Cellular Biology

Lateral Membrane-Specific MAGUK CASK Down-Regulates Na\textsubscript{v}1.5 Channel in Cardiac Myocytes

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**Rationale:** Mechanisms underlying membrane protein localization are crucial in the proper function of cardiac myocytes. The main cardiac sodium channel, Na\textsubscript{v}1.5, carries the sodium current ($I_{\text{Na}}$) that provides a rapid depolarizing current during the upstroke of the action potential. Although enriched in the intercalated disc, Na\textsubscript{v}1.5 is present in different membrane domains in myocytes and interacts with several partners.

**Objective:** To test the hypothesis that the MAGUK (membrane-associated guanylate kinase) protein CASK (calcium/calmodulin-dependent serine protein kinase) interacts with and regulates Na\textsubscript{v}1.5 in cardiac myocytes.

**Methods and Results:** Immunostaining experiments showed that CASK localizes at lateral membranes of cardiac myocytes, in association with dystrophin. Whole-cell patch clamp showed that CASK-silencing increases $I_{\text{Na}}$ in vitro. In vivo CASK knockdown similarly increased $I_{\text{Na}}$ recorded in freshly isolated myocytes. Pull-down experiments revealed that CASK directly interacts with the C-terminus of Na\textsubscript{v}1.5. CASK silencing reduces syntrophin expression without affecting Na\textsubscript{v}1.5 and dystrophin expression levels. Total Internal Reflection Fluorescence microscopy and biotinylation assays showed that CASK silencing increased the surface expression of Na\textsubscript{v}1.5 without changing mRNA levels. Quantification of Na\textsubscript{v}1.5 expression at the lateral membrane and intercalated disc revealed that the lateral membrane pool only was increased upon CASK silencing. The protein transport inhibitor brefeldin-A prevented $I_{\text{Na}}$ increase in CASK-silenced myocytes. During atrial dilation/remodeling, CASK expression was reduced but its localization remained unchanged.

**Conclusion:** This study constitutes the first description of an unconventional MAGUK protein, CASK, which directly interacts with Na\textsubscript{v}1.5 channel and controls its surface expression at the lateral membrane by regulating ion channel trafficking. ([Circ Res. 2016;119:544-556. DOI: 10.1161/CIRCRESAHA.116.309254.]

**Key Words:** calcium/calmodulin-dependent serine protein kinase • cardiac myocytes • dystrophin • ion channels • membrane-associated guanylate kinase • sodium channels • syntrophin

Cardiac myocytes are highly differentiated striated muscle cells possessing well-organized membrane domains with specialized roles in their electric and mechanical functions. The T-tubule system and intercalated discs (IDs) are the best characterized membrane domains and are crucial for the excitation–contraction coupling process and the propagation of action potentials (APs) from one myocyte to the other. Specialized membrane domains contain distinct populations of ion channels, pumps, exchangers, and receptors such as the voltage-gated L-type calcium channel and gap-junction protein connexin-43, for T-tubules and ID, respectively.\(^1\)

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organization of specialized membrane domains in structurally polarized cells such as neurons, epithelial cells, or cardiac myocytes. MAGUK proteins have emerged as key partners participating in this organization. For instance, in the central nervous system, MAGUK proteins organize synapses through their ability to establish specific macromolecular complexes. In cardiac myocytes, SAP97 is a key regulator of ion channel function and organization, whereas zonula occludens-1 (ZO-1) organizes the gap-junction protein connexin-43 at the ID. Similar to other MAGUK proteins, CASK (calcium/calmodulin-dependent serine kinase) displays a multidomain structure enabling interaction with multiple proteins including ion channels and receptors, cell-adhesion proteins, and the actin cytoskeleton. However, among MAGUK proteins, CASK differs because of the possession of an active CaMKII (Ca$^{2+}$/calmodulin-dependent protein kinase II) homolog domain in the N-terminal and a guanylate kinase domain in its C-terminus, which is involved in CASK translocation to the nucleus and subsequent regulation of gene expression. Despite previous works reporting the expression of CASK in the myocardium, its function has not been investigated. Therefore, we investigated the potential role of CASK in the regulation of Na$_{\text{v}}$1.5 channels in cardiac myocytes.

Here, we present the first detailed characterization of the localization and function of CASK in the heart. CASK was detected specifically in costameric structures of the lateral membrane (LM) of cardiac myocytes, in association with dystrophin. Using a combination of whole-cell patch-clamp, high-resolution 3-dimensional (3D) deconvolution microscopy and biochemistry, we showed that CASK interacts with Na$_{\text{v}}$1.5 and negatively regulates the corresponding current in cultured adult atrial myocytes. CASK knockdown in vivo revealed that CASK exerted the same regulatory function in freshly isolated ventricular myocytes. Pull-down assays showed that CASK directly interacts with the C-terminus of Na$_{\text{v}}$1.5. Biotinylation and Total Internal Reflection Fluorescence microscopy (TIRFm) revealed that CASK silencing increased Na$_{\text{v}}$1.5 expression at the cell surface without changing Na$_{\text{v}}$1.5 mRNA and protein levels. However, CASK silencing reduced the expression of syntrophin. The $I_{\text{Na}}$ increase induced by CASK silencing was completely prevented by the protein transport inhibitor brefeldin-A, suggesting the involvement of CASK in trafficking processes. Finally, we showed that CASK expression is reduced in remodeling/dilated atrial biopsies without changes in localization. These results not only strengthen the concept of differentially regulated pools of Na$_{\text{v}}$1.5 channels within the cardiac myocyte playing distinct roles in cardiac physiology but also suggest that CASK could participate in maintaining low levels of Na$_{\text{v}}$1.5 at the LM and consequently contribute to anisotropic conduction.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Cell Preparations, Transfections, and Adenoviral Infections**

Adult atrial or ventricular myocytes were obtained by enzymatic dissociation on a Langendorff column as previously described. Adult rat atrial myocytes were infected with adenovirus- scramble short hairpin RNA (Ad-shscr), adenoviral construct containing a short hairpin RNA for CASK (Ad-shCASK), Ad-green fluorescent protein (GFP), or Ad-CASK. Human embryonic kidney 293 (HEK293) cells stably expressing Na$_{\text{v}}$1.5 were transiently transfected with either shscr or shCASK and plasmid internal ribosome entry site (pIRES)-GFP or pIRES-GFP-CASK.

**Cardiac-Specific CASK Knockdown Mouse**

Mice were generated by Cre/loxP-mediated gene targeting through homologous recombination. The homozygous floxed CASK$^{\text{flx/flx}}$ mouse was bred with a cardiac myocyte-specific Cre-recombinase mouse, α myosin heavy chain (αMHC)-Cre.

**Semiquantitative Real-Time Polymerase Chain Reaction**

Details of specific primers designed to amplify cask, scn5a, and housekeeping genes are described in the Online Data Supplement.

**Protein Extraction, Western Blot, and Pull-Down Assays**

These procedures were performed as previously reported. See Online Data Supplement for other details.

**Deconvolution Microscopy, Evanescent Field Microscopy and Image Analysis**

Three-dimensional deconvolution epifluorescence and TIRFm images were acquired as described previously. Analysis was performed using ImageJ software (National Institutes of Health). Details are given in the Online Data Supplement.

**Electrophysiological Measurements**

Patch-clamp recordings were performed in the whole-cell configuration at room temperature. Details and protocols are available in the Online Data Supplement.

**Human Samples**

In accordance with approval of Institution Ethic Committee, biopsies of right atrial appendages were obtained from patients undergoing cardiac surgery (Online Data Supplement).

**Statistics**

Data are represented as mean±SEM; *P<0.05, **P<0.01, and ***P<0.001; ns: not significant.

**Results**

**MAGUK CASK Is Expressed at the LM and Excluded From the ID**

We first looked at the expression of CASK in adult myocardium by Western blot. CASK was expressed both in rat atria and ventricle lysates, with a ~2-fold larger expression in atria. CASK was also expressed in myocyte- and fibroblast-enriched fractions (Figure 1A and 1B). Second, we examined the localization of CASK in rat atrial and ventricular myocardial cryosections using high-resolution 3D deconvolution microscopy.
In ventricle, CASK was expressed along the LM stained with caveolin3 (Figure 1C, top), whereas no signal was detectable at the ID stained with N-cadherin (Figure 1C, middle). CASK also exhibited a striated pattern, facing the sarcomeric α-actinin corresponding to z-line (Figure 1C, bottom), suggesting either a T-tubular or a costameric localization. The distribution of CASK about the different compartments of cardiac myocytes was similar in the atrial myocardium (Online Figure I).

**CASK Is a New Member of the Dystrophin–Glycoprotein Complex at the LM of Cardiac Myocytes**

In freshly isolated myocytes, CASK staining was apparent along the LM and exhibited the z-line superposition reported above. To investigate whether CASK was located at the T-tubule, co-immunofluorescence experiments were conducted with Cav1.2 or ryanodine receptor 2 (RyR2). However, little colocalization was observed in myocytes (Online Figure IIA). In addition, after detubulation by formamide treatment, the striated pattern was still observed in live ventricular myocytes stained with the voltage-sensitive dye Di-8-ANEPS (di-8-aminonaphthalene-9-lysrenium; Online Figure IIB).

Next, the association of CASK with either vinculin or dystrophin costameric complexes was investigated in freshly isolated adult atrial myocytes (Figure 2A and 2B). CASK more closely associated with dystrophin ($R=0.68$) than with vinculin ($R=0.31$; Figure 2A). In contrast with vinculin, which is organized in
rib-like structures at the sarcolemma, CASK showed a continuous layering of the sarcolemma, similar to dystrophin (Figure 2B). In addition, CASK and dystrophin displayed a regular interval alignment of ≈2 µm, the interval between 2 z-lines, in the midsection of cardiac myocytes (Figure 2C). The association between CASK and dystrophin was further supported by coimmunoprecipitation experiments performed on whole-heart lysates (Figure 2D). Finally, immunostainings performed on dystrophin-deficient mdx mouse ventricular cryosections showed a loss of CASK staining, suggesting that CASK expression at the LM of cardiac myocytes depends on dystrophin expression (Figure 2E). Note that the striated distribution of CASK was not observed in mouse myocardium, suggesting distinct costameric organization between species.

Altogether, these results indicate that CASK is poorly expressed at the T-tubule under normal conditions and is a new member of the dystrophin–glycoprotein complex.

CASK Directly Interacts With Na$_v$1.5

Because Na$_v$1.5 associates with the dystrophin–glycoprotein complex at the LM, we explored the interaction of CASK with Na$_v$1.5. In freshly isolated atrial myocytes, CASK and Na$_v$1.5 exhibited both a sarcolemmal and striated expression and partially overlapped (R=0.31; Figure 3A and 3B). This partial overlap is likely because of high expression of Na$_v$1.5 channels at the ID where CASK is not present. Periodic intervals between 2 striations calculated using Fast Fourier Transform revealed a coherent distance of ≈2 µm (Figure 3A). Of note, in this selected focal plane, the ID was not visible. The interaction between CASK and Na$_v$1.5 in the rat myocardium was supported by coimmunoprecipitation experiments (Figure 3C).

To investigate whether CASK could specifically interact with Na$_v$1.5, pull-down experiments were performed using the wild-type (WT) Na$_v$1.5 C-terminus (Ct) or deleted for the PDZ-binding motif corresponding to the 3 last SIV residues (ΔSIV; Figure 3D). Western blot experiments performed using anti-CASK and anti pan-syntrophin antibody revealed that the Na$_v$1.5 WT Ct precipitated the 2 proteins from rat ventricle lysates. This interaction was dependent on the SIV motif of Na$_v$1.5 since interactions with CASK and syntrophin were, respectively, reduced by 94% and 61% with the truncated construct (Figure 3E). Finally, direct interaction between CASK and the Na$_v$1.5 Ct was investigated by performing pull-down
Figure 3. CASK (calcium/calmodulin-dependent serine protein kinase) is associated with and interacts with Na\textsubscript{v}1.5 channels. A, High-resolution 3-dimensional deconvolution microscopy images of CASK (top) and Na\textsubscript{v}1.5 (bottom) taken from freshly isolated atrial myocytes and enlargement of outlined squares. Intensity plots for CASK and Na\textsubscript{v}1.5 drawn from 12-µm lines in the midsection of the cell and interval distribution (middle; n=8). B, Coimmunostaining of CASK and Na\textsubscript{v}1.5 performed with another pair of antibodies and enlargement of outlined square. C, Representative coimmunoprecipitation assay of CASK and Na\textsubscript{v}1.5 performed on whole-heart lysates (n=4). D, Scheme of GST fusion proteins used to perform pull-down experiments. The 66 last amino acids (Ct) of wild-type (WT) Na\textsubscript{v}1.5 or deleted for the PDZ-interacting motif (SIV) were compared with GST alone. E and F, Representative Western blots after GST pull-downs performed with cardiac myocyte-enriched lysates (E, n=3) or purified protein CASK (F, n=3). CASK and syntrophin both interact with Na\textsubscript{v}1.5 C-terminus, and a part of this interaction is mediated by the SIV motif. Ponceau stainings served as loading controls.
experiments with the purified CASK protein (Figure 3F). At 10 ng of purified CASK protein, the interaction was reduced by 21% but not abolished in ΔSIV.

These results support that CASK interacts directly with Na,1.5 Ct, regardless of the presence of syntrophin, and that other residues than SIV are likely involved in this interaction.

**CASK Regulates the Sodium Current In Vitro**

To assess the functional consequence of the interaction between CASK and Na,1.5, $I_{Na}$ was recorded in adult atrial myocytes using whole-cell patch-clamp after modulating the expression of CASK. Representative images of adenoviral infection efficiency in cultured rat atrial myocytes are presented Online Figure IIIA. CASK was overexpressed using an adenoviral construct containing the GFP reporter (Ad-CASK) and compared with GFP alone (Ad-GFP). CASK mRNA was increased by 120% and protein level by 200% in Ad-CASK after 3 days in vitro (DIV; Online Figure IIIB through IIID). Average current–voltage ($I–V$) plots from voltage-clamp experiments showed a significant decrease in $I_{Na}$ density at different tested voltages in Ad-CASK (Figure 4A through 4C). CASK overexpression shifted the steady-state inactivation curve leftward by ~8 mV ($P<0.001$; Figure 4E), whereas no difference was found for activation properties between Ad-CASK and Ad-GFP–infected cells (Figure 4D). Next, we used adenoviral transfer technology to knockdown CASK in adult rat atrial myocytes. Silencing experiments were performed using GFP Ad-shCASK and compared with Ad-shscr. CASK mRNA was decreased by 60% and protein level by 40% in Ad-shCASK at 5 DIV (Online Figure IIIB through IIIID). Voltage-clamp experiments showed a significant increase in $I_{Na}$ at several tested voltages in Ad-shCASK (Figure 4B through 4F). No difference was found for activation properties between Ad-shCASK and Ad-shscr cells, whereas steady-state inactivation was ~8 mV more positive in Ad-shCASK cells ($P<0.05$; Figure 4G and 4H). It is worth noting that the CASK-dependent shift in steady-state inactivation cannot explain the 2-fold change in $I_{Na}$ density. These experiments clearly demonstrate that CASK exerted a repressive function on $I_{Na}$.

**Regulatory Function of CASK on $I_{Na}$ Is Conserved In Vitro From Polarized Cardiac Myocytes to Nonpolarized HEK293 Cells**

As CASK expression was restricted to the LM of cardiac myocytes, we investigated whether the regulatory function of $I_{Na}$ by CASK could be dependent on the loss of structural polarity and dedifferentiation under culture conditions.

We followed the distribution of CASK in cardiac myocytes in vitro in accordance with the distribution of the adherens junction protein N-cadherin. Immediately after dissociation, CASK was absent from the ID where N-cadherin was confined. At DIV1, N-cadherin started to be internalized and at DIV2, was randomly distributed in the cytoplasm. At the same stage, CASK was located at the cell membrane. After 1 week in culture (DIV7), cardiac myocytes had re-established contacts (ID-like structures) in which N-cadherin was located and CASK excluded (Online Figure IV). Therefore, CASK localization seemed related to the architecture/polarity of cardiac myocytes.

**Negative Regulation of NaV1.5 by CASK**

Thus, to investigate whether the regulation of $I_{Na}$ by CASK could be polarity dependent, we induced the formation of adherens junctions in vitro. Adult atrial myocytes infected with Ad-shscr or Ad-shCASK were seeded on Fc fragment plus N-cadherin (N-cadh) or Fc fragment alone, used as a control (Fc; Online Figure VA). Atrial myocytes seeded on N-cadh showed both increased spreading and increased N-cadherin staining as shown by TIRFm (Online Figure VA). Average $I–V$ plots from voltage-clamp experiments showed a significant increase in $I_{Na}$ density at several tested voltages in the 2 coating conditions (Fc and N-cadh) in Ad-shCASK–infected myocytes (Online Figure VB). No difference was found between Ad-shCASK–infected myocytes cultured on N-cadh or Fc for activation properties, whereas the steady-state inactivation curve was significantly shifted rightward in Ad-shCASK myocytes in the 2 coating conditions ($≈12$ and 10 mV, respectively, $P<0.05$; Online Figure VC and VD).

Next, $I_{Na}$ was recorded in the nonpolarized HEK293 cell line stably expressing cardiac Na,1.5 α subunits (HEK293-Na,1.5; Online Figure VIA). We first overexpressed CASK using a GFP plasmid construct (CASK), and control HEK293-Na,1.5 were transfected with GFP alone (GFP). CASK expression was 2-fold increased in CASK at 3DIV (Online Figure VIB and VIC). Average $I–V$ plots from voltage-clamp experiments showed a significant decrease in $I_{Na}$ density in CASK compared with control cells (Online Figure VID). CASK overexpression shifted the steady-state inactivation curve by ~6 mV ($P<0.05$; Online Figure VIE), whereas activation properties were unchanged (Online Figure VIE and VIF). We next used CASK sh-RNA plasmid with the red fluorescent protein (RFP) reporter (shCASK) to transfect HEK293-Na,1.5; scramble short hairpin RNA with RFP was used as control (shscr). CASK protein level was decreased by 45% in shCASK at 5 DIV (Online Figure VIB and VIC). Voltage-clamp experiments showed a significant increase in $I_{Na}$ density at several tested voltages in shCASK-transfected cells (Online Figure VIG). Steady-state inactivation was ~6 mV more positive in shCASK cells ($P<0.01$), whereas no difference was found for activation properties between groups (Online Figure VIH and VII).

These results confirmed that CASK is excluded from ID structures and established that the negative regulatory function of CASK is a conserved mechanism.

**Regulatory Function of CASK Is Conserved in Myocytes From CASK Knockdown Mouse**

To assess the functional regulation of sodium channels by CASK in vivo, $I_{Na}$ was recorded in freshly isolated ventricular myocytes from αMHC-Cre/CASK and αMHC-Cre/WT mice. The homozygous floxed CASK tm1Sud mouse was bred with an α-myosin heavy chain (αMHC)-Cre mouse to target cardiac myocytes. Recombination with αMHC-Cre leads to ~68% CASK knockdown in cardiac myocytes ($P<0.01$; $n=3$; Figure 5A). The incomplete extinction of CASK is likely because of contamination by nonmyocyte cells. Average $I–V$ plots from voltage-clamp experiments showed a significant increase in $I_{Na}$ density at several tested voltages in CASK knockdown compared with WT Cre myocytes (Figure 5B and 5C). No significant differences were found for activation properties...
and steady-state inactivation between CASK knockdown and WT Cre mouse myocytes (Figure 5D and 5E).

The results obtained in CASK knockdown ventricular myocytes together with the results obtained in HEK293-NaV1.5 and cultured atrial myocytes support that CASK-induced effect is not dependent on the experimental model used or on atrial versus ventricular myocytes.

CASK Affects Trafficking of NaV1.5

Because \( \text{I}_{\text{Na}} \) was regulated by CASK expression level, we dissected out the mechanisms of NaV1.5 regulation. Reverse transcription-quantitative polymerase chain reaction experiments were performed to investigate whether CASK influenced the expression level of \( \text{scn5a} \). However, the copy number of \( \text{scn5a} \) was not different between controls and CASK-overexpressing or CASK-silenced myocytes (Figure 6A). Western blots performed from lysates of Ad-shscr– and Ad-shCASK–infected atrial myocytes revealed that NaV1.5 protein expression was not modified by CASK silencing. Interestingly, CASK silencing impacted syntrophin level, reducing its expression by \( \approx 20\% \) (\( P<0.001 \)), whereas dystrophin levels remained unchanged (Online Figure VII). Biotinylation experiments were performed to assess NaV1.5 surface expression. In Ad-shCASK myocytes, NaV1.5 expression was reduced by \( \approx 20\% \) in the total fraction whereas increased by \( \approx 45\% \) in the membrane fraction (\( P<0.05 \); Figure 6B). TIRFm experiments also demonstrated a \( \approx 20\% \) increase in surface staining of NaV1.5 in Ad-shCASK myocytes (\( P<0.001 \); Figure 6C). These results showed that the increase in \( \text{I}_{\text{Na}} \) on CASK silencing was neither transcriptional nor translational and rather because of increased surface expression of NaV1.5. Therefore, a likely explanation was that CASK regulates anterograde trafficking and/or stabilization at the sarcolemma.

As the canonical early step in ion channel trafficking is the transport from the endoplasmic reticulum (ER) to the Golgi apparatus, the hypothesis of an increased forward trafficking was tested in silencing conditions. Twelve hour post infection with Ad-shscr or Ad-shCASK, cardiac myocytes were treated...
for 48 hours with brefeldin-A (BFA) before electrophysiological recordings. Voltage-clamp experiments showed that $I_{\text{Na}}$ density was not increased in Ad-shCASK–infected cells after BFA treatment (Figure 6D). TIRFm experiments also showed a reduced NaV1.5 surface staining in Ad-shCASK myocytes treated with BFA (Figure 6E). In addition, biotinylation experiments performed on HEK293-NaV1.5 cells showed that the increase in NaV1.5 surface expression was reduced after BFA treatment in shCASK cells (Figure 6F).

Therefore, these results indicate that alterations in trafficking processes are involved in the effect of CASK in the regulation of $I_{\text{Na}}$.

**CASK Silencing Modulates the Expression of Na$_{\text{v}}$1.5 Channels at the LM**

To understand further the mechanisms of NaV1.5 regulation by CASK, we investigated whether CASK silencing affected a specific Na$_{\text{v}}$1.5 channel population. Ad-shscr– or Ad-shCASK–infected atrial myocytes were cultured at half confluence, a situation that allows formation of cell contacts (ID-like structures) between some myocytes and the preservation of free membranes (LM-like structures; Figure 7A). Immunostainings of Na$_{\text{v}}$1.5 channels were performed and quantified by drawing lines in contact zones versus free membranes. As shown Figure 7B, the Na$_{\text{v}}$1.5 signal was increased by ≈21% at LM ($P<0.01$) and by ≈10% at ID (not significant) in CASK-silenced myocytes (Figure 7B). These results support that the LM membrane pool of Na$_{\text{v}}$1.5 channels is more susceptible to CASK modulation. In addition, it supports the concept that different Na$_{\text{v}}$1.5 pools coexist in cardiac myocytes.

**CASK Expression Is Downregulated in Remodeled Atria, Whereas Its Localization Is Unchanged**

Given the specific targeting of CASK to the costamere, we examined whether the expression and localization of the protein could be altered during conditions characterized by the remodeling of myocardial structure and of myocyte architecture such as dilated atria.18,20,21

In control human atrial tissue samples, CASK was expressed at the LM and excluded from ID as in rat (Figure 8A). In patients with dilated atria, CASK remained localized at the costamere whereas its expression level was reduced as evidenced by fainter fluorescence intensity ($P<0.001$; Figure 8B) and a by ≈42% decrease in total protein expression ($P<0.01$; Figure 8C). Chronically hemodynamic atrial overload associated with the development of ischemic heart failure in rat is also characterized by profound ultrastructural alterations of atrial myocytes.18,20 In this model too, CASK protein expression was decreased, whereas its localization in membrane subdomains remained unchanged (Online Figure VIII). These results indicate that CASK expression is downregulated during atrial remodeling.

**Discussion**

This study is the first characterization of CASK in cardiac myocytes. We found that CASK is the sole cardiac MAGUK protein identified to date excluded from the ID and strictly located at LM, where it associated with dystrophin. We showed that CASK directly interacts with Na$_{\text{v}}$1.5 C-terminus and downregulates the sodium current. CASK silencing decreases syntrophin protein levels without impacting Na$_{\text{v}}$1.5 or dystrophin
Figure 6. CASK (calcium/calmodulin-dependent serine protein kinase) regulates early anterograde trafficking of Na\textsubscript{v}1.5 channels. 

A, RT-qPCR histogram of CASK mRNA expression levels in myocytes infected with Ad-GFP (black), Ad-CASK (green), Ad-scramble short hairpin RNA (shscr; black), or Ad-short hairpin RNA for CASK (shCASK; red; N=4). B, Representative biotinylation assay performed in Ad-shscr– and Ad-shCASK–infected atrial myocytes and corresponding histogram showing the expression level of Na\textsubscript{v}1.5 normalized to GAPDH in total and surface fractions (n=3). C, Example Total Internal Reflection Fluorescence microscopy (TIRFm) images taken from fixed Ad-shscr (black) and Ad-shCASK (red) atrial myocytes and corresponding histogram showing the expression level of Na\textsubscript{v}1.5 normalized to GAPDH in total and surface fractions (n=3). D, Current density–voltage relationships (25 mmol/L [Na\textsuperscript{+}]) of I\textsubscript{Na} obtained from adult atrial myocytes infected with Ad-shscr (black) or Ad-shCASK treated with brefeldin-A (BFA; purple) or nontreated (red; N=3). E, Histogram of Na\textsubscript{v}1.5 EFF intensity from TIRFm images taken from fixed atrial myocytes infected with Ad-shscr (black) and Ad-shCASK treated with BFA (purple) and nontreated (red; N=3). F, Representative biotinylation assay performed in shscr and shCASK transfected HEK-Na\textsubscript{v}1.5 cells and corresponding histogram showing the expression level of Na\textsubscript{v}1.5 normalized to GAPDH in total and surface fractions (n=3). *P<0.05, **P<0.01, ***P<0.001, ns, not significant.
expression levels. We demonstrated that CASK modulates the early trafficking of Na\textsubscript{1.5} and prevents its functional expression at the cell surface and more specifically at the LM. Finally, we showed that CASK expression is reduced during atrial remodeling but its localization remained unchanged.

**Unique Localization of CASK in Cardiac Myocytes**

The 2 already known cardiac MAGUK proteins, SAP97 and ZO-1, are both preferentially expressed at the ID, a membrane domain specialized in the rapid propagation of APs along cardiac fibers. IDs, located at the ends of cardiac myocytes, are the sites of intense electric activity and concentrate numbers of ion channels, notably the main cardiac sodium channel, Na\textsubscript{1.5}. It has been proposed that this organization facilitates longitudinal propagation of the AP over transverse propagation and determines conduction velocity.\textsuperscript{22}

Na\textsubscript{1.5} channels located at the ID are associated with different partners such as connexin-43,\textsuperscript{2} plakophilin-2,\textsuperscript{2,4} ankyrin-G,\textsuperscript{5,6} and SAP97.\textsuperscript{7,8} All of these partners participate in anchoring/stabilization of Na\textsubscript{1.5} at the ID. In accordance, the sodium current recorded by macropatch in the midsection of cardiac myocytes (LM) is of smaller amplitude than the current recorded at the ID.\textsuperscript{23–25} At the LM, Na\textsubscript{1.5} has been shown to interact with the dystrophin/syntrophin complex.\textsuperscript{19} In the dystrophin-deficient mdx mouse model of Duchenne muscular dystrophy, the LM pool of Na\textsubscript{1.5} is strongly reduced and $I_{\text{Na}}$ is decreased by $\approx 30\%$.\textsuperscript{8,19} In agreement, transverse conduction velocity is more reduced than longitudinal conduction velocity in $\Delta$SIV mouse hearts.\textsuperscript{25} From these observations, a scheme can be drawn on the relative contribution of the populations of Na\textsubscript{1.5} in the propagation of the AP: the ID pool having a higher weight than the LM pool. Our results indicate that CASK could participate in the maintenance of low level of functional Na\textsubscript{1.5} at the LM.

**CASK, a New Member of the Dystrophin–Glycoprotein/Na\textsubscript{1.5} Macromolecular Complex**

Scanning ion conductance microscopy identified 3 topographical entities in the LM: T-tubules, crests, and z grooves, the latter corresponding to T-tubule openings.\textsuperscript{26} Like T-tubules, crests show a periodicity of $\approx 2\ \mu$m.\textsuperscript{27} Here, we focused on the LM distribution of CASK into microdomains using high-resolution 3D deconvolution microscopy. We showed that CASK was in closer association with dystrophin than with...
vinculin, 2 costameric proteins. In addition, we showed that CASK was able to precipitate dystrophin and that CASK staining at LM was extinct in mdx cardiac myocytes, suggesting that dystrophin is necessary for proper localization of CASK. However, in contrast to dystrophin which also extends along T-tubules in the rat myocardium, CASK was not found deep in the T-tubule because it did not colocalize with calcium channels or ryanodine receptors and its expression pattern was not affected by detubulation.

Interestingly, we showed that CASK silencing reduced syntrophin levels, whereas dystrophin and NaV1.5 levels were unchanged. In addition, we showed that CASK directly interacts with the Na,v,1.5 channel C-terminus using myocyte-enriched fraction from rat heart lysates. Moreover, Na,v,1.5 C-terminus also interacted with purified CASK protein, suggesting that Na,v,1.5 and CASK interaction is not dependent on the presence of syntrophin. Altogether, these results suggest that CASK is downstream dystrophin and upstream syntrophin and that syntrophin and CASK could compete for Na,v,1.5 channel binding, likely at early stages of trafficking.

Super-resolution scanning patch-clamp recordings showed that Na,v,1.5 channels do not distribute homogeneously along the LM but instead form clusters confined into functional microdomains, with the largest aggregated at crest regions. Here, we showed that CASK, Na,v,1.5, and dystrophin all displayed a periodicity of 2 µm and that CASK was associated with Na,v,1.5. In light of this distribution and poor expression in T-tubules, one could speculate that the macromolecular complex formed by Na,v,1.5, CASK, and dystrophin corresponds to crest structures identified by scanning ion conductance microscopy. The differential distribution of Na,v,1.5 in microdomains of the LM suggests distinct roles for crests and T-tubules in electric and contractile activities of cardiac myocytes. For instance, Na,v,1.5 located at the crest could play a preferential role in the propagation of the AP along the LM, whereas those located in T-tubules could be involved in excitation–contraction coupling. In this line, the specific localization of CASK at LM and its exclusion from T-tubules suggests a role for CASK in the regulation of the surface pool/crest population of sodium channels.

Mechanisms of Regulation of I Na by CASK

MAGUKs are multidomain scaffolding proteins known to be involved in trafficking, targeting, and signaling of ion channels and receptors. In neurons, differential sorting of glutamate receptor subtypes has been shown to start in the ER and to depend on SAP97 and CASK. Although AMPARs are trafficked to the plasma membrane via the classical secretory pathway, NMDARs are diverted toward a specialized ER subcompartment. This ER subcompartment is composed of NMDAR subunits, CASK and SAP97. Although CASK does not directly bind to NMDAR, it prevents the SAP97-dependent ER retention of NMDAR. When bound to CASK, SAP97 colocalizes with NMDAR, whereas when unbound, SAP97 preferentially associates with AMPAR. These studies suggest that CASK regulates SAP97 association with glutamate receptor subtypes and subsequent sorting and trafficking to correct location. In cardiac myocytes, Shy et al showed that the last 3 residues of Na,v,1.5 in C-terminus, the SIV motif, are essential for the localization of Na,v,1.5 at the LM, but not
required for its expression at the ID and T-tubules. The SIV motif interacts with syntrophin at the LM and with SAP97 at the ID, thereby defining distinct pools of NaV1.5 multiprotein complexes. However, the mechanisms regulating the differential sorting of NaV1.5 to specific membrane subdomains have not been elucidated.

In this context, the present study provides new insights in processes regulating the sorting of NaV1.5. Our results revealed that CASK exerts a repressive effect on the functional expression of NaV1.5. In vitro silencing or in vivo knockdown of CASK increased \( I_{Na} \). In addition, this negative regulatory function was conserved for both atrial and ventricular myocytes. We showed that the current increase observed after CASK silencing was because of increased surface expression of NaV1.5, and more specifically, at the LM. Treatment with the inhibitor of forward transport between ER and Golgi apparatus, BFA, prevented the effect. This result shows that CASK impedes NaV1.5 trafficking. Therefore, one could hypothesize that NaV1.5 sorting could be determined during anterograde processing steps of trafficking. Despite a common biosynthetic pathway, the early association of the channel with \( \beta \) auxiliary subunits and with other partners during biosynthesis could determine its sorting and final targeting to specific subdomains. Our results support that CASK plays a role in maintaining low level of NaV1.5 at the LM by repressing a yet unknown identified step of channel trafficking. In this regard, CASK is unique among MAGUK proteins investigated to date that usually favors anchoring/stabilization of ion channels at the sarcolemma.

**CASK Expression Is Reduced During Atrial Remodeling**

Costameres encircle myocytes perpendicularly to their longitudinal axis in alignment with z-lines, making the bridge between extracellular matrix, sarcolemma, and intracellular contractile sarcomeres. They reinforce the sarcolemma during cardiac workload and transmit contractile forces exerted by myocytes or extracellular matrix in a bidirectional manner.

Both in human and rat, diseased atria characterized by extensive structural alterations of atrial myocytes, CASK expression levels was reduced, whereas its LM localization was not modified. Whether this downregulation of CASK is associated with the redistribution of NaV1.5 subpopulations in diseased myocardium needs to be carefully examined.

In summary, this study constitutes the first description of an unconventional MAGUK protein both in terms of localization and function in the heart. CASK acts as a repressor of the population of NaV1.5 dedicated to the LM by preventing early trafficking. These results represent a substantial step forward in the concept of differentially regulated pools of NaV1.5 within the cardiac myocyte having distinct roles in cardiac physiology. Notably, CASK could participate in maintaining low level of NaV1.5 at the LM and consequently contribute to the anisotropic conduction.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is Known?**

- Cardiac sodium channels are located in distinct membrane domains of the cardiac myocyte and interact with several partners, defining different populations of sodium channels that could play different roles in cardiac electrophysiology.

- At the intercalated disc, a high concentration of sodium channels is found in interaction with several anchoring partners where they contribute to the fast and longitudinal propagation of the action potential.

- While recent studies have identified another subpopulation of sodium channels at the lateral membrane, which underlies a sodium current of small amplitude, the partners and mechanisms regulating this subpopulation are still largely unknown.

**What New Information Does This Article Contribute?**

- In atrial and ventricular myocardium, we identified a new MAGUK protein, CASK, which is restricted to the lateral membranes of cardiac myocytes and interacts with both the dystrophin–glycoprotein complex and sodium channels, defining a new multimolecular complex in this specific membrane domain.

- CASK negatively regulates the sodium current both in vitro and in vivo and reduces the surface expression of sodium channels at the lateral membrane by impeding channel trafficking.

- CASK seems to be a crucial determinant of the organization of the subpopulation of sodium channel of the lateral membrane; reduced CASK expression in diseased myocardium might contribute to the alteration of the normal anisotropic propagation of electric impulses.

Cardiac myocytes are highly organized cells, characterized by specific plasma membrane domains specialized in mechanical and electric functions. These domains are constituted by macromolecular complexes containing several ion channels and partners, particularly well described for the intercalated disc. Here, we describe a new macromolecular complex at the lateral membrane of cardiac myocytes that organizes a subpopulation of sodium channels. We found that the MAGUK protein CASK is an unconventional partner of Na+ channels in cardiac myocytes. CASK is specifically distributed in costamere structures of the lateral membrane in association with dystrophin. We show that CASK directly interacts with Na+1,5 and negatively regulates the corresponding current both in vitro and in vivo by impeding Na+1,5 trafficking to the plasma membrane. Finally, we show that CASK expression is reduced in remodeled/dilated atrial biopsies without changes in localization. These results not only strengthen the concept of differentially regulated pools of Na+1,5 channels within the cardiac myocyte but also suggest that CASK could participate in maintaining low levels of active Na+1,5 at the lateral membrane. Given the role of Na+1,5 in the fast activation and propagation of the action potential, the repressive effect of CASK on Na+1,5 could contribute to the anisotropic conduction of the depolarization wave.
Lateral Membrane-Specific MAGUK CASK Down-Regulates Na\textsubscript{\textit{v}} 1.5 Channel in Cardiac Myocytes

Catherine A. Eichel, Adeline Beuriot, Morgan Y.E. Chevalier, Jean-Sébastien Rougier, Florent Louault, Gilles Dilanian, Julien Amour, Alain Coulombe, Hugues Abriel, Stéphane N. Hatem and Elise Balse

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Animals
Adult male Wistar rats (Janvier) were treated in accordance with French Institutional guidelines (Ministère de l’Agriculture, France, authorization 75-1090) and conformed to the Directive 2010/63/EU of the European Parliament.

Cardiomyocyte Isolation
Adult atrial or ventricular cardiomyocytes were obtained by enzymatic dissociation on a Langendorff column as previously described. Briefly, hearts were retrogradly perfused at 5mL/min through the aorta, first with an enzymatic solution containing in mmol/L: NaCl 100, KCl 4, MgCl₂ 1.7, glucose 10, NaHCO₃ 5.5, KH₂PO₄ 1, HEPES 22, BDM 15 and 200UI/mL collagenase A (Roche Diagnostics) plus 0.5% bovine serum albumin, pH 7.4 (NaOH) at 37°C. Solution was bubbled with 95% O₂/5% CO₂ throughout. Tissue was cut into small pieces and gently minced with a glass pipette. Cells were plated on laminin-coated (Roche) 35mm Petri dishes or 18mm coverslips and maintained in Kraft-Brühe (KB) solution, in mmol/L: glutamate Acid Potassium Salt Monohydrate 70, KCl 25, Taurine 20, KH₂PO₄ 10, MgCl₂ 3, EGTA 0.5, Glucose 10, HEPES 10, until experiments. Isolated cells were used for immunofluorescence experiments within 2-8 hours after isolation (freshly isolated cells) or after 3-5 days in culture (immuno-fluorescence experiments or patch-clamp recordings). Cells were cultured under standard conditions (37°C, 5% CO₂) in M199 medium (Gibco) supplemented with 1% penicillin/streptomycin (Gibco), 5% fetal bovine serum (Gibco) and 1‰ insulin/transferrin/selenium cocktail (Sigma). Detubulation was achieved using formamide (1.5 mol/L) for 10 min. To induce formation of adherens junctions with the substrate, Petri dishes were coated with goat anti-human Fc antibody (50 μg/ml) before seeding the cells. Brefeldin-A was incubated for 48h (5 μg/ml) 12h after adenoviral infections.

Cardiac-specific CASK Knockdown mouse
Mice were generated by Cre/loxP-mediated gene targeting through homologous recombination. The homozygous floxed CASK<sup>tm1Sud</sup> mouse (The Jackson Laboratory, Atasoy et al., 2007) was bred with a cardiac myocyte-specific Cre-recombinase mouse, α myosin heavy chain (αMHC)-Cre (The Jackson Laboratory).

Plasmids and adenoviruses
ShRNA sequences for CASK 5’-tgagaatgtgaccagagttcgcctggta-3’ (shCASK) or its control (shscr) 5’-gcactaccagagctaactcagatagtact-3’ were provided by Origene into a pRFP-C-RS vector. For overexpression experiments, the CASK sequence (NM_001126055) was cloned into a pIRES-GFP vector (pIRES-GFP-CASK) and the pIRES-GFP vector served as control. HEK293 cells stably expressing Na<sub>v</sub>1.5 channels were transiently transfected using Lipofectamin™ (Lipofectamin 2000; Invitrogen) with 2 μg of either shscr or shCASK and pIRES-GFP or pIRES-GFP CASK. ShCASK or shscr under a U6 promoter (Ad-shscr and Ad-shCASK) and CASK sequence (Ad-CASK) were cloned into pShuttle vector. Expression cassettes were transferred into the adenovirus vector containing GFP reporter for selection of infected cells. Adenoviruses were then produced, amplified and concentrated for further infections. Rat adult cardiomyocytes were infected 24h after isolation with 5.10<sup>7</sup> particles/ml of Ad-shscr, Ad-shCASK, empty vector (Ad-GFP) or Ad-CASK. Overexpression studies were performed 3 days post infection while silencing studies were performed 5 days after infection.

Immunohistochemistry, immunocytochemistry and antibodies
Monoclonal anti-dystrophin (clone MANDYS8, 5.75 μg/ml), monoclonal anti-vinculin (clone hVIN-1, 23.3 μg/ml) and monoclonal anti-actinin (clone EA-53, 1:400) were obtained from
Sigma. Polyclonal antibody recognizing human/rat Na\textsubscript{v}1.5 was purchased from Alomone labs (ASC-005, 8 μg/ml). Monoclonal anti-Ca\textsubscript{v}1.2 (clone N263/31, 10 μg/ml) was obtained from UC Davis/NIH NeuroMab Facility. Monoclonal anti-CASK used for biochemical assays was from UC Davis/NIH NeuroMab Facility (clone S56A-50, 2-20 μg/ml) and polyclonal anti-CASK used for immunohistochemistry was from Abcam (ab3383, 10 μg/ml). Monoclonal anti-caveolin 3 (clone 26/Caveolin 3, 5 μg/ml) was purchased from BD Biosciences. Monoclonal anti-N-cadherin (clone 3B9, 5 μg/ml) was from Zymed Laboratories. Monoclonal anti-caveolin 3 (clone 26/Caveolin 3, 5 μg/ml) was purchased from BD Biosciences. Monoclonal anti-N-cadherin (clone 3B9, 5 μg/ml) was from Zymed Laboratories. Monoclonal anti-caveolin 3 (clone 26/Caveolin 3, 5 μg/ml) was purchased from BD Biosciences. Monoclonal anti-N-cadherin (clone 3B9, 5 μg/ml) was from Zymed Laboratories.

**Deconvolution microscopy**

Freshly isolated cells and myocardium cryosections were observed on an Olympus epifluorescence microscope (60X, UPlanSAPo, 1.35). Digital images were captured with a CoolSnap camera (Roper-Scientific) in Z-depth in 0.2 μm increments using Metamorph software (Molecular Devices). Images were deconvoluted using acquired point spread function with the Metamorph 3D-deconvolution module. For representation, 2 frames were z-projected into one frame. Fluorescence quantification and colocalisation analysis were performed using ImageJ software (NIH).

**Evanescent field microscopy and image analysis**

HEK293 and adult cardiac myocytes were cultured on glass bottomed dishes (170 μm thickness, Ibidi), fixed and stained with anti-Nav1.5 antibody. Cells were visualized with total internal reflection fluorescence (TIRF) microscopy using the Olympus CellR’ system. Cells were placed on an Olympus IX81-ZDC2 inverted microscope and TIRF illumination achieved with the motorized integrated TIRF illuminator combiner (MITICO-2L) using an APON 60X 1.49 numerical aperture (NA) oil immersion objective. GFP and RFP were visualized using a 50 mW solid state 491-nm and 561 lasers for excitation and dual band optical filter (M2TIR488-561). All image acquisition, TIRF angle adjustment and some of the analysis was performed using the xcellence software (Olympus). The lasers beams were focused on the periphery of the back focal plane of the objective in order to obtain an evanescent field of 75nm. Digital images were captured on a cooled charge-coupled device (CCD) camera (ORCA-ER, Hamamatsu). Control wide field and differential interference contrast (DIC) images were also acquired. Cells were selected on the basis of the GFP fluorescence and outlined manually using ImageJ software. Selected regions of interest (ROI) were pasted on the Na\textsubscript{v}1.5 images and the evanescent field fluorescence intensity was measured for each cell (mean intensity/area, in arbitrary unit).

**Real time RT-PCR**

Total mRNA was extracted from snap frozen hearts with TRIzol (Life Technologies) and quantified by NanoDrop 1000 Spectrophotometer (Thermo Scientific). cDNA was synthesized from 2 μg of total mRNA with random primers by Superscript™ III reverse transcriptase (Life Technologies). Quantitative real-time PCR was performed using the LightCycler 480 System (Roche Diagnostics) and the Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies), according to the manufacturer’s instructions. Specific primers were designed to amplify cask (cask-F: 3’ gtaggccccacctggctc5’ and cask-R: 5’ tcttgggccataaagtgag 3’) and scn5a (scn5a-F: 3’ tatgcctccagccccctc5’ and scn5a-R: 5’ gaacagcgcagttgctcag 3’). All data was normalized to mRNA level of housekeeping genes polr2a, rplp0, rpl32 (polr2a-F: 3’ cgtatccgcatcatgaacagt 5’ and polr2a-R: 5’ tcacatcttatcaccacaccttt 3’; rplp0-F: 3’ gcaacctggagaatccacatt 5’ and rplp0-R: 5’ gctgcttcctccacaaggg 3’; rpl32-F: 3’ ccagagcatgcaacaca 5’ and rpl32-R: 5’ cgcacctccagctcttg 3’) using the 2-ΔΔCT method.

**Protein Extraction**
Frozen tissues were homogenized in a lysis buffer, in mmol/L: NaCl 150, Tris-HCl 50 (pH7.5), EDTA 1 and 0.5% Triton 100-X and 1% protease cocktail inhibitor (Sigma) using a polytron. Solubilization was achieved by rotating the preparation 1h at 4°C. Soluble fractions obtained from 15 min centrifugation at 15000g and 4°C were used for experiments. Protein concentrations were measured by BCA Protein Assay (Pierce) using a BSA standard curve.

**Immunoprecipitation Assays**

Whole heart lysates were incubated with equal amount of antibodies or IgG (control) overnight at 4°C before pull-down with G-Sepharose beads (Sigma) for 1h. Material bound to washed-beads was eluted, boiled and separated by western blot.

**Biotinylation Assays**

Live cells were biotinylated for 30 minutes at 4°C using PBS containing 0.5 mg/ml of EZ Link Sulfo-NHS-SS-Biotin (Sigma). Cells were washed with PBS and incubated for 10 minutes in PBS containing 100 mmol/L Glycine to quench unlinked biotin. Cells were then lysed for 30 minutes at 4°C with RIPA buffer containing, in mmol/L: NaCl 150 and Tris-HCl 50 (pH7.5) supplemented with 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitor cocktail (Sigma). After centrifugation, supernatants were incubated with 50 μl of streptavidin-sepharose beads (Thermofisher Scientific) overnight at 4°C. After 5 washes with RIPA buffer, samples were warmed for 20 minutes at 37°C in 2X sample buffer and analyzed by western blot as described below.

**Pull-down assays**

cDNAs encoding the last 66 amino acids of Nav1.5 WT and S2014Stop were cloned into pGEX-4T1 (Amersham Bioscience, Piscataway, USA). Expression of GST-fusion proteins in E.Coli K12 cells was induced with 0.5 mmol/L IPTG for 2 h at 30°C. Cells were harvested by centrifugation and re-suspended in lysis buffer (in mol/L: Tris pH 7.5 mM, NaCl 250, EDTA 1, PMSF 1, and 0.5% NP40, 0.2 mg/ml DNASE I (Roche) and 0.2 mg/ml Lysozyme (Roche)). After bacterial lysis, fusion proteins were purified on Glutathione-Sepharose beads (Amersham Bioscience). Purified protein CASK obtained from OriGene (NM_003688) or soluble fractions obtained after applying the protein extraction protocol (see above) were incubated with GSH-sepharose beads containing either GST or the GST fusion proteins. After four washes in Lysis Buffer, samples were re-suspended with 2X Sample Buffer (NuPAGE® LDS Sample Buffer 4X, Life Technologies) containing 50 mM Dithiothreitol (NuPAGE® Sample Reducing Agent 10X, Life Technologies) and heated ten minutes at 60°C. Beads were removed from the samples and the resulting pulled-down proteins were analyzed by Western blot as described below.

**Western Blot**

Proteins were separated on NuPAGE® Novex® 10% Bis-Tris gels or NuPAGE® Novex® 3-8% Tris-Acetate gels for high molecular weight proteins (Life Technologies) and transferred on nitrocellulose membrane (Biorad). Incubation were performed with appropriate primary antibodies followed by HRP-coupled secondary antibodies (Cell Signaling). Proteins were revealed with ECL Prime (GE Healthcare) and images were acquired using a LAS4000 Camera (GE Healthcare).

**Electrophysiological measurements**

I_{Na} properties were studied using standard whole-cell patch clamp. For experiments performed on cultured cells, pipette solution contained in mmol/L: NaCl 5, CaCl2 2, MgCl2 2, CsCl 130, HEPES 10, EGTA 15 and MgATP 4, pH 7.2 with CsOH. Cells were perfused with a solution containing in mmol/L: NaCl 25, CaCl2 2, MgCl2 2.5, CsCl 108.5, HEPES 10, CoCl2 2.5 and glucose 10, pH 7.4 with CsOH (E_{Na} ~40 mV) or NaCl 135, CaCl2 2, KCl 4, MgCl2 2.5, HEPES 10, and glucose 20, pH 7.4 with NaOH (E_{Na} ~80 mV). For experiments performed on freshly isolated WT Cre and CASK KO ventricular myocytes, pipette solution contained in mmol/L: aspartic acid 60, cesium asparte 70, CaCl2 1, MgCl2 1, HEPES 10, EGTA 11, and Na2 ATP 5,
pH 7.2 with CsOH. Myocytes were bathed with a solution containing in mmol/L: NaCl 5, NMDG-Cl 125, CaCl₂ 2, MgCl₂ 1.2, CsCl 5, HEPES and glucose 5, pH adjusted to 7.4 with CsOH. 10 μmol/L nifedipine and 10 μmol/L CoCl₂ were added to the extracellular solution to prevent calcium current contamination of the recordings. For the current-Vₘ and activation (ₘₐₓ) protocol, currents were elicited by 50ms depolarizing pulses from a holding potential of -120 mV, in 5 or 10 mV increments, between -100 and +60 mV, at a frequency of 0.2 Hz. For the steady-state availability-Vₘ protocol (ₘₐₓ), currents were elicited by a 50ms test pulse to -20 mV following 1s pre-pulses applied in 5 or 10 mV increments, between -140 mV and -20 mV, from a holding potential of -120 mV, at 0.2 Hz.

**Human samples**
In accordance with approval of Institution Ethic Committee, biopsies of right atrial appendages were obtained from 28 patients undergoing cardiac surgery. Two groups of patients were defined. The control group was constituted of patients in sinus rhythm, preserved left ventricular function and a normal atria (N=14); the diseased group was constituted of patients with marked dilation of the right atria associated with chronic AF (up to 6 months duration) or with severe mitral and tricuspid valve regurgitations (N=14).

**Experimental model of atrial dilatation.**
Myocardial infarction was induced in 2 months-old male Wistar rats by ligating the left coronary artery (n=13). Sham rats underwent the same surgical procedure without left coronary artery ligation (n=14). The cardiopathy was characterized by transthoracic echography 2 months after surgery. Atria were collected, placed in lysis buffer, homogenized and frozen at -80°C for use in western blot experiments. Atria were fixed with 4% PFA for 10 min at RT, embedded in cryomatrix and 8μm-thick sections were prepared for IF.

**Statistics**
Data was tested for normality using the D’Agostino and Pearson normality test. Statistical analysis was performed using Student's t test or ANOVA followed by post-hoc Student-Newman-Keuls test on raw data. Results are given as means ± SEM and P values of less than 0.05 were considered significant. *: P<0.05, **: P<0.01 and ***: P<0.001. N corresponds to the number of independent experiments, and n to the number of samples.

**REFERENCES**
Online Figure I. CASK is expressed at the lateral membrane of cardiac myocytes and excluded from the intercalated disc in the atrial myocardium. High resolution 3-D deconvolution microscopy images and enlargements of outlined squares (right panels) obtained from atrial tissue sections stained with CASK and caveolin3 (top panel), N-cadherin (middle panel) and α-actinin (bottom panel). Note that the focal plane was selected in order to highlight the different structures of interest.
Online Figure II. CASK expression at the z-line does not correspond to T-tubule localization. (A) High resolution 3-D deconvolution microscopy images of CASK and \(\text{Ca}_v1.2\) channel (top panel) or ryanodine receptor 2 (\(\text{RyR2}\), bottom panel) in freshly isolated ventricular myocytes. Right panels correspond to enlargement of outlined squares. (B) Live images of Di-8-ANEPS staining (top panel) and post-staining of CASK after fixation (bottom panel) in control or formamide-treated cardiac myocytes.
Online Figure III. Modulation of CASK mRNA and protein levels by adenoviral transfer technology. (A) Representative bright field and epifluorescence images of adenoviral infection efficiency in cultured rat atrial myocytes for overexpression (top) and silencing (bottom) experiments (20X). Squares in the merged images outline representative isolated cell used for patch clamp experiments. (B) Histogram showing the expression level of CASK mRNA in adult atrial myocytes infected with Ad-CASK (green) or Ad-shCASK (red) compared to their respective control Ad-GFP and Ad-shscr (black). (C) Representative western blot showing increased CASK expression in Ad-CASK infected myocytes (endogenous CASK at ~110 kDa and CASK-EGFP at ~140 kDa) or decreased CASK expression in myocytes infected with either Ad-shCASK. (D) Histogram of the expression level of CASK normalized to GAPDH after modulation of its expression by shRNA or overexpressing adenoviruses (N=2). * P<0.05; ** P<0.01.
Online Figure IV. Distribution of CASK in cardiac myocytes in function of the time in culture. High resolution 3-D deconvolution microscopy images showing the distribution of CASK and the adherens junction protein N-cadherin at different days in vitro (DIV). N-cadherin was used as a marker of the intercalated disc (ID) or ID-like structures after reestablishment of cell-cell contacts. Arrowheads point the localization of ID in freshly isolated cells and cell-cell contacts stained with N-cadherin. Note that the two proteins are never associated and that CASK is absent at sites of contact.
Online Figure V. CASK negatively regulates the cardiac sodium current $I_{Na}$ in polarized cardiac myocytes. (A) Cartoon depicting the induction of adherens junctions with the substrate and corresponding TIRFm images of N-cadherin staining (right panels). (B) Current density-voltage relationships (135 mmol/L [Na$^+$]) of $I_{Na}$ obtained from adult atrial myocytes infected with Ad-shscr and seeded on Fc (black) or on N-cadh (grey) or from myocytes infected with Ad-shCASK and seeded on Fc (red) or N-cadh (orange) (N=2). (C-D) Voltage-dependent activation and steady-state inactivation curves obtained from adult atrial myocytes in the same four conditions (N=2). * P<0.05; ** P<0.01; *** P<0.001.
Online Figure VI. CASK regulates the cardiac sodium current $I_{\text{Na}}$ in non-polarized HEK293-Na$_V$1.5 cells. (A) Representative phase contrast and epifluorescence images of the transfection efficiency in HEK293-Na$_V$1.5 cells. (B) Representative western blots showing CASK expression in HEK293-Na$_V$1.5 cells transfected with either pIRES-GFP-CASK or RFP-shCASK. (C) Histogram of the expression level of CASK normalized to GAPDH after modulation of its expression by overexpression plasmids or shRNA (N=2). * P<0.05; *** P<0.001. (D) Current density-voltage relationships (25 mmol/L [Na$^+$]) of $I_{\text{Na}}$ obtained from GFP (black) and CASK (green) transfected HEK293 cells. (E-F) Activation and steady-state inactivation curves obtained from GFP (black) and CASK (green) HEK293 cells (N=3). (G) Current density-voltage relationships (25 mmol/L [Na$^+$]) of $I_{\text{Na}}$ obtained from shscr (black) and shCASK (red) transfected HEK293 cells. (H-I) Activation and steady-state inactivation curves obtained from shscr (black) and shCASK (red) HEK293 cells (N=3). * P<0.05; ** P<0.01; *** P<0.001.
Online Figure VII. CASK silencing inhibits syntrophin expression. (A) Representative western blot showing the expression of CASK, dystrophin, Na\(_{V1.5}\) and syntrophin in control (black) and CASK-silenced myocytes (red). GAPDH served as loading control. (B) Histogram showing the relative protein expression normalized to GAPDH in Ad-shscr and Ad-shCASK. n=7 independent cultures, N=3-5 western blots. ns, not significant; *** P<0.001.
Online Figure VIII. CASK expression is reduced but its localization is unchanged in a model of chronic hemodynamic overload and dilated atria. (A) Representative western blot and histogram of the expression level of CASK normalized to GAPDH in sham and MI rats (N=3). (B) High resolution 3-D deconvolution microscopy images showing the distribution of CASK and N-cadherin in sham and MI rats and corresponding histogram of CASK fluorescence intensity from the same rats (N=3) from serial tissue slices. Arrows indicate the localization of intercalated discs. ** P<0.01.