically pulled down from mouse heart lysate incubated with glutathione beads coupled to GST-F11 but not GST alone.

**Figure 5.** Colocalization of Na1.5 and 14-3-3 in rabbit adult cardiomyocytes. A, Actin filament labeling with phalloidin–Texas Red. B and F, 14-3-3 localization (green). C and D, Merge of F-actin and 14-3-3 labeling. E, Na1.5 localization (red). G and H, Merge of Na1.5 and 14-3-3 indicating their colocalization at intercalated discs (yellow). Scale bars = 10 μm. No signal was observed in the absence of primary antibody (not shown).
5.3-mV shift of the $I_{\text{Na}}$ steady-state inactivation curve and a 70% increase in the rate constants governing fast and slow recovery from $I_{\text{Na}}$ inactivation (see online data supplement). To validate this approach, we performed in silico voltage clamp experiments using the same protocols as in our experiments (Figure 7A through 7C). As illustrated in Figure 7A, these alterations did not result in significant changes in current density (for example, Figure 6A). When fitting Boltzmann curves to the in silico data (Figure 7B), no differences were observed in activation parameters ($V_{1/2}\text{act}: -42.1 \text{ mV}; \text{slope}: 5.0 \text{ mV}$). In contrast, the inactivation curve was shifted toward more positive values in the absence of 14-3-3 ($V_{1/2}\text{inact}: -75.4 \text{ versus } -79.9 \text{ mV}; +4.5\text{-mV shift}$) without change in slope ($-7.1 \text{ mV}$). Figure 7B (inset) shows that this shift resulted in increased window current in the absence of 14-3-3, as also seen experimentally (Figure 6B). Finally, the rate of inactivation was not changed, whereas recovery from inactivation was accelerated in the absence of 14-3-3 (Figure 7A and 7C). As in the experiments shown in Figure 6, the time course of recovery from steady-state inactivation was quantified by the time to reach 50% and 75% recovery: $t_{1/2}$ was 19.9 ms in the presence of 14-3-3 and 11.7 ms in its absence. Similarly, $t_{3/4}$ was 39.8 ms in the presence of 14-3-3 and 23.4 ms in its absence. Thus, recovery in the model, which was designed for physiological temperature, was not significantly faster than in the experiments performed at room temperature. This model feature is based on the experimental observation that the relative amplitude of the slow components of recovery increases at close to physiological temperature.14

Figure 7D shows the effects of the absence of 14-3-3 through its action on $I_{\text{Na}}$ at a pacing interval (basic cycle length [BCL]) of 800 ms (75 bpm). Changes to the shape of the action potential were mild. The 4- to 5-mV increase in
action potential overshoot in the absence of 14-3-3 reflected the increase in peak Na⁺ current (Figure 7D, bottom) attributable to the higher availability of Na⁺ channels. This also resulted in a steeper upstroke (higher maximum upstroke velocity) in the absence of 14-3-3 (402 versus 334 V/sec in its presence; 21% increase; Figure 7F, top). At BCL = 800 ms, the higher availability of Na⁺ channels was caused by the positive shift in steady-state inactivation rather than the accelerated recovery from inactivation (Figure 7F, bottom).

Because we expected the effects of faster recovery from inactivation to be augmented at higher pacing rate, we repeated our simulations with a pacing interval of 400 ms (150 bpm; Figure 7E). At this pacing interval, the control action potential (+14-3-3, solid line) had shorter action potential duration (APD) (222 versus 255 ms at a pacing interval of 800 ms) and lower maximum upstroke velocity (271 versus 334 V/sec). The combined effects of increased Na⁺ channel availability and accelerated recovery from inactivation in the absence of 14-3-3 resulted in an enhanced Na⁺ current (Figure 7E, bottom) associated with a 6-mV increase in action potential overshoot and a 34% increase in maximum upstroke velocity (Figure 7F, top). At BCL = 400 ms, the higher availability of Na⁺ channels in the absence of 14-3-3 was not only attributable to the positive shift in steady-state inactivation but also to the accelerated recovery from inactivation (Figure 7F, bottom).

14-3-3 Changes Cardiac Electrical Restitution Properties

Changes in APD and conduction velocity (CV) restitution curves, relating APD and CV to the preceding diastolic interval, have been implicated in the susceptibility to cardiac arrhythmias. The significant increase in Na⁺ current in the absence of 14-3-3, preferentially at high pacing rate, facilitates action potential formation and conduction, and may thus affect APD and CV restitution. Therefore, we deter-
mained APD and CV restitution curves in the absence and presence of 14-3-3 using standard protocols (see online data supplement). Figure 8A shows that the APD restitution curve in the absence of 14-3-3 was steeper than under control conditions (+14-3-3). Notably, its slope exceeded 1 at short diastolic interval, whereas the slope of the control curve was <0.7. The CV restitution curve is shown in Figure 8B. In the absence of 14-3-3, conduction velocity was higher, especially at short pacing intervals. As a result, the CV restitution curve was flattened. Also, the absence of 14-3-3 allowed successful conduction at higher pacing rates and shorter diastolic intervals. The shortest pacing interval (5-ms resolution steps) resulting in successful action potential conduction was 240 ms in the absence of 14-3-3 (diastolic interval of 40 ms) and 280 ms (diastolic interval of 72 ms) in its presence.

Kagan et al. have previously reported that the 14-3-3 protein also affects the rapid delayed rectifier current (I_{Kr}), ie, the current carried by the HERG channel. We have performed additional computer simulations to assess the effects of the 14-3-3 protein through its action on I_{Kr}. These effects appeared to be relatively modest (supplemental Figures II and III).

Discussion

In the present study, a direct interaction between the cardiac voltage-dependent Na⁺ channel Na1.5 and 14-3-3 protein was identified using a 2-hybrid screen. The 14-3-3-binding region was determined in the first interdomain of Na1.5 between amino acids 417 and 467. We also showed colocalization of 14-3-3 and Na1.5 at intercalated discs of cardiomyocytes and both communoprecipitation and pulldown experiments confirmed their physical association. Functional studies using the whole-cell patch-clamp configuration brought further light to the regulation of Na⁺ channel activity by 14-3-3.

14-3-3-interacting consensus sequences have been extensively analyzed in mammalian systems (for review, see Bridges and Moorhead[20]). Two consensus phosphopeptide motifs (ie, RSPXpSXP and RXXXpSXP, where X is any amino acid and pS is phosphoserine) have been uncovered. 14-3-3 binding to its target has been shown to depend on phosphorylation of a serine or threonine. None of these motifs is present in the Na1.5 417 to 467 sequence interacting with 14-3-3. However, many target proteins do not contain sequences conforming precisely to these motifs or do not need phosphorylation to bind. Noteworthy, the protein kinase A (PKA)-dependent mechanism of β-adrenergic stimulation regulating functional expression of I_{Kr}, by phosphorylation of S525 and S528 is located in I to II linker, ie, close to the site interacting with 14-3-3. These residues could be protected by 14-3-3 from dephosphorylation, as proposed for HERG. In addition, I_{Kr} of native cardiomyocytes or recombinant I_{Kr} show a shift in channel inactivation and slower recovery from inactivation on adrenergic stimulation, as we observed with 14-3-3. However, unlike adrenergic stimulation, 14-3-3 induced neither an increase in current density nor a shift in voltage dependence of channel activation. Therefore, 14-3-3 effects on Na1.5 differ from those expected of a protection of PKA sites from phosphatase, as shown for HERG.

Our results suggest that 14-3-3 dimers regulate Na1.5 channels. If so, 14-3-3 may contribute to the Na⁺ channel clustering at the membrane as for the H⁺-ATPase at the plant plasma membrane. The number of 14-3-3 targets and its effects are plethoric. Various interactions with ion transporters or channels have been reported in animals and plants. Among cardiac channels, the α subunit of the ATP-dependent K⁺ channel, Kir6.2, and the voltage-dependent K⁺ channel HERG are regulated by 14-3-3. Dimeric 14-3-3 binding on the Kir6.2 C terminus containing the RKR motif, known as an endoplasmic reticulum localization signal, prevented channel retention. On the other hand, Kagan et al. observed that 2 interaction sites exist on the HERG channel and that the association requires phosphorylation of the channel by PKA. In heterologous systems, PKA-dependent phosphorylation of HERG leads to a decrease in current amplitudes, whereas coexpression and dimerization of 14-3-3 increases and accelerates the current activation. According to our results, no RKR retention signal is detected in the 14-3-3-binding site in Na1.5 ID I. In addition, the Na⁺ channel trafficking to the cell membrane was not impacted by 14-3-3 expression.
because the current amplitude was not affected by the presence of 14-3-3.

Na1,5 Na+ channel is crucial for coordinating cardiac muscle contraction and critical for the vulnerability of the heart to abnormal rhythm. Alterations in Na+ channel expression and function are known to have severe effects on cardiac excitability and conduction. The SCN5A gene encoding Na1,5 is mutated in 4 different forms of congenital disorders: long QT3 syndrome, Brugada syndrome, progressive cardiac conduction disorder (Lenègre-Lev disease), and sick sinus syndrome.31–34 Our computer simulations demonstrate that APD restitution is steepened in the absence of 14-3-3, with a slope >1 at short diastolic interval. Such steeply sloped APD restitution curve may have strong proarrhythmic effects.15–19 We also observed that propagating action potentials could be elicited at shorter pacing intervals and diastolic intervals in the absence of 14-3-3, thus further enhancing the susceptibility to arrhythmias. The effects of increased conduction velocity and flattening of the CV restitution curve in the absence of 14-3-3 are less clear cut. Some studies suggest that these effects are proarrhythmic,15,18 whereas others suggest that they are antiarrhythmic.16,17 Cherry and Fenton have recently shown that CV restitution can have both proarrhythmic and antiarrhythmic effects.19 One can suspect that mutations of a Na1,5 cofactor may induce cardiac disorders. In the same line, mutations in KCNE1, a regulator of KCNQ1, a voltage-dependent K+ channel, or mutations of ankyrin B, which interferes with Ca2+ homeostasis proteins, have been implicated in inherited cardiac arrhythmias.35,36 Using in silico models, we have shown that the absence of 14-3-3 could result in proarrhythmic changes in cardiac electrical restitution properties. Our computation data suggest that loss-of-function mutations in 14-3-3 could result in cardiac arrhythmias. Mice expressing 14-3-3Y dominant negative double mutant under the control of the α-myosin heavy chain promoter have been generated.37 As in NIH 3T3 cells transfected with DN-14-3-3, the activity of JNK1 and p38 mitogen-activated protein kinase is enhanced in cardiomyocytes.38,39 We have observed that 14-3-3 interact with the cardiac Na+ channel. However, further studies in cardiac myocytes are needed to assess whether the different 14-3-3 isoforms could functionally replace each other to regulate Na1,5.

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Disclosures
None.

References


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MATERIALS AND METHODS

Plasmids – To proceed to the two-hybrid screen, five baits corresponding to various cytosolic Na\textsubscript{v}1.5 fragment cDNAs were constructed. They are all localized in the sequence coding for ID I fragment of human Na\textsubscript{v}1.5 (see Supplemental Table 1). The constructions were made using the PCR products obtained from SP64T Na\textsubscript{v}1.5 (1), EcoR1-Sal1 digested and then inserted into pVJL10 to generate the five baits in frame with lexA-binding domain (lexA BD). A cDNA library from mouse BALB/c poly(A)+ RNA was fused to the GAL4 activation domain (GAL4 AD), by insertion into pGAD1318 (2) using the Stratagene cDNA synthesis kit. pGAD-14-3-3\eta, \tau and \zeta plasmids were constructed as previously described (3).

pCI 14-3-3\eta was obtained by inserting pGAD-14-3-3\eta EcoR1/Xho1 digested into ECOR1/Sal1 pCI site.

pcDNA3.1 plasmids coding for human wild-type 14-3-3\eta and R56,60A double mutant (pcDNA3-14-3-3\eta and pcDNA3-DN-14-3-3\eta, respectively) were a generous gift from Dr Andrey Shaw (Washington University, St Louis, MO). pcDNA3-F1 was constructed by insertion of F1 PCR product (HA-tagged, see primers in Supplemental Table 2) Spe1-Sal1 digested into Nhe-Xho1 pcDNA3 multicloning site.
For pulldown assays, a PCR amplified cDNA fragment encoding the F11 Na\textsubscript{v} 1.5 region was cloned in frame with Glutathione-S-Transferase (GST) into pGEX-3X vector (Amersham Biosciences) using BamH1 and EcoRI sites.

For pIRES plasmids, HA tag sequence containing PCR products of full length human 14-3-3\texteta and of Na\textsubscript{v}1.5 F1 fragment cDNAs (see Supplemental Tables 1 and 2) were digested with Spe1/Xho1 and inserted into Nhe1/Xho1 site of the first pIRES multicloning site (MCSA; Clontech). C-terminus GFP-tagged Na\textsubscript{v}1.5 (Na\textsubscript{v}1.5-GFP) digested with Pme1/Not1 from the plasmid containing the GFP-tagged human Na\textsubscript{v}1.5 cDNA (pCI Na\textsubscript{v}1.5-GFP, 4) was inserted into Sma1/Not1 site of the second pIRES multicloning site (MCSB; pIRES-14-3-3-Na\textsubscript{v}1.5 and pIRES-F1-Na\textsubscript{v}1.5, respectively).

EcoR1 digested R56,60A-14-3-3\texteta was introduced into pIRES MCSA, and Na\textsubscript{v}1.5-GFP into the same vector MCSB as described above (pIRES-DN-14-3-3\texteta-Na\textsubscript{v}1.5). For control, Nhe1/Xho1 EGFP sequence digested from pEGFP-C1 plasmid (Clontech), was inserted into Nhe1/Xho1 site of MCSA, and Na\textsubscript{v}1.5-GFP into MCSB (pIRES-GFP-Na\textsubscript{v}1.5).

All constructs were checked by direct sequencing.

Yeast two-hybrid screening – The yeast reporter strain L40, which contains the reporter gene HIS3 downstream of the binding sequence for LexA, was sequentially transformed with the pVJL10-ID I plasmid and with a mouse cDNA library, using the lithium acetate method (5) and subsequently treated as previously described (6). Cells were plated on synthetic medium lacking histidine, leucine and tryptophan; the plates were incubated at 30°C for 2-6 days. His+ colonies were patched onto selective plates. Plasmid DNA was prepared from colonies displaying a His+
phenotype and used to transform E. coli DH5α. Clones were screened by PCR with pGAD1318 specific primers and plasmids from positive cells were tested for specificity by co-transformation into L40 with pVJL10 ID I-LexA BD-hybrid (Supplemental Table 1) or pVJL10-LexA BD (control; 3). Sequence identification was performed using the Basic Local Alignment Search Tool (BLAST) provided by NCBI Entrez protein site.

**Co-immunoprecipitation, pulldown and Western blotting** – The African green monkey kidney derived COS-7 cells (obtained from ATCC), were transiently co-transfected with pCI Naᵥ1.5-GFP and pCI 14-3-3η expression vectors using BGTC-DOPE (7). For each experimental condition, confluent cells from two 30 mm dishes were harvested after 2 days and incubated in 500 µL ice-cold lysis buffer (in mmol/L: 150 NaCl, 1% IGEPAL, 50 tris-HCl, pH 8.0; all from Sigma) with 1 minitab of protease inhibitors (Roche) and 1 mmol/L PMSF (phenylmethylsulfonyl fluoride, Sigma) for 1 h at 4°C. The same procedure was used for mouse hearts except that tissues were homogenized in lysis buffer on ice in a glass potter. The insoluble fraction was pelleted by centrifugation at 20,000 x g for 30 minutes at 4°C. The supernatant was incubated with 1 µg anti-14-3-3 mouse monoclonal antibody (Santa Cruz Biotechnology) or anti-Naᵥ1.5 rabbit polyclonal antibody raised against a peptide corresponding to residues 493-511 of rat Naᵥ1.5 (ASC-005, Alomone Labs) for 16 h at 4°C. For the control, 1 µg anti-14-3-3 antibody was pre-adsorbed with 10 µg of the blocking peptide (Santa Cruz Biotechnology) during one hour before addition to the supernatant. Fifty µL of protein-G sepharose beads (Amersham Biosciences) were added and left for 1 h with constant rotation at 4°C. Beads were centrifuged and washed three times with lysis buffer. The resin was suspended in gel loading buffer,
heated for 5 min at 95°C, and subjected to SDS/PAGE. The precipitated proteins were detected by immunoblotting using ECL detection (Amersham Bioscience).

For pulldown assays, Glutathione-S-Transferase-F11 fusion protein (GST-F11) and GST were produced in BL21 bacteria treated for 1 h with 1 mM isopropyl-β-D-1-thiogalactopyranoside. Following induction, the bacterial cultures were harvested, the bacterial pellets washed with ice-cold phosphate-buffered saline (PBS) (in mmol/L: 135 NaCl, 5 Na₂HPO₄, 0.8 NaH₂PO₄, 4.1 KCl, pH 7.3) and sonicated on ice for 6 minutes in lysis buffer (PBS, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride and antiprotease Complete (Roche). The suspension was cleared by two centrifugations at 2,600 x g and 5,400 x g for 15 min. GST and GST-F11 were purified using Glutathione Sepharose 4 Fast Flow™ (Amersham Biosciences) and then covalently bound to the resin with disuccinimidyl suberate (DSS) (Pierce). The purity and amount of bound GST and GST-F11 was then determined by SDS-PAGE and Coomassie staining. 700 mg of mouse heart were homogenized on ice with a glass-Teflon homogenizer in 3.5 mL of cold lysis buffer (TL) (in mmol/L: 150 NaCl, 50 tris-HCl, pH 7.4 with 1 mmol/L PMSF and antiprotease Complete, Roche), and centrifuged at 5,400 x g for 10 min. The supernatant was then centrifuged at 21,000 x g for 30 min and proteins were quantified with a Nanodrop® spectrophotometer.

Pulldowns were performed by incubating heart protein lysates (6 mg) with GST or GST-F11 (80 µg) coupled to glutathione beads overnight at 4°C. The beads were then washed twice with TL buffer. The bound proteins were released by boiling in Laemmli buffer and then separated by SDS/PAGE. After transfer onto HybondTM-C Extra nitrocellulose membranes (Amersham Bioscience), the blots were probed with anti-14-3-3 mouse monoclonal antibody (1/2,000) overnight at 4°C. 14-3-3 proteins
were visualized using anti-mouse peroxidase-coupled secondary antibody (1/50,000 Santa Cruz Biotechnology) and Western Lightning® detection kit (PerkinElmer).

**Immunocytochemistry** – Thirty six to 48 h after transfection, COS-7 cells grown on glass coverslips were rinsed in PBS buffer (in mmol/L: 4 KH\(_2\)PO\(_4\), 16 Na\(_2\)HPO\(_4\), 115 NaCl) and fixed in freshly prepared 3.7% formaldehyde in PBS. Cells were rinsed in PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min. After 30 min incubation in PBS containing 1% BSA, cells were incubated overnight at 4°C with 0.5 µg anti-HA rabbit polyclonal antibody (Clontech). Following incubation, cells were rinsed in PBS and stained for 1 h at room temperature with a 1/5,000 dilution of Alexa A568-conjugated anti-rabbit antibody (Molecular Probes). Coverslips were rinsed with PBS and mounted with Vectashield immunofluorescence medium. Observations were made with a Leica TCS-SP1 confocal scanning laser microscope.

Cardiomyocyte immunostaining was performed as described by Mohler *et al.* (8). Freshly isolated rabbit cardiomyocytes (9) were fixed in ethanol for 10 min. Cells were rinsed with PBS (pH 7.4) and incubated during 3 h in blocking buffer (PBS containing 0.025% Triton X-100/ 3% gelatin/ 0.001% sodium azide). Cells were incubated overnight in the presence of primary antibodies with 5 µg anti-Na\(_v\)1.5 (Alomone Labs) and 1 µg anti-14-3-3 (Santa Cruz Biotechnology) or 1 unit Phalloïdin-Texas-Red in 1 mL blocking buffer at room temperature. After washes in blocking buffer, cells were stained with fluorescent secondary antibodies (Alexa Fluor 488 and 568) for 1 h at room temperature in blocking buffer. For the control, incubation of the cells was done with the secondary antibodies only. After washes cells were mounted and observed as described above.
Electrophysiology – For patch-clamp experiments, COS-7 cells were transfected with the plasmids [2 µg per 35-mm diameter Petri dish: 30% pIRES Na\textsubscript{v}1.5 containing plasmid; 20% of pcDNA3 plasmid when needed (see results for details), 20% pRC h\textbeta{} 1 (4) and completed with pEGFP; Clontech] complexed with JetPEI (Polyplus-tranfection). Eight to 24 h post-transfection, cells were isolated and plated at low density on glass coverslips. Twenty-four to 72 h post-transfection, Na\textsuperscript{+} currents were recorded at room temperature using an Axopatch 200A amplifier (Axon Instruments, Inc.). The cells were continuously superfused with Tyrode solution containing (in mmol/L): NaCl 145, KCl 4, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1, HEPES 5, glucose 5, pH adjusted to 7.4 with NaOH. Wax-coated patch pipettes (tip resistance: 1.2 to 3.5 M\textOmega{}, Kimble) were filled with intracellular medium containing (in mmol/L): NaCl 10, CsCl 64.5, aspartic acid 70.5, HEPES 5, pH adjusted to 7.2 with CsOH. During data recording, the studied cell was locally superfused with extracellular medium containing (in mmol/L): NaCl 145, CsCl 4, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 1, HEPES 5, glucose 5, pH adjusted to 7.4 with NaOH. All products were purchased from Sigma. Junction potential, capacitance and series resistance were electronically compensated. Stimulation, data recording through an A/D converter (Tecmar TM100 Labmaster, Scientific Solutions; 5 kHz filtering, 25 kHz sampling) and analysis were performed with Acquis1 software (Bio-Logic).

All current measurements were normalized using the cell capacitance. Peak inward current density was measured during a depolarizing test pulse to -20 mV (20-ms prepulse to -120 mV; frequency: 0.5 Hz).
Computer simulations – The functional role of the 14-3-3 protein in shaping the cardiac action potential was assessed by computer simulations using the human ventricular cell model by ten Tusscher, Noble, Noble and Panfilov (10). This model ('TNNP model') is largely based on recent experimental data on individual ionic currents, including the fast sodium current (I_{Na}), i.e. the current carried by the Na_v1.5 channel, and the rapid delayed rectifier current (I_{Kr}), i.e. the current carried by the HERG channel. Of note, the restitution curves (see below) of the TNNP model closely match experimentally obtained restitution curves (10).

The effects of the absence of the 14-3-3 protein on I_{Na} were implemented by a +5.3 mV shift of the I_{Na} steady-state inactivation curve and a 70% increase in the model rate constants governing fast and slow recovery from I_{Na} inactivation (\( \alpha_h \) and \( \alpha_j \), respectively; 10), in accordance with our patch-clamp observations. The effects of the absence of the 14-3-3 protein on I_{Kr} were implemented by a +11 mV shift of the I_{Kr} steady-state activation curve and a 50% decrease in the rate constant governing I_{Kr} activation (\( \alpha_{vr1} \); 10), based on experimental observations in HERG-expressing Chinese hamster ovary (CHO) cells by Kagan et al. (11). Action potentials were elicited by repetitive stimulation with a 1-ms, 6-nA (=25% suprathreshold) stimulus current. The model cell was paced for 4 min in order to reach steady-state behavior.

Restitution of action potential duration (APD restitution) was determined using a standard S1-S2 stimulus protocol (12; 10). Following a period of pacing at a basic S1-S1 pacing interval of 1,000 ms, S2 was applied after a variable S1-S2 interval. The strengths of the 1-ms S1 and S2 stimuli were fixed at two times threshold, and APD was defined using a threshold voltage of -74.2 mV, which is near the voltage at which the action potential is 90% repolarized. The APD restitution curve was then
constructed by plotting APD of the S2 action potential against the preceding diastolic interval.

Restitution of conduction velocity (CV restitution) was determined by probing action potential propagation in a linear strand of 60 longitudinally coupled TNNP model cells. Entire cell length was used as the spatial discretization element with elements connected by the lumped gap junctional resistance (calculated from the selected gap junctional conductance) and myoplasmic resistance (calculated from the model cell dimensions and the myoplasmic resistivity of 150 $\Omega\cdot$cm) (13). The gap junctional conductance was set to 10 $\mu$S, which is within the estimated range for human ventricular myocardium (13) and yields a conduction velocity of $\approx$60 cm/s at normal pacing rate, in accordance with clinical observations (14). The strand was paced at one end with a $\approx$25% suprathreshold stimulus, and CV and diastolic interval were measured at a point in the middle of the strand. The diastolic interval at that point was varied by pacing at different intervals ranging from 240 to 800 ms. The CV restitution curve was then constructed by plotting CV against diastolic interval (12; 15; 10). As for the single cell simulations, the strand was paced for 4 min in order to reach steady-state behavior.

Computational models were coded using Compaq Visual Fortran 6.6C and run on a 3-GHz Intel Pentium-4 processor workstation, applying an Euler-type integration scheme with a 5-µs time step.
RESULTS

**14-3-3 interacts with Na\textsubscript{v}1.5 in transfected COS-7 cells**

We determined the subcellular distribution of Na\textsubscript{v}1.5 and 14-3-3 using confocal microscopy. Immunostaining revealed co-localization of Na\textsubscript{v}1.5 and 14-3-3 in the plasma membrane of transfected COS-7 cells (Supplemental Fig. 1).

**14-3-3 affects the cardiac action potential**

Kagan et al. (11) have previously reported that the rapid delayed rectifier current (I\textsubscript{Kr}), *i.e.* the current carried by the HERG channel, is also affected by the 14-3-3 protein. They compared the biophysical properties of HERG channels in CHO cells transfected with HERG alone or co-transfected with HERG and 14-3-3\(\varepsilon\). They found that overexpression of 14-3-3\(\varepsilon\) shifted the steady-state activation curve by \(\approx -11\) mV and decreased the activation time constant by a factor of \(\approx 2\), without affecting current density, reversal potential, steady-state inactivation or kinetics of deactivation. We carried out computer simulations to test the functional effects of these changes *per se* or in combination with the changes in sodium current. In accordance with the experimental observations by Kagan et al. (11), we implemented the effects of the absence of the 14-3-3 protein on I\textsubscript{Kr} by a +11 mV shift of the I\textsubscript{Kr} steady-state activation curve and a 50% decrease in the rate constant governing I\textsubscript{Kr} activation (\(\alpha_{x1}\); 10).

Before carrying out computer simulations to assess the functional role of the 14-3-3 protein in shaping the cardiac action potential, we checked that our *in silico* voltage clamp data on I\textsubscript{Kr} resemble the afore-mentioned data of Kagan et al. (11). As illustrated in Suppl. Fig. 2, A–C, the Boltzmann fits to the *in silico* data reveal a +10.8 mV shift of the I\textsubscript{Kr} steady-state activation curve (\(V_{1/2}: -14.7\) versus -25.5 mV) without
change in slope (6.8 mV), whereas activation is slowed. It should be noted that, compared to the data of Kagan et al. (11), this slowing is somewhat underestimated at −10 and −20 mV but not at more positive potentials.

Supplemental Figure 2, D–F, shows the effects of the absence of 14-3-3 on the action potential through its action on $I_{Na}$ and $I_{Kr}$, both separately and in combination, at a pacing interval of 800 ms (75 beats/min). Data on upstroke velocity are summarized in Suppl. Fig. 2G. As already shown in Fig. 7, the main effect of 14-3-3 through its action on $I_{Na}$ is an increase in peak Na$^{+}$ current, resulting in a higher maximum upstroke velocity in the absence of 14-3-3 (402 versus 334 V/s in the presence of 14-3-3, 21% increase). We also tested the effects of the absence of 14-3-3 through its action on $I_{Kr}$ per se (Suppl. Fig. 2E). The slower activation of $I_{Kr}$ results in delayed repolarization and a 6-ms increase in APD. If both $I_{Na}$ and $I_{Kr}$ are affected, the action potential both shows the increased upstroke velocity and the increased APD (Suppl. Fig. 2F). The differences in the initial transient peak $I_{Kr}$ upon depolarization (Suppl. Fig. 2E, bottom, arrows) have no noticeable effect on maximum upstroke velocity (Suppl. Fig. 2G), because peak $I_{Na}$ is much larger than initial peak $I_{Kr}$ (note different current scales in Suppl. Fig. 2, D and E).

In the absence of 14-3-3, $I_{Na}$ shows faster recovery from inactivation. Since we expected the effects thereof to be augmented at higher pacing rate, we repeated the simulations of Suppl. Fig. 2, D–G, with a pacing interval of 400 ms (150 beats/min; Suppl. Fig. 2, H–K). As already shown in Fig. 7, the effects of increased Na$^{+}$ channel availability and accelerated recovery from inactivation in the absence of 14-3-3 are stronger at this pacing interval, with a 34% increase in maximum upstroke velocity (Suppl. Fig. 2K). With a 10-ms increase in APD, the effects of the absence of 14-3-3 through its action on $I_{Kr}$ per se (Suppl. Fig. 2I) are also more
pronounced than at a pacing interval of 800 ms. Again, the action potential both shows the increased upstroke velocity and the increased APD if both $I_{Na}$ and $I_{Kr}$ are affected (Suppl. Fig. 2J).

**14-3-3 changes cardiac electrical restitution properties**

As shown in Suppl. Fig. 2, the effects of 14-3-3 on APD, through its action on $I_{Kr}$, are minor. As a result, the APD and CV restitution curves do not show any major changes if not only the effects of 14-3-3 on $I_{Na}$ (as in Fig. 8) but also on $I_{Kr}$ are taken into account (Suppl. Fig. 3). The APD restitution curve in the absence of 14-3-3 ($\emptyset$ 14-3-3) is again steeper than under control conditions (+14-3-3; Suppl. Fig. 3A). Its slope also exceeds 1 at short diastolic interval, whereas the slope of the control curve is <0.7. The CV restitution curve is again flattened in the absence of 14-3-3 (Suppl. Fig. 3B). The shortest pacing interval, as determined with 5-ms resolution, that resulted in successful action potential conduction in the absence of 14-3-3 is now 245 ms (diastolic interval of 32 ms).

**REFERENCES**


2  Benichou S, Bomsel M, Bodeus M, Durand H, Doute M, Letourneur F, Camonis J, Benarous R. Physical interaction of the HIV-1 Nef protein with $\beta$-COP, a
component of non-clathrin-coated vesicles essential for membrane traffic. 


**Supplemental Table 1:** Primers used for two-hybrid baits construct

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*lexA BD: lexA-binding domain. *underlined*:* EcoR1 and Sal1 restriction sites*

**Supplemental Table 2:** PCR primers used for pIRES constructs

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*underlined,* same as Tab. 1; *bold,* HA tag coding sequence.
SUPPLEMENTAL FIGURE LEGENDS

Suppl. Fig. 1. Co-localization of Na\textsubscript{v}1.5 and 14-3-3\eta in mammalian cells. COS-7 cells were transfected with full-length Na\textsubscript{v}1.5-GFP and HA-14-3-3\eta pIRES expression vector. Cells were probed with anti-HA antibodies followed by fluorescent secondary antibodies. \textit{a} and \textit{b}, Na\textsubscript{v}1.5- (\textit{a}) 14-3-3-related (\textit{b}) fluorescence; \textit{c}, merge. No signal was observed in the absence of primary antibody (not shown).

Suppl. Fig. 2. A-C, Effects of the absence of the 14-3-3 protein on I\textsubscript{Kr} in simulated voltage clamp experiments. \textit{A}, Current traces elicited using the indicated voltage clamp protocol. \textit{B}, I\textsubscript{Kr} activation curves as determined from the tail currents at \textasciitilde40 mV following a 3.5-s step to test potentials between \textasciitilde60 and +40 mV from a holding potential of \textasciitilde70 mV (11). Solid lines are Boltzmann fits to the simulated data. \textit{C}, I\textsubscript{Kr} activation time constant as determined from a mono-exponential fit to the current trace elicited by a voltage clamp step to test potentials between \textasciitilde20 and +40 mV from a holding potential of \textasciitilde70 mV (11). D-K, Simulated effects of the absence of the 14-3-3 protein on the human ventricular action potential at pacing intervals of 800 ms (D-G) and 400 ms (H-K). \textit{D and H}, Action potential (top) and associated Na\textsuperscript{+} current (I\textsubscript{Na}, bottom) if only I\textsubscript{Na} is affected by the absence of 14-3-3. Solid lines represent the control situation (+ 14-3-3). Arrows indicate peak Na\textsuperscript{+} current. \textit{E and I}, Action potential (top) and associated rapid delayed rectifier current (I\textsubscript{Kr}, bottom) if only I\textsubscript{Kr} is affected by the absence of 14-3-3. Arrows indicate initial transient peak I\textsubscript{Kr}. \textit{F and J}, Action
potential if both $I_{Na}$ and $I_{Kr}$ are affected by the absence of 14-3-3. $G$ and $K$,
Maximum upstroke velocity of the action potential ($dV/dt_{max}$) in the presence and
in the absence of 14-3-3.

Suppl. Fig. 3. Simulated effects of the absence of the 14-3-3 protein on cardiac
electrical restitution properties if both $I_{Na}$ and $I_{Kr}$ are affected by the absence of
14-3-3. $A$, Restitution of action potential duration (APD) in the presence (+ 14-3-3,
solid lines with closed symbols) and absence of 14-3-3 (Ø 14-3-3, dashed
lines with open symbols). $B$, Restitution of conduction velocity (CV) in the
presence and absence of 14-3-3. Numbers near symbols indicate the associated
pacing interval in ms.
Supplemental Figure 1