Cytoskeletal Protein 4.1R Affects Repolarization and Regulates Calcium Handling in the Heart

Mark A. Stagg, Edward Carter, Nadia Sohrabi, Urszula Siedlecka, Gopal K. Soppa, Fiona Mead, Narla Mohandas, Pamela Taylor-Harris, Anthony Baines, Pauline Bennett, Magdi H. Yacoub, Jennifer C. Pinder, Cesare M.N. Terracciano

Abstract—The 4.1 proteins are a family of multifunctional adaptor proteins. They promote the mechanical stability of plasma membranes by interaction with the cytoskeletal proteins spectrin and actin and are required for the cell surface expression of a number of transmembrane proteins. Protein 4.1R is expressed in heart and upregulated in deteriorating human heart failure, but its functional role in myocardium is unknown. To investigate the role of protein 4.1R on myocardial contractility and electrophysiology, we studied 4.1R-deficient (knockout) mice (4.1R KO). ECG analysis revealed reduced heart rate with prolonged Q-T interval in 4.1R KO. No changes in ejection fraction and fractional shortening, assessed by echocardiography, were found. The action potential duration in isolated ventricular myocytes was prolonged in 4.1R KO. Ca\(^{2+}\) transients were larger and slower to decay in 4.1R KO. The sarcoplasmic reticulum Ca\(^{2+}\) content and Ca\(^{2+}\) sparks frequency were increased. The Na\(^{+}/Ca\(^{2+}\) exchanger current density was reduced in 4.1R KO. The transient inward current inactivation was faster and the persistent Na\(^{+}\) current density was increased in the 4.1R KO group, with possible effects on action potential duration. Although no major morphological changes were noted, 4.1R KO hearts showed reduced expression of NaV1.5 and increased expression of protein 4.1G. Our data indicate an unexpected and novel role for the cytoskeletal protein 4.1R in modulating the functional properties of several cardiac ion transporters with consequences on cardiac electrophysiology and with possible significant roles during normal cardiac function and disease. (Circ Res. 2008;103:855-863.)

Key Words: cardiac cytoskeleton ▪ ion transporter regulation ▪ EC coupling

The cardiac cytoskeleton is important in conferring stability to the myocardium, in sensing the mechanical stretch, and in coordinating the assembly of cellular structures and intercellular signaling.\(^1\) One group of cytoskeletal proteins, the spectrin- and ankyrin-associated system, is involved in the complex interplay between actin, spectrin, and various ion transporters in relation to the regulation of intracellular [Ca\(^{2+}\)].\(^2\) Beta II spectrin, muscle LIM-only protein, and ankyrin B and G have all been associated with cardiac function and regulation of the electrophysiological properties of the myocardium.\(^3\) The 4.1 protein family is also part of the spectrin-associated cytoskeleton. It promotes the interaction between spectrin and F-actin and, thus, membrane stability.\(^2\) In mammals, the 4 genes, EPB41, EPB41L1, EPB41L2, and EPB41L3, encode proteins 4.1R, 4.1G, 4.1N, and 4.1B. mRNA transcripts from all 4 genes are found in mouse myocardium.\(^4\) All 4.1 proteins have a FERM membrane-binding domain, a spectrin–actin binding (SAB) domain, and a C-terminal domain.\(^2\) A FERM-adjacent domain regulates the activities of both FERM and SAB domains.\(^5\) The FERM and C-terminal domains bind to membrane proteins,\(^6\) whereas the SAB domain binds the spectrin–actin cytoskeleton.\(^7\) Multiple ion channels, pumps, and exchangers located in the plasma membrane are believed to directly interact with members of the 4.1 protein family. The cytoskeletal docking complex not only regulates cell shape, confers mechanical stability, and permits communication across the cell membrane but may additionally function to cluster and stabilize ion transporters to regions of the plasma membrane to form microdomains with specialized roles.\(^8\)

Protein 4.1R has been associated with the progression of heart failure. We have previously reported that EPB41 mRNA expression is increased in patients with deteriorating heart failure undergoing ventricular assist device surgery compared with patients with stable heart failure.\(^9\) Given the limited information available on the roles of this protein in the normal and diseased myocardium, whether protein 4.1R takes direct part in the mechanical and electrophysiological changes associated to cardiac disease is unclear. It is known that protein 4.1R, like other members of this family, is
compartmentalized within myocytes. It has been identified at subcellular locations that include the Z/I-band, M-line, intercalated disks, and lateral plasma membrane and within the nucleus of mouse myocytes. 4.1R is required for cell surface expression of PMCA4, a plasma membrane Ca\(^{2+}\)/H\(^{+}\) ATPase. However, the functional role of protein 4.1R in the myocardium is unknown.

To study the role of protein 4.1R on myocardial function and regulation, we used protein 4.1R–deficient (knockout) mice (4.1R KO) previously characterized by Shi et al. Only 6-month-old male mice were used in these experiments, and C57bl/6 male mice from Harlan (UK) were used as controls. Echocardiography was performed using a 15-MHz probe on an Acuson Sequoia 256 system in M-mode. ECG recording was performed in conscious mice using a radiotelemetry system (Data Science International, St Paul, Minn).

**Materials and Methods**

**4.1R-Deficient Mice**

4.1R KO, bred in C57bl/6 background, were kindly provided by Dr Phillipe Gascard (Life Science Division, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, Calif). Only 6-month-old male mice were used in these experiments, and C57bl/6 male mice from Harlan (UK) were used as controls. Echocardiography was performed using a 15-MHz probe on an Acuson Sequoia 256 system in M-mode. ECG recording was performed in conscious mice using a radiotelemetry system (Data Science International, St Paul, Minn).

**Cellular Studies**

Cardiomyocytes were enzymatically dissociated and examined with a \( \times 60 \) objective. Cell planimetry was performed using ImageJ software (http://rsb.info.nih.gov/ij). For Ca\(^{2+}\) transient experiments, myocytes loaded with indo-1-acetoxymethyl ester (Molecular Probes) were field-stimulated at 37°C. Confocal studies were performed in myocytes loaded with fluo-4 acetoxymethyl ester. To measure Ca\(^{2+}\) transient synchronicity, time-to-peak of Ca\(^{2+}\) transients at each pixel was measured and the variance calculated and taken as a measure of synchronicity of Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). Action potentials were measured in current-clamp mode. Measurement of ion transporter current density was carried out using the whole-cell configuration of the patch-clamping technique. Pipette resistance was 1.5 to 2.5 MΩ.

**Electron Microscopy, Immunofluorescence, and Western Blotting**

Thin sections for electron microscopy and 0.2-μm frozen sections for immunofluorescence were prepared and analyzed as described previously. Heat homogenates were prepared for Western blotting...
A total of 100 μg of protein was loaded per lane of SDS polyacrylamide gels.

Statistical Analysis

Statistical comparison of data was performed using a 2-way ANOVA or Student’s t test where appropriate. Data are expressed as means±SEM (n) unless otherwise specified. For cellular studies, n is the number of myocytes. In the figures, * indicates P<0.05; **, P<0.01; and ***, P<0.001.

Results

The in vivo assessment of cardiac function in 4.1R KO mice was performed using M-mode echocardiography and ECG recording. Left ventricular ejection fraction and fractional shortening were not significantly different between control (wild type [WT]) and 4.1R KO (left ventricular ejection fraction [%]: WT, 67.7±2.7 [6]; 4.1R KO, 64.6±2.8 [6];
fractional shortening [%], WT, 32.8±2 [6]; 4.1R KO, 30.8±2 [6]). The ECG analysis (Figure 1 and Table I in the online data supplement, available at http://circres.ahajournals.org) showed a significant reduction in heart rate in 4.1R KO compared with control (WT) (Figure 1B and 1C). This was accompanied by a significantly prolonged Q-T interval (Figure 1D through 1F). At rest, the changes in ECG parameters were not accompanied by increased arrhythmic events, which remained very low in both groups (number of premature ventricular contractions per hour: WT, 1.75±0.81 [6]; 4.1R KO, 2.16±0.87 [6]; number of supraventricular arrhythmias per hour [including AV blocks and atrial ectopic beats], WT, 4.65±0.5 [6]; 4.1R KO, 5±0.5 [6]).

Heart weight and heart weight-to-body weight ratio were unchanged in 4.1R KO mice (heart weight [g] WT, 0.277±0.01 [9]; 4.1R KO, 0.245±0.01 [10]; heart weight-to-body weight ratio (g/kg): WT, 9.04±0.56 [8]; 4.1R KO, 7.99±0.3 [8]). Cardiomyocyte size measured with planimetry (supplemental Figure IA through IC), and cell capacitance (supplemental Figure ID) was also unchanged in the 4.1R KO heart.

The different proteins 4.1 have been localized in close proximity to Ca²⁺ handling proteins in heart muscle,² but whether they are implicated in the regulation of intracellular [Ca²⁺] is unknown. Ca²⁺ transients were recorded from 4.1R KO and WT myocytes. Indo-1 transient amplitude was increased by approximately 33% (Figure 2B), and Indo-1 decay was delayed by approximately 16% (Figure 2C) in 4.1R KO. There was no difference in the time-to-peak or diastolic levels.

To investigate the causes for these changes in Ca²⁺ transients, a number of specific studies were performed. Firstly, the role of the SR was investigated as previously described.¹² SR Ca²⁺ content was significantly increased by approximately 28% in the 4.1R KO myocytes (Figure 2D). No difference could be detected in fractional release (4.1R KO, 0.74±0.03 [16]; WT, 0.7±0.08 [7]; P>0.05) nor the relative contribution of the SR Ca²⁺ uptake to Ca²⁺ removal from the cytoplasm (4.1R KO [%], 92±1.5 (9 mice); WT, 95±1.1 [6]; P>0.05).

The ability of the unitary ryanodine receptor clusters to spontaneously release Ca²⁺ during rest (Ca²⁺ sparks), as an indication of SR Ca²⁺ release function, was also investigated. Ca²⁺ spark frequency and duration were increased in the 4.1R KO myocytes (Figure 3A through 3C), without changes in fluorescence peak and width (Figure 3D and 3E). Whether this is the consequence of the increased SR Ca²⁺ content or a direct effect of the protein 4.1R deficiency on the SR Ca²⁺ release unit is unclear.

Local Ca²⁺-induced Ca²⁺ release (CICR) in protein 4.1R KO myocytes was further investigated by measuring the synchronicity of Ca²⁺ release during the Ca²⁺ transient (Figure 3F). Despite the observation that the global time-to-peak of the Ca²⁺ transients was unchanged, the variance of the time-to-peak values measured at each pixel on line scan was significantly increased in 4.1R KO myocytes (Figure 3G).

We have previously described a colocalization of protein 4.1R with the plasmalemmal Ca²⁺ ATPase (PMCA) in cardiac myocytes.³ Ca²⁺ extrusion was carried out by PMCA in the 4.1R KO and WT myocytes was therefore investigated. Using experimental conditions where SR Ca²⁺ uptake, Na⁺/Ca²⁺ exchanger (NCX) and mitochondrial uptake are inhibited⁴ no significant difference between the groups could be detected (time constant of decline: 4.1R KO, 8.1±0.6 seconds [19]; WT: 9.8±0.8 seconds [14]; P=NS), suggesting that PMCA is not involved in the changes in Ca²⁺ transients observed in protein 4.1R KO myocytes.

The prolonged Q-T interval observed in the ECG from 4.1R KO mice suggests electrophysiological modifications when protein 4.1R is deficient. Figure 4 shows that action potentials recorded in 4.1R KO mice were significantly prolonged. This prolongation was detected at all frequencies studied (1, 3 and 5 Hz). The resting membrane potential was not changed (4.1R KO, −69±1.2 mV [28]; WT, −68±1.8 mV [21]).

Action potential prolongation can result from modifications of most ion transporters function.¹⁵ Given the importance of NCX and Na⁺/K⁺ ATPase (NKP) function in determining Q-T prolongation in mice with ankyrin B mutations,¹⁶ the electrophysiological properties of these 2 ion transporters were studied. The NCX current was significantly reduced in 4.1R KO myocytes compared with WT (Figure 5A and 5B), suggesting a relationship between protein 4.1R and NCX. In contrast, the NKP function was not affected by the deficiency of protein 4.1R (supplemental Figure II).

The reduction in NCX function, together with the prolonged action potential, can partially explain the increased SR Ca²⁺ content and the slower Ca²⁺ decline observed in protein 4.1R KO myocytes; however, other ion transporters must be responsible for the action potential prolongation observed in 4.1R KO mice. We investigated 3 other ion currents which contribute to the plateau phase of the action potential: the

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**Figure 4.** A, Action potential traces recorded from a 4.1R KO and a WT myocyte. Action potential duration, assessed as the time to 75% repolarization (B) and 90% repolarization (C), was significantly prolonged in 4.1R KO myocytes. The prolonged action potential was observed at all 3 frequencies of stimulation studied (D). Data from 21 WT (5 mice) and 28 (6 mice) 4.1R KO myocytes.
Transient outward current $I_o$ for its predominant role in repolarization of the mouse myocardium, the L-type Ca$^{2+}$ current $I_{Ca}$ and the persistent Na$^+$ current $I_{pNa}$. Despite a similar steady-state current–voltage relationship in the 2 groups, $I_o$ inactivated more rapidly in 4.1R KO myocytes (Figure 5C through 5F). $I_{Ca}$ was not affected by the 4.1R deficiency ($I_{Ca}$ density at 0 mV: 4.1R KO, 0.25 pA/pF [45]; WT, 0.25 pA/pF [27]; $P$ = NS). The voltage–current relationship and the voltage-dependent activation and inactivation of $I_{Ca}$ were all unchanged (data not shown).

$I_{pNa}$ density, calculated as the 30 μmol/L tetrodotoxin–sensitive current (Figure 6) was significantly increased at 20 mV in 4.1R KO myocytes. In addition, the integral of this current between 50 and 300 ms was also significantly increased in the 4.1R KO myocytes. The combined faster inactivation of $I_o$ and increased $I_{pNa}$ can contribute to the prolongation of the action potential. WT, $n$ = 35 (5 mice); 4.1R KO, $n$ = 32 (6 mice).

Immunofluorescence (Figure 7C) revealed the same pattern for 4.1R in WT myocardium as described previously, namely staining at intercalated discs and lateral plasma membrane, plus internal striations not separable by light microscopy from α-actinin (ie, Z-disks or T-tubules) (Figure 7D and 7E). Similarly, staining for NaV1.5 and NCX1 was present at intercalated discs, lateral plasma membrane and internal striations (presumably T-tubules) as described by others.

In the 4.1R KO hearts, some residual 4.1R immunoreactivity was detectable, in the same locations as in the WT hearts (Figure 7L). We attribute this to the way the 4.1R KO mice were constructed: of the 3 initiation codons in 4.1R, only AUG1 and 2 are eliminated. It might be expected that our anti-4.1R antibody recognizes products initiated at AUG3. Figure 7N through 7S reveals no significant disorganization of NCX1 or NaV1.5; in each case, staining is detectable at lateral plasma membranes, intercalated disks, and internal striations.

Finally, we investigated the abundance of proteins that might be relevant to the phenotype we describe above using Western blotting. Figure 8A shows (left) comparative Coomassie blue–stained gels of the homogenates (100 μg protein per lane) and (right) immunoblots probed with an anti-4.1R antibody directed against epitopes in the spectrin–actin binding domain. As expected, the major 80-kDa isoform (translated from AUG2) is completely lost. In the generation of these 4.1R KO mice, the third initiation codon, AUG 3, was not eliminated: an immunoreactive band of lower molecular mass was detected consistent with expression from AUG3.

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**Figure 5.** A, Example traces of Ni$^{2+}$-subtracted currents in the 2 experimental groups, elicited by a voltage ramp protocol (middle) and ascribed to NCX. B, The NCX current–voltage relationship, with a significant reduction in $I_{NCX}$ in the 4.1R KO group (data from 14 WT [5 mice] and 16 4.1R KO myocytes [6 mice]). C through F describe the assessment of $I_o$ in WT and 4.1R KO myocytes. C, Example traces. The amplitudes of the currents were not different between the 2 groups (D). The fast (E) and slow (F) time constants of inactivation for the $I_o$ current were both quicker in 4.1R KO myocytes, and this can contribute to the prolongation of the action potential. WT, $n$ = 35 (5 mice); 4.1R KO, $n$ = 32 (6 mice).
This 40-kDa band was not significantly altered relative to WT tissue.

Protein 4.1G was increased in 4.1R KO myocardium 1.9-fold relative to WT (Figure 8B), but there was no significant change in either 4.1B or 4.1N (Figure 8C and 8D). Figure 8E through 8G show that the expression of the actin-binding proteins α-actinin, spectrin, and tropomyosin (TM) is also unaltered: of these, spectrin and TM also bind 4.1R.19,20

We also tested whether alterations in ion transporter activity were associated with changes in protein expression. Figure 8H shows that the level of the α-subunit of NaV1.5 is reduced by approximately 40% in 4.1R KO myocardium. The expression of NCX1 (Figure 8I) and PMCA2 (Figure 8J) were not significantly changed.

Discussion

In this study, we show that deficiency of protein 4.1R in mice results in Q-T prolongation without apparent changes in left ventricular function at rest. At myocyte level, deficiency of protein 4.1R is accompanied by prolongation of the action potential duration, increased amplitude, and slower decay of Ca2+ transients. At the subcellular level, the deficiency of protein 4.1R affects local calcium-induced calcium release, reduces the NCX activity, hastens the inactivation of Ito, and increases I\textsubscript{pNa}. The functional changes are associated with increased expression of protein 4.1G and decrease expression of NaV1.5α subunit.

These results indicate a role of protein 4.1R on the function of several ion transporters affecting cardiac electrophysiology.

Cytoskeleton and Ion Transporter Function

There is increasing evidence that ion transporters exist in the context of macromolecular complexes formed not only by the ion channel subunits but also by regulatory kinases and phosphatases, proteins from the extracellular matrix, from trafficking, other ion channels, and the cytoskeleton.21 The importance of the cytoskeleton in the regulation of cardiac ion transporter function and generation of cardiac arrhythmias is becoming progressively established.22 The specific
role of the spectrin–ankyrin cytoskeleton is of particular interest for the relationship between some of its components and cardiac arrhythmias in humans.23 Mutations of ankyrins for example have been clearly associated to congenital arrhythmias.16,18 In general, beta II spectrin and ankynin B and G have all been associated with cardiac dysfunction.5

The functional roles of proteins 4.1 in heart have not been investigated previously. Protein 4.1R in particular is of interest because its gene expression changes during the progression of heart failure in patients.9 Protein 4.1R is localized in the proximity of ion transporters in cardiac cells and, more specifically, is required for cell surface targeting of PMCA4.3 Although PMCA has a minimal role in Ca2+ extrusion in normal adult cardiac myocytes,24 it can be important in Ca2+ regulation when compensation for dysfunction in other Ca2+ regulatory mechanisms is required.25 However, 4.1R deficiency did not appear to affect PMCA-mediated Ca2+ extrusion function. Other aspects of PMCA function, particularly signal transduction pathways, may be altered in the 4.1R KO mice, and more studies are required.26

**Defects of Ca2+ Handling in Protein 4.1R KO Myocytes**

Protein 4.1R KO myocytes showed increased Ca2+ transients amplitude and slower Ca2+ extrusion. Whereas these changes were significant at myocyte level, they did not influence whole heart basal function. Whether this is attributable to compensatory mechanisms present in this transgenic model is unclear. Also whether these changes may become relevant during exercise, stress, or disease is unknown, and further studies in this direction are warranted.

The increased amplitude of the Ca2+ transients could be ascribed to increased SR Ca2+ content in the absence of changes in the trigger for Ca2+ release I_{Ca}. Two possible causes for the increased SR Ca2+ content can be identified from this study: the prolonged action potential27 and the reduced NCX activity. Despite a normal NKp function, an increased cytoplasmic [Na+] as the consequence of the increased I_{Na}, is expected in protein 4.1R KO myocytes.28 The combined effects of reduced intrinsic NCX activity, the prolonged action potential, and the increased cytoplasmic [Na+] can favor SR Ca2+ uptake during diastole, resulting in increased SR Ca2+ content and slower Ca2+ extrusion.

Changes in the microarchitecture of the cardiac myocyte have been implicated in defects of excitation–contraction coupling during hypotrophy and failure, resulting in inefficient CICR.29 The role of the cytoskeleton in this process is unclear. Protein 4.1R KO myocytes showed an increased variance of the local CICR and disruption to the synchronous Ca2+ release. This may suggest disruption of the spatial relationship between L-type Ca2+ channels and ryanodine receptors and incorrect coupling. The increased Ca2+ spark frequency may also suggest alteration in local SR Ca2+ release, as it has been suggested for defects of ankyrins.30 The increased SR Ca2+ content observed in this study, however, can be responsible for increased Ca2+ spark frequency.13 The prolonged repolarization observed in these myocytes can also be responsible for impaired synchronicity of CICR.30 Therefore, a direct relationship between protein 4.1R deficiency and ryanodine receptor activity cannot be concluded from our results. In general, as for other ion transporters, the relationship may be indirect, resulting from disruption of mechanisms involved in regulating the ion transporters, and this point warrants further studies.

**Defects of Repolarization in Protein 4.1R KO Myocytes**

Among the different currents implicated in shaping the action potential in murine myocytes, we studied I_{Ca}, I_{Na}, and I_{NaC}, I_{Ca} was unaffected in all parameters studied. Whereas the I_{Na} density–voltage relationship was unchanged in protein 4.1R KO myocytes, the inactivation of I_{Na} was faster with possible effects on repolarization. Furthermore, the increased depolarizing current I_{pNa} can also be responsible for delayed repolarization.31 The relationship between the Na+ current and Q-T prolongation is well established and seems to be important in both congenital and acquired cardiomyopathies.32

**Interactions Between Protein 4.1R and Ion Transporters**

Our results indicate the existence of primary functional effects of the lack of protein 4.1R on NCX, I_{NaC} and I_{pNa}. The effects on SR Ca2+ handling and local CICR may be secondary, but this needs to be tested. The exact molecular mechanisms involving protein 4.1R, and capable of regulating these functions, are unclear at this stage, and further studies are
required. Protein 4.1R, like other members of this family, is compartmentalized within myocytes. It has been located at several subcellular locations that include the Z/I-band, M-line, intercalated disks, and lateral plasma membrane and within the nucleus of mouse myocytes. Little is known about its precise role at these locations. One interesting aspect is that the SAB domain is preserved in cardiac 4.1R but is lacking in brain isoforms. This may reflect a role in maintaining strong interactions between the cytoskeleton and the contractile apparatus in the heart. As a consequence, the ability of protein 4.1R to regulate ion transporter function could be of the links between mechanical load and regulation of function.

In patients with deteriorating heart failure, mRNA for 4.1 proteins were differentially overexpressed. Given this, it was possible that 1 or more 4.1 protein was upregulated to compensate for the loss of 4.1R in the knockout mice. We noted nearly double the intensity of the 4.1G band in the 4.1R KO mice (Figure 8B). Expression of 4.1G may be linked to that of 4.1R.

Of the ion currents we analyzed, it was striking that 0 Na was increased (Figure 6). Because this current is linked to NaV1.5, we analyzed the channel-forming α-subunit by immunoblot (Figure 8H). This was reduced by approximately 40% in the 4.1R KO heart. This result was surprising, because it indicates that 4.1R has a role in modulating the cellular content of the protein and also its activity. It is not clear whether this is a direct or indirect effect. 4.1 proteins bind numerous ion channels (eg, the erythrocyte anion exchanger and both ionotropic and metabotropic glutamate receptors). 4.1R is required for the stable cellular accumulation of glycophorin C in erythrocytes, through its direct binding to the glycophorin C cytoplasmic domain. 4.1R-NaV1.5 interaction may be analogous. However, in erythrocytes, glycophorin C is lost, unlike NaV1.5 in heart. Because 4.1G is upregulated in 4.1R KO heart (Figure 8B) it might be hypothesized that 4.1G compensates in part for the loss of 4.1R in this tissue.

It is also interesting that NaV1.5 binds ankyrin, and interaction with ankyrin is required for its stable cellular accumulation. 4.1R and ankyrin have an overlapping spectrum of interactions with membrane proteins: for example, both the erythrocyte anion exchanger and CD44 binds 4.1R and ankyrin. In these cases, there are distinct sites for interaction with each protein.

NCX activity is also altered in the 4.1R KO mice. Again, NCX1 binds ankyrin, and ankyrin interaction is required for its retention in cardiac membranes. However, we find no evidence for loss of NCX1 protein from the 4.1R KO hearts (Figure 8I). Whether 4.1R has a regulatory role in NCX1 activity through direct interaction remains an open question.

In conclusion, deficiency of protein 4.1R in mice results in defects of repolarization and Ca2+ regulation in the heart that are, at least in part, explained by changes in transient outward current, Na+ currents, and NCX function. This study has shown that protein 4.1R has an important role in determining the functional properties of several ion transporters, with possible significance during normal cardiac function and disease.

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Disclosures
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Supplemental information
Supplemental Methods

4.1R-deficient mice:

4.1R-deficient mice (4.1R KO), bred in C57bl/6 background, were kindly provided by Dr Phillipe Gascard, Berkeley, CA, USA. Dr Mohandas Narla, NY Blood Centre, supplied the original 4.1R-deficient line bred in mixed strain. These mice were generated by deleting two of the three initiation codons in the 4.1R gene\(^1\). This gene (EPB41) has initiation codons in exons 2, 4 and 8\(^2\). Of these, only those encoded in exons 2 (AUG1) and 4 (AUG1) were deleted in these mice, potentially allowing products to be expressed from AUG3 (encoded in exon 8). The data acquired in preliminary studies from this line in mixed strain are not presented in this manuscript. The 4.1R-deficient mice are viable, with modest anemia but without gross abnormalities\(^4\). The gene targeting was shown to be uniform in the cardiac tissue of these mice\(^4\). At the planning stage of this study there had been preliminary unpublished reports that 4.1R hearts develop more slowly and that cardiac myocytes are smaller in the first few weeks after birth. For this reason we decided to study hearts after full growth to avoid confounding effect of differences in maturation. 4.1R KO mice were bred to six months of age in C57bl/6 strain background. Only male mice were used in these experiments and 6 months old C57bl/6 male mice from Harlan, UK, were used as controls.

Echocardiography:

Animals were anesthetized by administering 1.5% isoflurane in 100% oxygen. Using a 15 MHz probe on an Acuson Sequoia™ 256 system transthoracic echocardiography was performed to obtain parasternal short-axis views at the
mid-papillary muscle level. M-mode was used for the measurements of the left ventricular ejection fraction and fractional shortening.

**ECG recording in conscious mice using radiotelemetry:**

ECG recording was performed in conscious mice using a radiotelemetry system (Data Science International, St. Paul, MN, U.S.A). Animals were anesthetized by administering 1.5% isoflurane in 100% oxygen and a radiotelemetry transmitter (Data Science International, model EA-F20) for the recording of electrical activity was implanted in the scruff of the neck. The electrode wires were tunnelled under the skin with the negative lead sutured in the muscle just under the right clavicle and the positive lead placed left of the xyphoid process and below the rib cage. Recording was performed at least 3 days after implantation, continuously for 2 hours. Analysis of the recording was performed using ECG-auto software (EMKA technologies, Paris, France).

To correct for the Q-T interval (Q-Tc) the formula described by Mitchell et al. (1998)\(^5\) which takes into account the average heart rate and the physiological rate-dependent Q-T variation was used:

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Q-T_c = Q-T/(R-R/R-R_{avg})^S
\]

Where the average R-R interval (R-R\(_{avg}\)) was 111.6 ± 0.48 ms (calculated from 1282 QRS complexes from 6 C57bl/6 male mice aged six months old); S is the slope of the linear regression between the natural logarithms of R-R/R-R\(_{avg}\) and the Q-T interval, and was 0.371 ± 0.01 (n=1275 QRS complexes from 6 animals) (r\(^2\) = 0.33; p<0.001).
**Cell isolation and cell area planimetry:**

Hearts were removed for cardiomyocyte isolation by standard enzymatic digestion using protease, hyaluronidase and type-2 collagenase as previously described. Isolated cells were examined using an Olympus IX-71 inverted epifluorescence microscope with a ×60 objective. Photographs were taken and the projected 2-dimensional area for each cell was measured using ImageJ software (http://rsb.info.nih.gov/ij/).

**Indo-1 fluorescence:**

Isolated cells were loaded with 10 μM indo-1 AM (Molecular Probes™) for 20 minutes, and allowed to de-esterify for at least 1 hour. They were field-stimulated whilst being superfused with normal Tyrode solution (NT) containing (in mM) NaCl 140; KCl 6; glucose 10; MgCl₂ 1; CaCl₂ 1; HEPES 10; pH 7.4 at 37°C. Excitation wavelength was 385 nm, and emissions at 405 nm and 485 nm were acquired after steady-state contraction was reached. After subtracting background levels the indo-1 fluorescence ratio was calculated (F405/485). For analysis, 10-20 transients were averaged with reference to the field-stimulation signal. Transient peak amplitude and time-to-peak (TTP) were calculated from the field-stimulation signal baseline, and decay times at 50% and 90% (T₅₀ and T₉₀) were calculated from TTP.

To elicit a caffeine-induced indo-1 transients, field-stimulation was stopped and then caffeine was rapidly applied using a solenoid switcher device. Immediately before application of caffeine, the superfusate was switched to a Na⁺-free/Ca²⁺-free solution containing (in mM) LiCl 140; glucose 10; HEPES 10; EGTA 0.75; MgCl₂ 1; KOH 6; pH 7.40, for 100 ms to prevent Ca²⁺
extrusion by NCX. This was followed by a 1 s application of 20 mM caffeine dissolved in the same Na⁺-free/Ca²⁺-free solution, and then 20 mM caffeine in normal Tyrode for 5 s. The amplitude of the caffeine-induced transient was taken as a measure of total SR Ca²⁺ content. The amplitude of the preceding field-stimulated indo-1 transient divided by the amplitude of the caffeine-induced transient was taken as the fractional release. The relative contribution of SR Ca²⁺ uptake towards Ca²⁺ removal was derived by dividing the decay rate constant of the caffeine-induced transient with that of the preceding field-stimulated indo-1 transient ⁷.

Ca²⁺ extrusion carried out by the sarcolemmal Ca²⁺ ATPase was assessed by measuring the rate of decline of the caffeine-induced indo-1 transient in the Na⁺-free/Ca²⁺-free solution containing 20 mM caffeine, 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 1 µM oligomycin (Sigma, Poole UK) ⁸. FCCP and oligomycin were added to the superfusing solution at least 15 s before the application of caffeine.

**Measurement of Ca²⁺ sparks:**

The Ca²⁺-sensitive, single excitation, single emission fluorescent dye, fluo-4 was used to monitor localized changes in intracellular [Ca²⁺]. Aliquots of cells were incubated with fluo-4 AM (10 µM) for 20 min and cells allowed to de-esterify for at least 30 minutes prior to use. The experimental chamber was mounted on the stage of a Zeiss Axiovert microscope with LSM 510 confocal attachment and myocytes observed through a Zeiss EC Plan-NeoFluar x 40 oil-immersion lens (N.A. = 1.3). Fluo-4 was excited using the 488 nm line of
an argon laser and the emitted fluorescence collected through a 505 nm long-pass filter.

Following a period of 30 s quiescence, after 1 Hz stimulation, line scans were collected (up to 30,000 lines ranging from 50-173 µm were scanned at speeds between 546-964 µs, n = 384). Analysis was performed using custom written routines in Matlab R2006b (The MathWorks, Inc, MA, USA) following the algorithm for automatic Ca\textsuperscript{2+} spark detection of Cheng et al.\textsuperscript{9}. Detection criteria for Ca\textsuperscript{2+} sparks were set at 3.8 times above the standard deviation of the background noise. Ca\textsuperscript{2+} spark frequency was obtained from the line scans and Ca\textsuperscript{2+} spark amplitude defined as the peak fluorescence over background fluorescence (F/F\textsubscript{o}). Morphometric analysis of Ca\textsuperscript{2+} sparks elucidated the full-width half-maximal (FWHM) and full-duration half-maximal (FDHM).

To measure Ca\textsuperscript{2+} transient synchronicity, Ca\textsuperscript{2+} transients were elicited by field stimulation at 1 Hz and recorded in line scan mode. Time-to-peak of Ca\textsuperscript{2+} transients at each x pixel was measured using custom written routines in Matlab R2006b (The MathWorks, Inc, MA, USA) and the variance calculated and taken as a measure of synchronicity of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the SR.

**Measurement of action potential:**

Cells were superfused with normal Tyrode solution as above and studied using an Axon 2B amplifier (Axon Instruments, CA, USA) in discontinuous (switch-clamp) mode. The pipette resistance was approximately 30 MΩ, and
the pipette-filling solution contained (in mM) KCl 2000, HEPES 5, EGTA 0.1; pH 7.2. Action potentials (AP) were measured in current-clamp mode after stimulation at 1, 3, and 5 Hz using a 1 ms, 1.2-1.4 nA pulse. The action potentials measured were analyzed using pClamp™ software (Molecular Devices, USA). Traces were averaged with reference to the stimulation signal, and the time to 75% repolarization (APD75) was taken as a measure of the AP duration for comparison between groups.

**Measurement of ion transporter current density:**

The whole-cell configuration of the patch-clamping technique was employed using a Multiclamp 700A amplifier (Molecular Devices, USA). Pipette resistance was 1.5-2.5 MΩ. All the experiments were performed at 37°C. Data were analyzed using pClamp 10 software (Molecular Devices, USA). All the currents were normalized to the whole-cell capacitance for cross-group comparisons.

**NCX current:** Cells were superfused at 37°C with K⁺-free solution containing (in mM) NaCl 140; HEPES 10; glucose 10; MgCl₂ 1; CaCl₂ 1; CsCl 6; pH 7.4. The solution contained 10 μM strophanthidin (Sigma-Aldrich, UK) and 10 μM nifedipine (Sigma-Aldrich, UK). The pipette-filling solution contained (in mM) CsCl 45; HEPES 20; MgCl₂ 11; Na₂ATP 10; CsOH 100; EGTA 50; CaCO₃ 25; pH 7.2 ([Ca²⁺], calculated to be ~200 nmol/l, as previously described ¹⁰. After achieving the whole-cell configuration, a period of at least 5 minutes was allowed for dialysis of the pipette-filling solution into the cardiomyocyte. From a holding potential of -40 mV, a 3 s descending ramp was applied from +80 mV to -120 mV at 0.1 Hz. The ramp was applied repeatedly until a
steady-state was reached, and then acquired 5 times to yield an average current for analysis. The superfusate was then changed to that with 5 mM Ni^{2+} added, and further current traces were acquired once a new steady state was reached. The NCX current (I_{NCX}) was taken as the 5 mM Ni^{2+}-sensitive component of the active current.

**NKP current:** A technique modified from Gao et al., \(^{11}\) was used. Cells were superfused at 37°C with NT solution containing 2 mM BaCl\(_2\) and 1 mM CsCl. The pipette-filling solution contained (in mM): Na-aspartate 50; K-aspartate 20; CsOH 30; HEPES 5; TEACl 20; NaH\(_2\)PO\(_4\) 10; MgATP 5; EGTA 11; glucose 10; CaCl\(_2\) 1; pH 7.2 ([Ca\(^{2+}\)]\(_i\) calculated to be \(\sim 314\) nmol/l)). The cell was held at 0 mV and 300 \(\mu\)M strophanthinidin (Sigma, Poole UK) was applied. The NKP current (I_{NKP}) was taken as the strophanthinidin–sensitive outward current.

**Transient outward current:** The transient outward current \(I_{to}\) was measured using a technique previously described \(^{12}\). Briefly cells were superfused with NT solution containing 300 \(\mu\)M Cd\(^{2+}\) and the pipette-filling solution contained (in mM): K-glutamate 120; KCl 10; MgCl\(_2\) 2, HEPES 10, EGTA 5; Mg-ATP 2, pH 7.2 with KOH at 37\(^\circ\) C. Pipette resistance was 1.5-2.5 M\(\Omega\). \(I_{to}\) was elicited by increasing voltage steps (1s duration) from – 40 mV to +80 mV at 0.5 Hz in increments of 10 mV. \(I_{to}\) was taken as the peak of the transient outward current \(I_{to,peak}\) subtracted of the steady-state current \(I_{ss}\). \(I_{to}\) inactivation was calculated by fitting a two exponential function on the decay portion of the current using pClamp 10 software (Molecular Devices, USA).
**L-type Ca^{2+} current:** Cells were superfused at 37°C with NT solution (K^+ substituted with Cs^+). The pipette resistance was 1.5-2.5 MΩ, and the pipette-filling solution contained (in mM): Cs-Aspartate 115, TEA-Cl 20, EGTA 10, Hepes 10, MgATP 5, pH 7.2. Current-voltage (I-V) relationships for $I_{Ca}$ were built using 450 ms depolarization steps from a holding potential of −50 mV (range: −50 mV to +50 mV, in 10 mV increments at 1 Hz). 200 μM Cd^{2+} was applied and the protocol repeated. Subtracted currents obtained were normalised to cell capacitance.

**Persistent Na^{+} current ($I_{pNa}$):** Cells were superfused at 37°C with NT solution (K^+ substituted with Cs^+) and containing 10 μM nifedipine (Sigma, Poole, UK), 100 μM strophantidin (Sigma, Poole, UK) and 30 μM niflumic acid (Sigma, Poole, UK). The pipette filling solution contained (in mM): Cs-Aspartate 115, TEA-Cl 20, EGTA 10, Hepes 10, MgATP 5, pH 7.2. From a holding potential of -100 mV increasing voltage (20 mV) steps from -80mV to +20 mV for 1 s were applied at 0.33 Hz. The protocol was repeated in the presence of 30 μM tetrodotoxin (TTX) (Alomone Lab, Israel), and the TTX-sensitive current was analysed. $I_{pNa}$ density was taken as the average current between 50 and 100 ms after depolarisation. The currents recorded at -20 mV were also integrated between 50 and 300 ms after depolarisation and normalised to cell capacitance. This period was chosen because this is approximately the range of action potential durations measured in our experiments. The fast component of the Na^{+} current was not measured, given the possible poor voltage control during this phase.

**Antibodies**

Antibodies to brain spectrin, proteins 4.1R, 4.1G, 4.1B and 4.1N, have been
described previously\textsuperscript{13-15}. The following antibodies were obtained commercially: $\alpha$-actinin (EA53, Sigma-Aldrich, Poole, UK), tropomyosin (T2780, Sigma-Aldrich, Poole, UK), NaV1.5 (ASC-005, Alomone Labs, Jerusalem, Israel), NCX1 (p11-13, Swant, Bellinzona, Switzerland). Anti-PMCA2 antibody was prepared by immunization of rabbits with a peptide representing residues 5-19 of human PMCA2 (SwissProt:Q01814), and specific IgG isolated by standard procedures.

**Electron Microscopy**

Thin sections for electron microscopy and 0.2 $\mu$m frozen sections for immunofluorescence were prepared and analysed as described previously\textsuperscript{16}.

**Immunoblot procedure**

Heart homogenates were prepared for immunoblot analysis as previously described\textsuperscript{17}. 100 $\mu$g of protein were loaded per lane of SDS polyacrylamide gels. Immunoblots methods were as described previously\textsuperscript{15}. The blots were developed using Enhanced Chemiluminescence reagents (GE, Amersham, UK) and exposed to X-ray film for varying lengths of time. Films were scanned as 16-bit Tiff images, and bands quantified using ImageJ (NIH, US), taking care to ensure the blots used for quantification gave signals within the linear range of the film response.
Statistical analysis

Statistical comparison of data was performed using a two-way analysis of variance or Student’s t-test where appropriate. All statistical analyses were performed using Prism 4 (GraphPad Software, Inc. USA) and p<0.05 was considered significant. Data is expressed as Mean ± SEM (n) unless otherwise specified. For cellular studies, n is the number of myocytes; all the experiments were performed from a minimum of 4 cell isolations per group. In figures * indicates p<0.05, ** p<0.01 and *** p<0.001.
Online Fig. I. Assessment of cardiomyocyte size by planimetry to determine area (A), length (B) and width (C) in 188 WT and 303 4.1R KO myocytes. Cell capacitance, an index of cell surface area, was also measured during the electrophysiology experiments (D) in 31 WT and 40 4.1R KO myocytes. Both techniques demonstrated that no difference in cardiomyocytes size exists between WT and 4.1 R KO mice.
Online Fig. II. The 300 μM strophanthidin-sensitive INKP currents recorded in a WT (left) and 4.1R KO myocyte (right) are shown (upper traces). No significant difference was detected in the two groups (panel C, WT n=12 (5 mice) and 4.1R KO n=15 (6 mice); p>0.05).
Online Table I. ECG parameters recorded in conscious 4.1R KO and wild type mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>4.1R KO</th>
<th>Student’s t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>536 ± 27 (6)</td>
<td>440 ± 18 (6)</td>
<td><em>p = 0.01</em></td>
</tr>
<tr>
<td>R-R interval (ms)</td>
<td>113 ± 5.9 (6)</td>
<td>138 ± 5.9 (6)</td>
<td><em>p = 0.01</em></td>
</tr>
<tr>
<td>R-R dispersion (ms)</td>
<td>0.109 ± 0.02 (6)</td>
<td>0.172 ± 0.03 (6)</td>
<td><em>p = 0.15</em></td>
</tr>
<tr>
<td>PR interval (ms)</td>
<td>35.3 ± 1.4 (6)</td>
<td>37 ± 1.4 (6)</td>
<td><em>p = 0.41</em></td>
</tr>
<tr>
<td>QRS duration (ms)</td>
<td>13.9 ± 0.7 (5)</td>
<td>18.2 ± 0.7 (6)</td>
<td><em>p = 0.002</em></td>
</tr>
<tr>
<td>QT interval (ms)</td>
<td>47.6 ± 2.7 (6)</td>
<td>57 ± 1.8 (6)</td>
<td><em>p = 0.01</em></td>
</tr>
<tr>
<td>QTc interval (ms)</td>
<td>47 ± 2.2 (6)</td>
<td>53.9 ± 1.2 (6)</td>
<td><em>p = 0.02</em></td>
</tr>
</tbody>
</table>

Data expressed as MEAN ± SEM (n animals)
Reference List


