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Original Paper

CaMKII Modulates the Cardiac Transient Outward K^+ Current through its Association with Kv4 Channels in Non-Caveolar Membrane Rafts

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Key Words
Calmodulin • I_{to} • Kv4.3 • Kv4.2 • Lipid rafts • Caveola

Abstract
Background/Aims: To test whether the physiological regulation of the cardiac Kv4 channels by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is restricted to lipid rafts and whether the interactions observed in rat cardiomyocytes also occur in the human ventricle. ***Methods:*** Ventricular myocytes were freshly isolated from Sprague-Dawley rats. I_{to} was recorded by the whole-cell Patch-Clamp technique. Membrane rafts were isolated by centrifugation in a discontinuous sucrose density gradient. The presence of the proteins of interest was analysed by western blot. Immunogold staining and electron microscopy of heart vibrosections was performed to localize Kv4.2/Kv4.3 and CaMKII proteins. Protein-protein interactions were determined by co-immunoprecipitation experiments in rat and human ventricular mycoytes. ***Results:*** Patch-Clamp recordings in control conditions and after lipid raft or caveolae disruption show that the CaMKII-Kv4 channel complex must associate in non-caveolar lipid rafts to be functional. Separation in density gradients, co-immunoprecipitation and electron microscopy show that there are two Kv4 channel populations: one located in caveolae, that is CaMKII independent, and another one located in planar membrane rafts, which is bound to CaMKII. ***Conclusion:*** CaMKII regulates only the Kv4 channel population located in non-caveolar lipid rafts. Thus, the regulation of cardiac Kv4 channels in rat and human ventricle depends on their subcellular localization.

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Introduction

The transient outward potassium current, I_{to}, modulates the duration of the plateau phase of the cardiac action potential and, therefore, determines the amount of calcium entry into the myocyte. Thus, changes in the amplitude and/or kinetics of I_{to} current have serious effects on both the strength and the duration of cardiac contraction, as well as on the refractory period. At a molecular level, I_{to} current is carried through a tetrameric channel composed by Kv4.2 and/or Kv4.3 proteins [1-3].

Ca^{+2}/Calmodulin-dependent protein kinase II (CaMKII) is a major regulator of the transient outward potassium current in physiological conditions. We and others have described in heterologous expression system, murine colonic cells, rat ventricular myocytes and human atrial myocytes, that CaMKII-dependent phosphorylation is necessary to slow down the otherwise very fast inactivation kinetics of I_{to} [4-7]. In a previous work, we observed that in cardiac myocytes CaMKII is bound to and phosphorylates Kv4 channels in physiological conditions [4].

Moreover, the interaction between I_{to} and CaMKII not only affects the channel, but also determines the behaviour of the enzyme. As CaMKII is a cytosolic protein, its association to membrane proteins such as Kv4 channels is a mechanism to guide and operate the kinase towards targets in the membrane. Thus, in cardiac myocytes the CaMKII-induced increase in L-type Ca^{2+} current ($I_{Ca\text{-}L}$) depends on the association between the kinase and the Kv4 channels in the cell membrane [8].

In the plasma membrane, there are discrete regions termed membrane rafts or lipid rafts, rich in cholesterol and sphingolipids, with a rigid structure that floats in the membrane [9]. Lipid rafts localize many transduction molecules and may serve as cell-surface compartmentation microdomains. The best-known specific subtype of lipid rafts is the caveolae. These are flask-shaped membrane invaginations characterized by the presence of the scaffolding protein caveolin [10]. Caveolin-3, the myocyte-specific caveolin isoform, is found in both surface and T-tubular sarcolemma in cardiomyocytes [11].

Voltage-gated potassium channels differentially associate with caveolar and non-caveolar lipid rafts [12]. Regarding the Kv4.2 and 4.3 channels, we demonstrated the existence of two different channel populations in cardiac myocytes. Thus, the Kv4.2/Kv4.3 channel forms a supramolecular complex with protein kinase A (PKA) through A-kinase anchoring protein 100 and is attached to caveolae by interacting with caveolin-3. However, a second Kv4 channel population localizes outside the caveolae and is not sensitive to PKA regulation [13].

The aim of this work was to investigate the mechanisms of assembly of the Kv4 channels and CaMKII in rodent and human ventricular myocytes. We propose that, in adult ventricular myocytes, the CaMKII-Kv4 pathway is compartmentalized within non-caveolar membrane rafts.

Materials and Methods

The investigation with rats fulfils the Spaniard (RD 1201/2005) and European (D2003/65/CE and R2007/526/CE) rules for care of animals used for experimental and other research purposes, and has been approved by the Ethics Committee for Animal Care of the University of the Basque Country (CEBA/45b/2010/CASIS SAENZ).

Human right ventricular samples (n=8) were obtained from the Basque BioBank for Research (Fundación Vasca de Innovación e Investigación Sanitarias), application code CBVI194/1M. The study with human samples conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Clinical Research of the Basque Country (CEIC-E oheun 12-34).

Cell isolation

Sprague-Dawley rats (200-220 g) were anaesthetized with ketamine and xylazine (50/15 mg/kg, respectively, i.p.). The hearts were removed and perfused as previously described [14] with a Tyrode solution containing (in mmol l^{-1}): NaCl 118, KCl 5.4, $NaHCO_3$ 24, $MgCl_2$ 1.02, $CaCl_2$ 1.8, NaH_2PO_4 0.42, dextrose 12 and taurine 20, bubbled with 95% O_2 and 5% CO_2, pH 7.4 at 37°C, followed by the same solution without Ca^{+2}, and by the same nominally Ca^{+2}-free solution containing collagenase Type I (0.5 mg ml^{-1}) and protease Type XIV (0.03 mg ml^{-1}). The hearts were finally perfused with a KB solution (in mmol l^{-1}): taurine 10, glutamic acid 70, creatine 0.5, succinic acid 5, dextrose 10, KH_2PO_4 10, KCl 20, HEPES-K^+ 10, EGTA-K^+ 0.2, adjusted to pH 7.4 with KOH. Single cells were obtained by mechanical agitation.

Human ventricular samples were minced in small pieces (<1mm) and gently agitated for 30 minutes at 37°C in the collagenase containing solution. The cell suspension was centrifuged and the pellet resuspended in RIPA buffer (Tris-HCl 50 mmol l^{-1}, NaCl 150 mmol l^{-1}, EDTA 1 mmol l^{-1}, Igepal 0.5%, Sodium deoxycholate 1 %, 2.5 µl ml^{-1} of the Sigma Protease inhibitor cocktail and the crosslinker DTBP 0.5 mg ml^{-1}, pH 7.4).

Patch-Clamp

I_{to} was recorded using the whole-cell variation of the Patch-Clamp technique [14] with an Axopatch 200B patch-clamp amplifier (Molecular Devices). Recording pipettes were obtained from borosilicate tubes (Sutter Instruments), and had a tip resistance of 1-3 MΩ when filled with the internal solution (in mmol l^{-1}): L-aspartic acid (potassium salt) 80, KH_2PO_4 10, $MgSO_4$ 1, KCl 50, HEPES-K^+ 5, ATP-Na_2 3, EGTA-K^+ 10, adjusted to pH 7.2 with KOH.

The voltage-clamp experimental protocols were controlled with the "Clampex" program of the "pClamp" software (Molecular Devices). The bathing solution was (in mmol l^{-1}): NaCl 86, $MgCl_2$ 1, HEPES-Na^+ 10, KCl 4, $CaCl_2$ 0.5, $CoCl_2$ 2, dextrose 12, TEA-Cl 50, adjusted to pH 7.4 with NaOH. Previous works showed that M-β-CD shifts the inactivation half voltage of some K^+ channels [15, 16], and so I_{to} was recorded applying depolarizing pulses to +50 mV, starting from a holding potential of -90 mV, at which there are no inactive I_{to} channels. The TEA-resistant time-independent sustained current, I_{ss}, was digitally subtracted.

Perfusing the cells with KN93, a selective inhibitor of CaMKII [17] typically decreases the peak current amplitude and increases the rate of inactivation [7]. These effects are due in part to a direct effect of KN93 on the extracellular side of K^+ channels, acting as an open channel blocker [18]. This direct effect of the drug on the extracellular side of the channels can be avoided by adding KN93 to the solution of the recording pipette [4, 19]. These experimental conditions have demonstrated that KN93 added to the recording pipette has a selectivity similar to either selective peptides added in the recording pipette or permeable forms of these peptides added in the bathing solution [4].

2% M-β-CD, 5 µmol l^{-1} Colchicine or 100 µM Cytochalasin-D was added to the KB medium two hours before the start of the experiments.

Membrane isolation

All procedures were performed at 4°C in a homogenization buffer (HF) containing Tris-HCl 20 mmol l^{-1} pH 7.4, EDTA 1 mmol l^{-1}, and 2.5 µl ml^{-1} of the Sigma Protease inhibitor cocktail (Sigma Chemical Co.). Either rat or human ventricular myocytes were homogenized 1 minute on ice. Nuclei and debris were pelleted by centrifugation at 500 g for 5 minutes. The supernatant was centrifuged at 20000 g for 30 minutes. The obtained new pellet was resuspended in HF and centrifuged again at 20000 g for 30 minutes. The final pellet was stored at –80ºC.

Raft Isolation

Due to their lipidic composition, rich in cholesterol and sphingolipids, lipid rafts are resistant to membrane solubilisation. Thus, there are two main procedures to isolate membrane rafts based on the buoyancy of unsolubilised rafts after membrane solubilisation: one uses for solubilisation a detergent-containing medium and another one uses a detergent-free medium [20, 21]. We found that in cardiac myocytes the detergent-free method gives a better membrane raft isolation than the detergent based method [13]. Cell membranes isolated from ventricular myocytes were resuspended into 1.5 ml of Mes-buffered saline (MBS) containing 25 mmol l^{-1} Mes, pH 6.5, 150 mmol l^{-1} NaCl, 250 mmol l^{-1} Na_2CO_3, and 2.5 µl ml^{-1} of the Sigma Protease inhibitor cocktail. Homogenization was carried out with three 10 second bursts on a Polytron tissue grinder (Sanyo Gallenkamp 23 Hz) and a 5 minute burst on a sonicator (Ultrasons, Selecta). The homogenate was then adjusted to 40% sucrose by adding 1.5 ml of an 80% sucrose solution prepared in MBS, and placed at the bottom of an ultracentrifuge tube. A 5-35% discontinuous sucrose gradient was formed above (3 ml of 5% sucrose / 3ml of 35% sucrose, both in MBS containing 250 mmol l^{-1} Na_2CO_3), and centrifuged at 200000 g for 18 hours in a Swinging-Bucket rotor (Kontron Instruments). The solubilization-resistant membrane rafts remained at the top of the gradient (5-35% sucrose interface), whereas the soluble lysate was observed at the bottom of the centrifuge tube (high density fraction of the gradient, 40%). The first 2 ml were discarded because they do not contain any detectable protein, and seven 1 ml gradient fractions were collected. Proteins from each fraction were precipitated with trichloroacetic acid and resuspended in SDS sample buffer. Caveolin-3 was used as marker of the membrane raft isolation, as it only appears at the low density fractions.

Co-immunoprecipitation

Membranes from ventricular myocytes were solubilised in RIPA buffer. 250 µg of proteins were centrifuged at 20000 g for 30 min. The pellet was resuspended in 150 µl of RIPA buffer and incubated for 1 hour at 4°C. Samples were cleared by centrifugation at 15000 g for 25 min, and supernatants were incubated for 3 hours at 4°C with 40 µl of protein G-sepharose to rule out non-specific pulldown of target proteins. After that, samples were centrifuged 2 minutes at 4, 000 g. 150 µl of the supernatant were obtained and incubated overnight with 2 µg of anti-CaMKII or anti-Caveolin-3 antibodies (Chemicon and Santa Cruz Biotechnologies respectively). 100 µl of 50% protein G-sepharose was added, and the mixture was incubated for 3 hours at 4°C. The beads were pelleted and washed three times in RIPA buffer. The bound proteins were eluted using 50 µl of SDS sample buffer, and both the pellet and the supernatant were examined by western blot.

Western blot

Gradient fractions or immunoprecipitation samples were fractionated on 10% SDS-polyacrylamide gels and transferred to Nitrocellulose membranes (Amersham Biosciences). Nitrocellulose membranes were blocked in TTBS solution (Tris-HCl 50 mmol l^{-1} pH 7.5, NaCl 150 mmol l^{-1}, Tween-20 0.05%) containing 3% BSA. Blots were incubated with primary antibodies specific to: Caveolin-3 (mouse, 1:1000), Na^+/K^+ ATPase (mouse, 1:50), CaMKII (rabbit, 1:200), Kv4.2 (goat, 1:200) and Kv4.3 (goat, 1:200) from Santa Cruz Biotechnology. Secondary antibodies were: donkey anti-rabbit IgG (1:5000, Amersham Biosciences), anti-mouse IgG (1:3300, Sigma Chemical Co) and anti-goat (1:5000, Santa Cruz Biotechnology). Blots were developed using the West Pico chemiluminescence reagent (Pierce).

Electron microscopy

Animals were transcardially perfused with PBS (0.1 M; pH 7.4) and then fixed by 500 ml of a fixative made up of 0.1% glutaraldehyde and 0.2% picric acid in PBS. Perfusates were used at 4°C. Tissue blocks were extensively rinsed in 0.1 M PBS, pH 7.4. 50 µm thick heart vibrosections were collected in 0.1 M PBS, pH 7.4, at room temperature. Sections were preincubated in 10% blocking BSA prepared in PBS for 1 hour at room temperature. The localization of Kv4.2, Kv4.3 and CaMKII were carried out by means of the preembedding silver intensified immunogold method. Following incubation with the primary monoclonal mouse antibody (1:1000 in 1.5% BSA/PBS; NeuroMab) for 2 days, heart sections were incubated with 1.4 nm gold-labelled rabbit anti-mouse IgG (Fab' fragment, 1:100, Nanoprobes Inc.) diluted in 1% BSA/PBS for 4 hours at room temperature. Tissue was subsequently postfixed in 1% glutaraldehyde for 10 minutes, rinsed extensively in double-distilled water, and gold particles were silver intensified with an HQ Silver kit (Nanoprobes Inc.,) for about 8 minutes. Stained sections were osmicated (1% OsO_4 in 0.1 M PBS, pH 7.4, 20 minutes), dehydrated in

graded alcohols to propylene oxide and plastic-embedded flat in Epon 812. Ultrathin sections were collected on mesh nickel grids, stained with uranyl acetate and lead citrate. The preparations were finally examined in a Philips EM208S electron microscope at the Microscopy Core Facility of the University of the Basque Country.

Data analysis

Current recordings were analysed using the Clampfit program of pClamp 10.1 software (Axon Instruments Inc.). Western blot bands were acquired with a Kodak Gel Logic 2200 and analysed with the Kodak Molecular Imaging software (Eastman Kodak Company). Paired student's t test was used for statistical analysis. The data are expressed as the mean ± S.E.M. A p value of less than 0.05 was considered to be statistically significant.

Results

CaMKII and Kv4 channels are functionally coupled in non-caveolar membrane rafts

We performed electrophysiological experiments to analyse the role of lipid rafts integrity in the functional regulation of the transient outward potassium current by CaMKII. In control conditions, I_{to} is a fast inactivating current (Fig. 1A-D). The kinetic model developed to explain the behaviour of Kv4 channels assumes at least six different inactivated states of the channel and involves two inactivation components of the macroscopic current [22, 23]. Thus, typical I_{to} inactivation fits to a biexponential function, $F(t) = A_s\, e^{-t/\tau s} + A_f\, e^{-t/\tau f}$, with a slow component (τ_s = 77±3.4 ms) and a fast component (τ_f = 18.4±2.6 ms). The slow component of inactivation slightly predominates over the fast one (A_s = 57±6.7 vs A_f = 44.7±2.7 %, respectively; Fig. 1E).

It has been reported that KN93 causes nonspecific inhibitory effects on K^+ currents acting on the extracellular side as an open channel blocker [7]. We and others have demonstrated that adding KN93 to the solution of the recording pipette avoids this unwanted effect and has similar selectivity and more potency than the selective peptide AIP [4, 19]. Addition of the specific CaMKII blocker KN93 to the internal solution resulted in an increase in the inactivation rate of I_{to}, with no effect on peak current amplitude as reported [4]. Fig. 1A depicts that when KN93 was included in the recording pipette, the time course of inactivation was faster because of a decrease in the contribution of the slow component (57.8±6.5% in control vs 44.7±2.5% after CaMKII inhibition, $p<0.05$; Fig. 1E).

Because of their lipid composition, lipid rafts are very sensitive to cholesterol-modifying agents. To test whether CaMKII-I_{to} complex localizes in rafts, we extracted the cholesterol from the membrane of the cardiomyocytes by incubating the cells with 2% Methyl-β-Cyclodextrin (M-β-CD). The electrophysiological experiments showed that membrane cholesterol depletion accelerated I_{to} inactivation rate, indicating that CaMKII is no longer regulating the channel. Consistent with this, the current did not further respond to CaMKII inhibition by KN93. These results suggest that the CaMKII-I_{to} complex must be associated in rafts in order to be functional (Fig. 1B, E).

On the other hand, cytoskeleton disruption alters caveolae localization in the membrane and [24, 25]. Therefore, although cytoskeleton disruption can also redistribute the localization of non-caveolar raft located proteins [26], microtubule-disrupting agents like colchicine are often used to discriminate between caveolar and non-caveolar lipid rafts. Incubation of the cells with 5 μmol l^{-1} colchicine did not affect I_{to} inactivation rate, meaning that regulation by CaMKII remained intact. Addition of KN93 to the recording pipette reduced the slow component and, as a result, accelerated I_{to} inactivation (Fig. 1C, E). These data suggest that the CaMKII-I_{to} complex is not located in caveolar rafts.

To confirm these results, we used a different mechanism to disrupt caveolae: the depolymerisation of actin filaments with cytochalasin-D [26]. As expected, cytochalasin-D had no effect on CaMKII-mediated regulation of I_{to} inactivation (Fig. 1D, E), further supporting the non-caveolar localization of the complex.

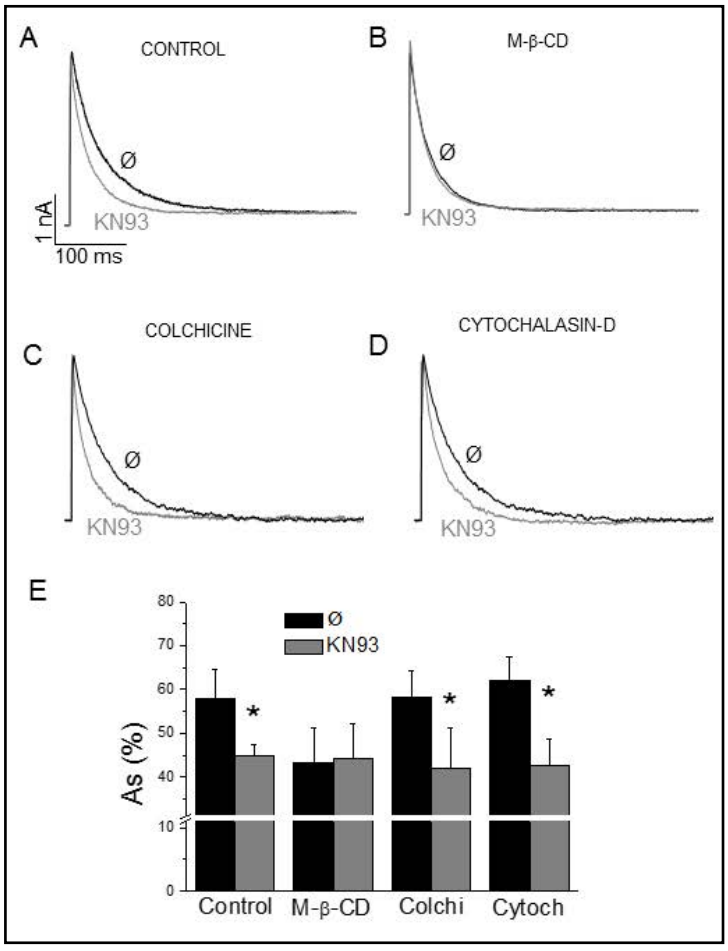

Fig. 1. CaMKII slows down the I_{to} inactivation kinetics only in intact non-caveolar membrane rafts. Superimposed I_{to} recordings from two representative myocytes: one recorded in control conditions (Ø) and the other one with KN93 in the internal solution. A) Myocytes in control conditions, B) Myocytes lacking membrane rafts after M-β-CD incubation, C) Myocytes lacking caveolae due to incubation with colchicine or D) Cytochalasine-D. E) Relative contribution of the slow component of I_{to} inactivation (A_s) in the same experimental groups. n=6-10. *p<0.05 (compared to control).

In order to corroborate that finding, we performed membrane fractionation experiments in sucrose density gradients to isolate lipid rafts, and then looked for the kinase and the I_{to}-channel forming proteins. Since caveolin is the major structural component of caveolae, we used the myocyte-specific isoform, caveolin-3, as a marker of the membrane raft and Na^+/K^+ ATPase as non-raft marker (Fig. 2A) [13]. Immunoblotting for Cav-3 and Na^+/K^+ ATPase after treatment with 2% MβCD and 5 μmol l^{-1} colchicine confirm effective disruption of lipid rafts.

As previously described for rat ventricular myocytes [13], we detected Kv4.2 and Kv4.3 only in the lower density fractions of the gradient, corresponding to the fractions enriched in lipid rafts. However, we found CaMKII throughout all gradient fractions, showing the existence of raft-located and non-raft-located populations of the kinase (Fig. 2B). Thus, in control conditions, lipid rafts contain both the I_{to} channel-forming proteins and some of the CaMKII molecules. When we depleted cholesterol thus dissociating rafts by incubation with 2% M-β-CD, the buoyancy of the raft associated proteins was altered, and all raft associated proteins, Kv4.2, Kv4.3 and CaMKII, were shifted towards high density fractions (Fig. 2B, C). These results confirm that there are components of the CaMKII-Kv4 regulatory complex localized in membrane rafts.

In order to distinguish whether these rafts are caveolar or non-caveolar, we incubated the myocytes with 5 μmol l^{-1} colchicine before membrane extraction, so we disrupted caveolae structure. Fig. 2 shows that, in colchicine-treated myocytes, part of the Kv4.2 and Kv4.3 proteins migrated towards higher density fractions, whereas another part remained in the lower density fractions. Therefore, we found channel proteins in both caveolar and non-caveolar rafts. Conversely, colchicine treatment did not modify CaMKII distribution across the density gradient, indicating its absence from caveolae (Fig. 2B, C). These results suggest

that the non-caveolar CaMKII would have access to and would regulate only non-caveolar Kv4 channels.

CaMKII and Kv4 channels are associated in non-caveolar membrane rafts

Since CaMKII is a cytosolic protein, we next analysed the nature of the association between the non-caveolar population of Kv4 channels and CaMKII by immunoprecipitation experiments using an anti-CaMKII antibody (Fig. 3, left). The presence of CaMKII in the pellet, and its absence in the supernatant was confirmed by inmunoblot. Kv4.2 and Kv4.3 proteins were observed in the CaMKII immunoprecipitate, demonstrating the association between them in a complex. This interaction could by direct or through other proteins in the complex. Some channel proteins were also found in the supernatant, showing that not all the channel proteins interact with CaMKII.

We probed the membrane with an anti-Caveolin 3 antibody, and found no trace of the protein in the CaMKII immunoprecipitate. This indicates absence of interaction between these two proteins, and supports the idea that CaMKII-Kv4 complex is absent from caveolae. Similarly, when we performed immunoprecipitation assays using an anti-Caveolin-3 antibody CaMKII was not found in the pellet (Fig. 3, right). Regarding the channels, Kv4.2 and Kv4.3 were found both in the pellet and in the supernatant, thus indicating that Cav-3 interacts with some but not all the channel proteins.

Next, we used silver-enhanced immunogold staining method and electron microscopy technique to visualize the Kv4.2, Kv4.3 and CaMKII proteins in the sarcoplasmic membrane. Caveolae were identified as flask shaped invaginations of the membrane. The electron micrographs clearly showed the previously described two different Kv4.2 and Kv4.3 populations: the one located in caveolae and the one located in planar membrane. As expected, CaMKII was not found in caveolae (Fig. 4).

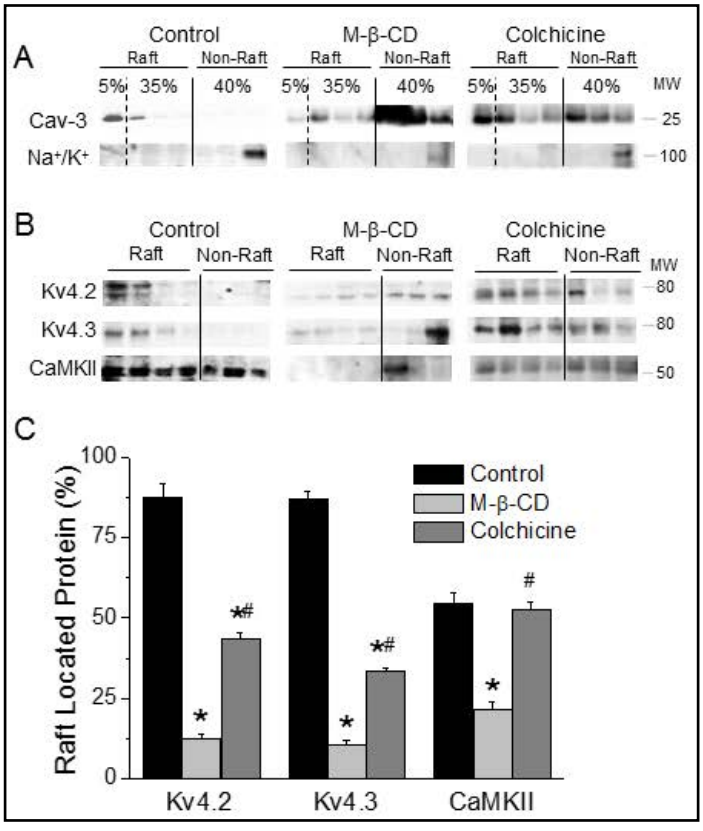

Fig. 2. Kv4 channels and CaMKII are differentially located in membrane rafts. A) Sucrose density gradient fractions of detergent-free extracted membrane rafts from rat ventricular myocytes. The presence of Caveolin-3 only in the low density fractions (5-35%) and Na^+/K^+ ATPase only at the bottom of the gradient demonstrate effective separation of raft from non-raft plasma membrane. B) Representative sucrose density gradients obtained from ventricular samples in control conditions or after pre-incubation with 2% M-β-CD or 5 μM colchicine. C) Quantification of the percentage of raft-located proteins in control conditions and after each treatment. Each western blot shown is representative of 8 different gradients.

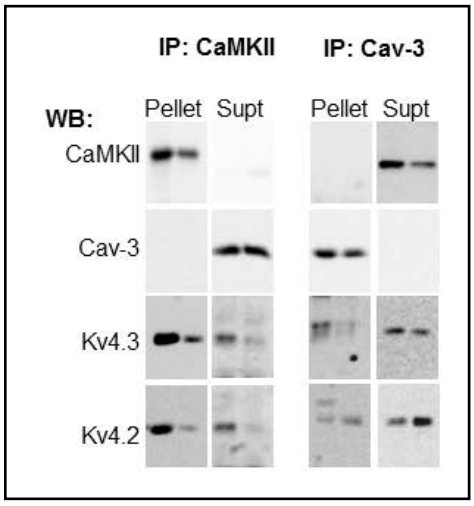

Fig. 3. Caveolin-3 and CaMKII bind to Kv4 channels but not to each other in rat cardiomyocytes. CaMKII (left) or Caveolin-3 (right) were immunoprecipitated in myocytic membranes and CaMKII, Cav-3, Kv4.2 and Kv4.3 immunodetected in the pellet and the supernatant. Each image shows 2 independent experiments, representative from 7 immunoprecipitations.

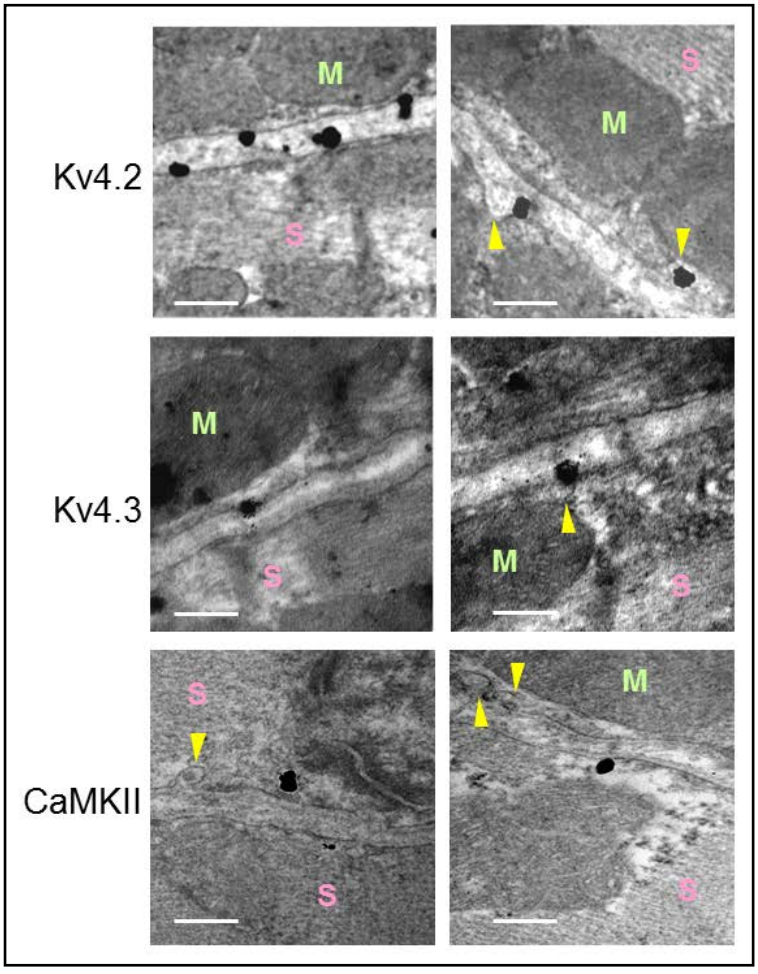

Fig. 4. Kv4.2 and Kv4.3 locate in caveolae and in planar membrane, whereas CaMKII only in non-caveolar regions. Caveolae (yellow arrowheads) were identified because of its flask shaped structure. Silver-enhanced immunogold staining showing Kv4.2 and Kv4.3 proteins in caveolae and in planar regions of the membrane, and CaMKII only in non-caveolar regions. Representative electron microscope images of 4 rat ventricles (Calibration Bar = 200 nm; M = Mitochondria; S = Sarcomere).

CaMKII-Kv4 complex also exists in human ventricular myocytes

Finally, we explored if the physical association between Kv4 channels and CaMKII in non-caveolar membrane rafts observed in rodents also occurred in the human heart. For that, we immunoprecipitated CaMKII in cardiomyocytes obtained from post-mortem human ventricular samples (Fig. 5, left). In human ventricle, the transient outward potassium current is driven only by Kv4.3 homotetramers [27]. As described in rat cardiomyocytes, we found Kv4.3 in the pellet and in the supernatant, indicating that CaMKII interacts with some, but not all, Kv4.3 proteins. Caveolin-3 was not observed in the CaMKII immunoprecipitate, thus showing the lack of interaction between the two proteins. We obtained similar results by immunoprecipitation with the anti-Caveolin-3 antibody: binding to some, but not all, Kv4 channel proteins, and absence of interaction with the kinase (Fig. 5, right).

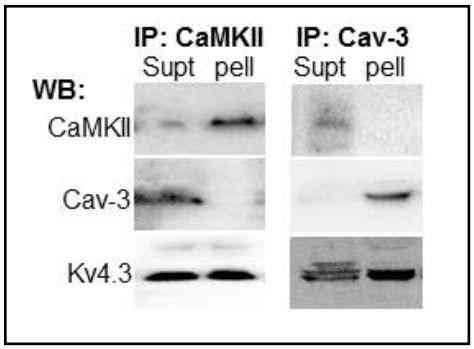

Fig. 5. Caveolin-3 and CaMKII bind to Kv4.3 but not to each other in human ventricular cardiomyocytes. CaMKII (left) and Caveolin-3 (right) were immunoprecipitated in cardiomyocytic membranes obtained from post mortem samples of human ventricles. Supernatants and pellets probed as indicated. Each image is a representative western blot from 4 immunoprecipitation experiments.

Discussion

Unlike PKA, PKC or ERK1/2-dependent Kv4 channels phosphorylation, which modifies I_{to} current amplitude [28-30], in cardiac myocytes CaMKII-mediated I_{to} regulation affects the inactivation characteristics of the current. As a consequence the action potential duration can be altered and, subsequently, the magnitude of $I_{Ca\text{-}L}$, the Ca^{2+} loading of the sarcoplasmic reticulum (SR) and the Ca^{2+} release from sarcoplasmic reticulum [31, 32]. One example of the pathophysiological relevance of the I_{to} regulation by CaMKII is the increased predisposition to episodes of ventricular arrhythmia in type 1 diabetic patients. Diabetic cardiomyopathy reduces I_{to} amplitude and [33, 34], interestingly, accelerates the current inactivation because of a defective regulation of the current by CaMKII [35]. Thus, it is important to elucidate the molecular mechanisms of interaction between the channel and the kinase.

In the heart, CaMKII is present in the plasma membrane, the cytoplasm and the nucleus of cardiac myocytes [36]. Therefore, to respond properly to a given signal, compartmentalization of signal transduction pathways seems essential. In this sense, Kv4 channels [13] and CaMKII [37-39] have been described in lipid rafts.

Three main approaches are used to identify the association of ion channels with lipid rafts: i) Functional assays after destroying membrane rafts to study the effects on channel activity; ii) Biochemical techniques to detect protein of interest in lipid rafts isolated from the bulk of the plasma membrane; and ii) Microscopic techniques to directly visualise caveolae [40]. In the present work we have combined the three approaches to clarify the mechanisms of compartmentalization of I_{to} regulation by CaMKII.

Our working hypothesis was that the CaMKII and the I_{to} channel proteins are bound in lipid rafts. This being the case, raft disruption by cholesterol extraction would result in the dissociation of the CaMKII-channel complex. Functional experiments showed that I_{to} inactivation is already accelerated in myocytes preincubated with M-β-CD, and it does not further respond to CaMKII inhibition. Therefore, CaMKII is not able to modulate I_{to} in ventricular cardiomyocytes lacking lipid rafts. The presence of the CaMKII/Kv4 complex in the low density fractions of the gradient fractionation experiments and their migration

to higher density fractions after cholesterol extraction confirmed its localization in these membrane microdomains.

However, the gradient fractionation experiments do not differentiate between planar and caveolar rafts. Chemical compounds that alter the cytoskeleton structure result in the disruption of caveolae and so, although cytoskeleton disruption can also redistribute the localization of non-caveolar raft located proteins [26], they are often used to discriminate the two types of rafts. In our experiments, colchicine or cytochalasin resulted in no effect on either the I_{to} channel regulation by CaMKII or the CaMKII distribution in the density gradient. In addition, we found no CaMKII in the Caveolin-3 immunoprecipitate, which also excluded the kinase from caveolae, placing it in planar rafts as previously reported [39].

Consistent with the biochemical approaches, electron microscopy showed no signal of CaMKII in caveolae, even when the kinase was in proximity to these structures. In our experiments, CaMKII was always observed in the internal face of the membrane, as expected from a cytoplasmic protein.

As mentioned, sucrose-gradient experiments showed Kv4.2 and Kv4.3 proteins located in lipid rafts. When caveolae were disrupted by colchicine incubation, some of the channel proteins were forced to migrate to higher density fractions, whereas another pool of proteins, undisturbed, stayed in the lower density fraction. Therefore, we can conclude that there are two different KV4.2/Kv4.3 channel populations: one located in non-caveolar lipid rafts (that would be regulated by CaMKII) and another one located in caveolae (CaMKII-independent). Two more results support this conclusion: i) the presence of the channels both in the pellet and in the supernatant of the Caveolin-3 immunoprecipitates and ii) electron micrographs clearly showing immunogold staining of channels inside and outside caveolae. These findings are consistent with our previous report demonstrating a caveolar pool of Kv4.2/Kv4.3 channels regulated by PKA, and a non-caveolar pool insensitive to regulation by PKA [13].

We next explored how the cytosolic protein CaMKII associates to lipid rafts. Post-translational modifications, such as acylation, target cytosolic proteins to lipid domains. However, some works showed that the presence of CaMKII in rafts does not depend on the activation state of the kinase, and that acylation is not necessary. Instead, protein-protein interactions seemed to be crucial [37-39]. In this line, CaMKII and Kv4 channels have been reported to couple at a distance of a few nanometres, which is compatible with a direct interaction between the channel proteins and the kinase [41]. In the present work, we demonstrate physical interaction between the Kv4 channels and CaMKII. Thus, in the CaMKII-Kv4 complex, the transmembrane channels integrated in planar rafts interact with the kinase, tethering it to the membrane. This allows the phosphorylation of the Kv4 channel, and might also facilitate the phosphorylation of other proteins available in the membrane, like the L-type calcium channel.

Finally, we confirmed that the association between Kv4 channels and CaMKII in non-caveolar membrane rafts observed in rodents also occurred in human ventricular cardiomyocytes. This is of special importance since, as recently published, the long QT type 9 syndrome (LQTS9) associated mutation in Caveolin-3 (Cav3-F97C) slows down the inactivation kinetics of Kv4.2 and Kv4.3 [42]. As Kv4 channel phosphorylation by CaMKII slows down their inactivation kinetics, we can hypothesize that the reduction of functional caveolin would increase the non-caveolar raft associated Kv4 proteins, where they could be now accessible for CaMKII.

Conclusion

We report here that Kv4 channels are found in different regions of the cell membrane both in rat and in human cardiomyocytes, but are differentially regulated depending on their localization. Only the non-caveolar population of Kv4 channels contribute to the physiological regulation of the inactivation rate of the cardiac transient outward potassium current. In addition, only these non-caveolar pool of Kv4 channels tether the CaMKII to the membrane. This allows the phosphorylation of Kv4 channels, and might also facilitate the phosphorylation of other proteins available in the membrane.

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Disclosure Statement

The authors declare that they have no competing interests.

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