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Role for SUR2A in Coupling Cardiac K_{ATP} Channels to Caveolin-3

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Key Words

Kir6.2/SUR2A channel • Caveolin-3 • Caveolin scaffolding domain peptide

Abstract

ATP-sensitive K^+ channels in the heart are localized in the caveolae. However, little is known about the molecular mechanism underlying the caveolar targeting of those ion channels. The present study was designed to explore the molecular compositions involved in the interaction between cardiac K_{ATP} channels and caveolin-3. The HA-tagged wild-type (Kir6.2/SUR2A) or mutant K_{ATP} channel (Kir6.2C4A or Kir6.2Δ36) subunits were transiently transfected into COS-7 cells with or without caveolin-3. Both Kir6.2C4A and Kir6.2Δ36 are able to form tetrameric Kir6.2 channels on the cell membrane without SUR subunit. We demonstrated that caveolin-3 co-immunoprecipitated Kir6.2 in COS-7 cells transfected with Kir6.2/SUR2A/caveolin-3, whereas Kir6.2 was not detected in the caveolin-3 immunoprecipitates in cells transfected with either caveolin-3 or Kir6.2/SUR2A alone. In cells transfected with Kir6.2C4A/caveolin-3, Kir6.2C4A was detected with anti-HA antibody but at a significantly lower level in the caveolin-3 immunoprecipitates when compared with Kir6.2 detected in cells transfected with the Kir6.2/SUR2A/caveolin-3. Kir6.2C4A was not co-immunoprecipitated with caveolin-3 in cells transfected with caveolin-3 or Kir6.2C4A alone. The application of caveolin-3 scaffolding domain peptide, corresponding to amino acid residues 55-74 of caveolin-3, largely blocked the co-immunoprecipitation of caveolin-3 and Kir6.2/SUR2A octameric channels but did not prevent the co-immunoprecipitation of caveolin-3 and the Kir6.2 tetrameric channels. Disrupting caveolae with methyl-β-cyclodextrin significantly attenuated association of tetrameric Kir6.2 channels with caveolin-3. Immunofluorescence microscopy revealed that a higher percentage of cells showed significant colocalization of caveolin-3 with Kir6.2 than colocalization of caveolin-3 with Kir6.2C4A or Kir6.2Δ36. We further confirmed that in adult rat cardiac myocytes the association of endogenous octameric K_{ATP} channels with caveolin-3 was largely prevented by caveolin-3 scaffolding domain peptide but not control peptide. We concluded that SUR2A is important for coupling cardiac K_{ATP} channels to caveolin-3, possibly through the caveolin-3 scaffolding domain.

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Introduction

The ATP-sensitive potassium channels (K_{ATP}) are a highly abundant plasma membrane proteins responsible for linking the cellular energy levels to membrane potentials and cellular excitability [1, 2]. They were first described in the heart by Noma [1] in 1983 and found in several different cell types. The major function of the K_{ATP} channel is to couple the cell metabolic state to its membrane potential by sensing changes in intracellular adenine nucletide concentration [3, 4]. The K_{ATP} channels are heterooctamers composed of four pore-forming subunits (Kir6.2 or Kir6.1) and four regulatory subunits (SUR1, SUR2A or SUR2B). It is currently known that cardiac K_{ATP} channels on the sarcolemmal membrane of cardiac ventricular myocytes consist of an octomeric complex of two subunits: Kir6.2 and SUR2A. These ion channels have been shown to protect against ischemia and contribute to adaptive responses to metabolic stress [5, 6].

Many signaling molecules are shown to interact with caveolins [7]. However, little is known about molecular interaction of caveolins with transmembrane proteins such as ion channels. Giving that several ion channels in the heart have been localized in caveolae or lipid rafts [8-16], it is important to understand the molecular mechanism underlying the caveolae-targeting of these ion channels. We have recently shown that K_{ATP} channels in the heart are primarily localized in the caveolae of cardiac myocytes and regulated by caveolin-3 or caveolae-related adenosine receptor signaling [17]. Giving that both Kir6.2 and SUR2A are required for functional K_{ATP} channels expressed on the plasma membrane of ventricular myocytes [18], we sought to explore the role of K_{ATP} subunit(s) that dictates caveolar localization of K_{ATP} channels. In the present study, we examined which K_{ATP} subunit (Kir6.2 or SUR2A) is mainly responsible for caveolar association of octameric cardiac K_{ATP} channels in cardiac mycotyes and COS-7 cells expressing recombinant K_{ATP} channels. We demonstrated that the regulatory subunit SUR2A is critical for caveolar association of cardiac K_{ATP} channels, possibly via the caveolin-3 scaffolding domains.

Materials and Methods

Peptides and cDNA clones

The wild-type cardiac K_{ATP} channel subunits contain HA-tagged Kir6.2 and SUR2A. The HA-tagged Kir6.2C4A is a Kir6.2 mutant without ER retention signal (LRKR- AAAA, 368-371), while the HA-tagged Kir6.2Δ36 is a deletion mutant of Kir6.2 lacking the last 36 amino acids at the c-terminus of Kir6.2. Both mutants of Kir6.2 have been shown to be able to traffic to the cell membrane without SUR subunit. Mammalian expression constructs for Kir6.2 and its mutants were all in pcDNA3 and SUR2A was in pCMV (Invitrogen). The caveolin-3-ECFP [13] was in pECFP vector. The caveolin-3 scaffolding domain peptide (SDP) containing the putative scaffolding domain of caveolin-3 (amino acids 55–74, DGVWRVSYTTFTVSKYWCYR) was purchased from AnaSpec Inc. (San Jose, CA, USA). The scrambled peptide (WGVDRVFYTTSTVSYKWCRY) was synthesized in Peptide 2.0 (Chantilly, VA, USA).

Cell culture and transfections

COS-7 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were plated onto 35-mm culture dishes and transfected with HA-tagged Kir6.2/SUR2A, HA-tagged Kir6.2C4A or HA-tagged Kir6.2Δ36, with or without caveolin-3 by using FuGENE6 (Roche). Experiments were conducted 2-3 days after transfection.

Immunoprecipitation

Cells were rinsed with ice-cold PBS (pH 7.4) and homogenized in ice-cold lysis buffer (25 mM Tris-HCl, 250 mM NaCl, 10 mM EDTA, 5 mM EGTA, pH 7.6) containing 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride with protease inhibitor tablets (Roche) [17]. The lysates were incubated with primary antibody for 2 hours at 4°C. For the caveolin-3 SDP and scrambled peptide, cell homogenates were incubated with the caveolin-3 SDP (10 μM) or scrambled peptide before they were immunoprecipitated with anti-caveolin-3 antibody. Antigen-antibody complexes were captured with r-protein-A agarose (4°C, 30 minutes). Agarose beads were washed 4-times with lysis buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE (10% acrylamide gel) and transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies.

Western blot

Immunoblot analysis was carried out as described previously [19]. The cell lysates or immunoprecipitates were denatured in a sample buffer, electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) and incubated for 1 hr at room temperature with primary antibodies in TBS, 0.1% Tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system.

Immunofluorescence

COS-7 cells were fixed with 4% formaldehyde in PBS for 30 min, blocked, permeablized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labeled with primary antibody for 2 hr [20]. Cells were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 hr.

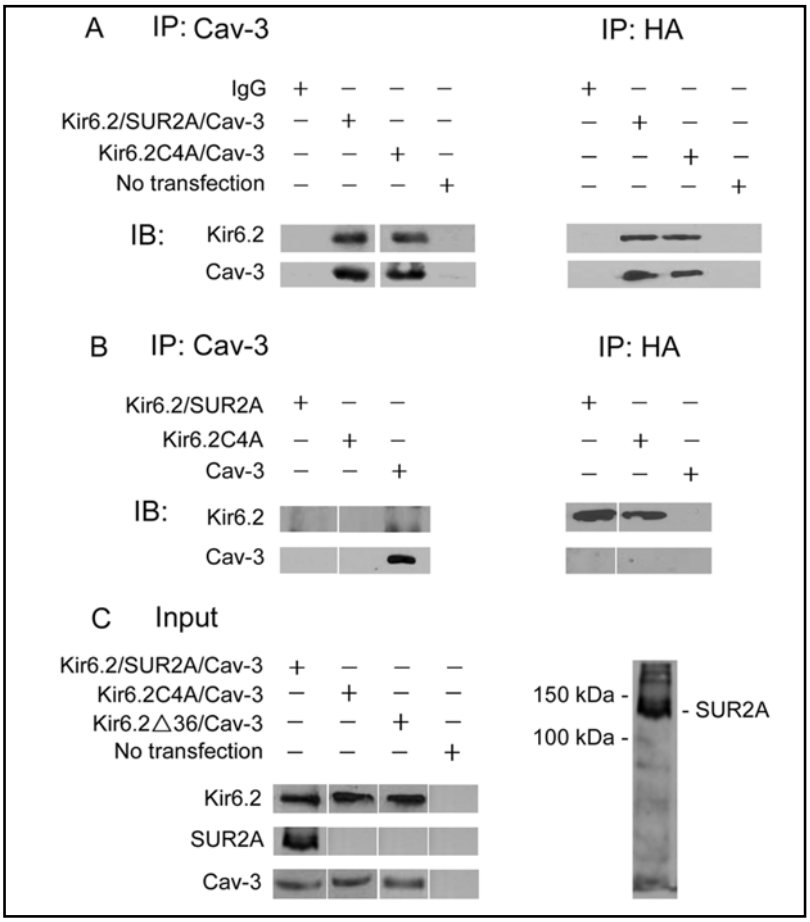

Fig. 1. Caveolin-3 co-purifies with Kir6.2C4A in COS-7 cells. A. The protein lysates from non-transfected cells or cells transfected with HA-tagged Kir6.2/SUR2A/caveolin-3-CFP or HA-tagged Kir6.2C4A/caveolin-3-CFP were immunoprecipitated with anti-caveolin-3 or anti-HA antibody followed by immunoblotting with anti-HA and anti-caveolin-3 antibody. IgG denotes immunoprecipitation with control IgG from the protein lysate of COS-7 cells transfected with Kir6.2/SUR2A/caveolin-3. B. Immunoprecipitation was performed with anti-caveolin-3 or anti-HA antibody from the protein lysates of COS-7 cells transfected with HA-tagged Kir6.2/SUR2A, HA-tagged Kir6.2C4A or caveolin-3. C. Western blot was performed in the protein lysates of non-transfected COS-7 cells or COS-7 cells transfected with HA-tagged Kir6.2/SUR2A/caveolin-3-CFP, Kir6.2C4A/caveolin-3-CFP or Kir6.2Δ36/caveolin-3-CFP. Western blot was conducted with antibodies against HA, SUR2A or caveolin-3. Results are representative of six independent experiments.

Immunofluorescence was visualized with immunofluorescence microscope. All images were analyzed using a background subtraction method offline.

Isolation of cardiomyocytes

Adult Rat ventricular myocytes were isolated from adult Wistar rats (250 to 300 g) by enzymatic dissociation [21, 22]. In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O_2) Tyrode's solution containing (mmol/L) NaCl 126, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES 10 and glucose 10 at 37°C. The perfusate was then changed to a Tyrode's solution that is nominally Ca^{2+} free but otherwise has the same composition. The hearts were perfused with the same solution containing collagenase for 20 minutes. Softened ventricular tissues were removed, cut into small pieces and mechanically dissociated by trituration. The digested cell suspension was gently centrifuged, after which the supernatant was removed and the remaining pellet was resuspended in lysis buffer for subsequent experiments.

Data analysis

Group data were presented as means ± SE. Multiple group means were compared by ANOVA followed by LSD post hoc test. Differences with a two-tailed $P<0.05$ were considered statistically significant.

Results

We have previously shown that cardiac K_{ATP} channels associate with caveolin-3 in cardiac myocytes. To determine the role of SUR subunit in coupling of K_{ATP} channel with caveolin-3, we examined a possible association between caveolin-3 and tetrameric Kir6.2 channels (without SUR2A expression). Kir6.2 C4A is a mutant of Kir6.2 where the RKR signal for ER retention/retrieval is replaced by alanine. Unlike Kir6.2, Kir6.2C4A is able to exit ER and to be expressed as tetrameric channels without co-expressing SUR subunit [18]. COS-7 cells were transfected with HA-tagged Kir6.2/SUR2A or HA-tagged Kir6.2C4A, with or without caveolin-3. The cell lysates overexpressing these proteins were subjected to immunoprecipitation. Immunoprecipitates were then subjected to immunoblot analysis. As a negative control, a lysate from cells overexpressing caveolin-3, HA-Kir6.2/SUR2A or HA-Kir6.2C4A alone was used. Caveolin-3 was detected with a specific rabbit polyclonal antibody. Kir6.2 was detected

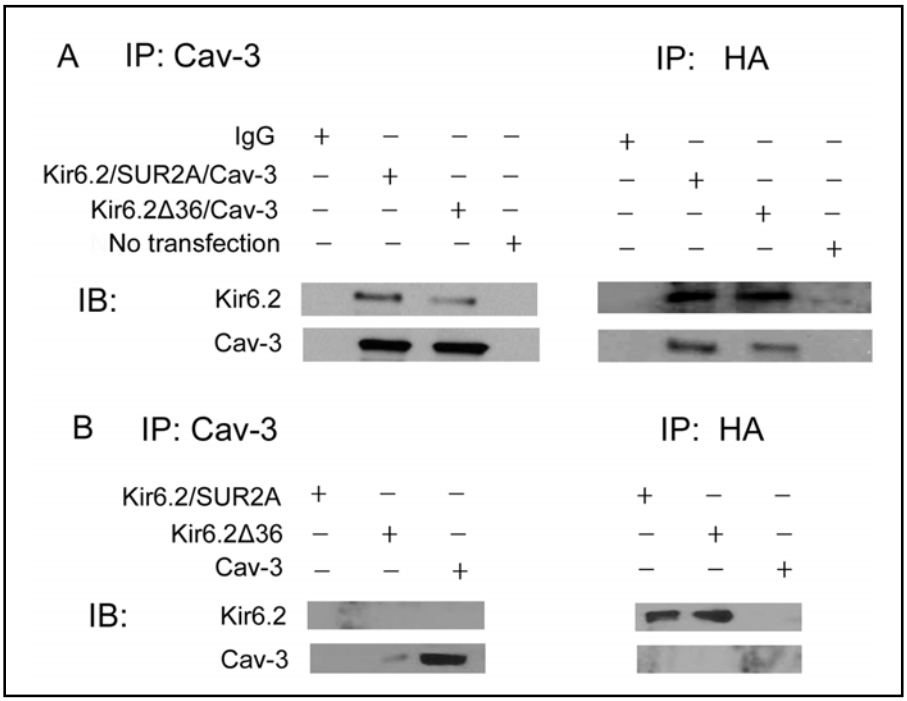

Fig. 2. Caveolin-3 co-purifies Kir6.2Δ36 in COS-7 cells. A. The protein lysates from non-transfected cells or transfected with HA-tagged Kir6.2/SUR2A/caveolin-3-CFP or HA-tagged Kir6.2Δ36/caveolin-3-CFP were immunoprecipitated with anti-caveolin-3 or anti-HA antibody followed by immunoblotting with anti-HA antibody and anti-caveolin-3 antibody. IgG denotes immuno-precipitation with control IgG from the protein lysate of COS-7 cells transfected with HA-Kir6.2/SUR2A/caveolin-3-CFP. B. Immuno-precipitation was performed with anti-caveolin-3 or anti-HA antibody from the protein lysates of COS-7 cells transfected with Kir6.2/SUR2A, Kir6.2Δ36 or caveolin-3. Western blot was conducted with anti-HA and anti-caveolin-3 antibodies. Results are representative of three independent experiments.

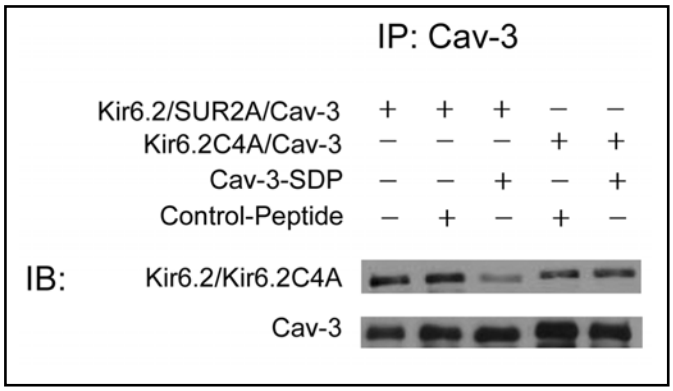

Fig. 3. The caveolin-3 scaffolding domain peptide (Cav-3-SDP, 10 μM) inhibits the co-imunoprecipitation of Kir6.2/SUR2A and caveolin-3 but not co-immunoprecipitation of Kir6.2C4A and caveolin-3 in COS-7 cells. The cell lysates from COS-7 cells transfected with HA-Kir6.2/SUR2A/caveolin-3-CFP or Kir6.2C4A/caveolin-3 were treated without or with Cav-3-SDP or control peptide prior to immunoprecipitation with anti-caveolin-3 antibody. Western blot was performed with anti-HA and anti-caveolin-3 antibodies. Results are representative of three independent experiments.

with a rat polyclonal antibody that recognized the HA epitope. In cells transfected with Kir6.2/SUR2A and caveolin-3, Kir6.2 and caveolin-3 were detected in the immunoprecitates with anti-caveolin-3 and anti-HA antibodies, respectively. The control IgG did not immunoprecipitate either Kir6.2 or caveolin-3 (Fig. 1A). Neither Kir6.2 nor caveolin-3 was detected in non-transfected cells. In cells transfected with Kir6.2C4A and caveolin-3, Kir6.2C4A and caveolin-3 were co-immunoprecipitated. The amount of Kir6.2C4A co-immunoprecipitated with caveolin-3 appeared to be significantly less than that of Kir6.2 co-immunoprecipitated with caveolin-3 whereas caveolin-3 was recovered at the same level. In the Kir6.2 immunoprecipitate with anti-HA antibody, more caveolin-3 was detected in the cells transfected with Kir6.2/SUR2A/caveolin-3 than that in the cells transfected with Kir6.2C4A/caveolin-3. Densitometric analysis revealed that the band intensity of Kir6.2 co-precipitated with caveolin-3 from cells co-transfected with Kir6.2C4A was about 18% of Kir6.2 from cells co-transfected with Kir6.2/SUR2A (18.3±0.14%, n=6, $p<0.01$). When cells were transfected with Kir6.2/SUR2A, Kir6.2C4A or caveolin-3 alone, no Kir6.2 band was detected in the caveolin-3 immuno-precipitate and caveolin-3 was recovered only in caveolin-3 transfected cells (Fig. 1B). No caveolin-3 band was found in the Kir6.2 or Kir6.2C4A immunoprecipitate with anti-HA antibody. Western blot analysis was performed to examine the expression of recombinant proteins (Fig. 1C). The total cellular expression level of octameric Kir6.2 is similar to tetrameric Kir6.2 including Kir6.2C4A and Kir6.2Δ36. SUR2A was detected as one major band at 140 kDa in cells expressing Kir6.2/SUR2A. Thus, caveolin-3 can associate with tetrameric Kir6.2 channels as well as octameric K_{ATP} channels. The stronger association of K_{ATP} channel with caveolin-3 was observed in cells expressing Kir6.2/SUR2A.

It has been shown that the distal c-terminus of some

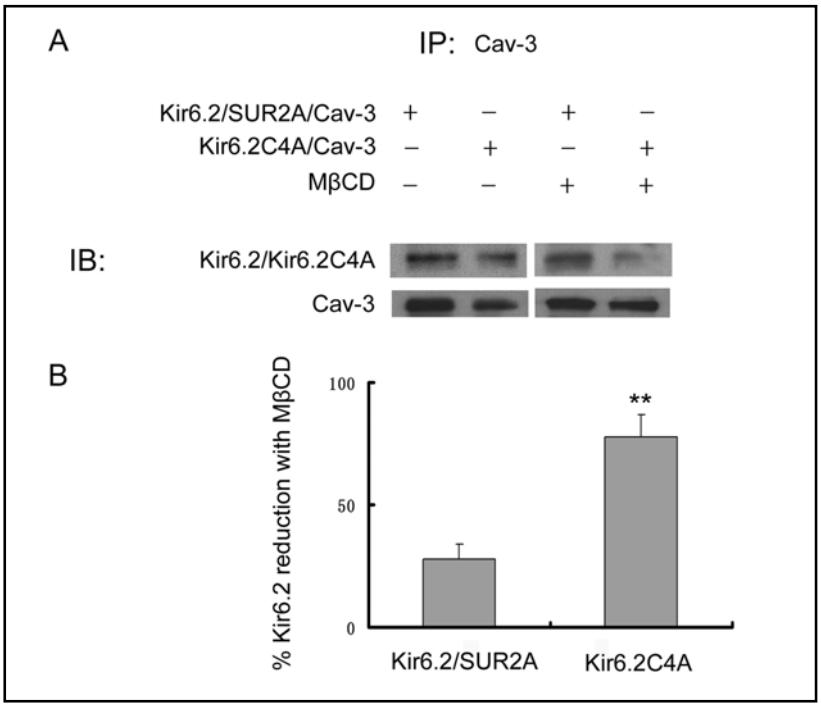

Fig. 4. The pretreatment with MβCD reduces co-immunoprecipitation of caveolin-3 and Kir6.2/SUR2A or Kir6.2C4A in COS-7 cells. COS-7 cells transfected with HA-Kir6.2/SUR2A/caveolin-3-CFP or Kir6.2C4A/caveolin-3-CFP were treated with 10 mM MβCD for 30 minutes before they were homogenized and subjected to immuno-precipitation. A. Co-imunoprecipitation was performed with anti-caveolin-3 antibody and Western blotting was conducted with anti-HA and caveolin-3 antibodies. B. Percent reduction of Kir6.2 detected in the caveolin-3 immuno-precipitates by MβCD. **p<0.01, n=3.

caveolar proteins directly interact with caveolin [23]. We reasoned that the same might be true for Kir6.2. To determine whether the distal region of the c-terminus of Kir6.2 is critical for K_{ATP} channel coupling with caveolin-3, we performed co-immunoprecipitation experiments in COS-7 cells transfected with HA-tagged Kir6.2Δ36 and caveolin-3. Kir6.2Δ36 is a truncated form of Kir6.2 with deleting the last 36 residues of Kir6.2 [24]. Since the RKR signal for ER retention in Kir6.2 is within the last 36 amino acid of Kir6.2 c-terminus, Kir6.2Δ36, when expressed alone, can also exit ER and be expressed on the cell membrane as tetrameric channels. As we can see from Figure 2, Kir6.2Δ36 and caveolin-3 were detected in the caveolin-3 and Kir6.2Δ36 immuno-preciptiates, respectively, in COS-7 cells transfected with Kir6.2Δ36/caveolin-3. Both subunits were not detected in the protein lysate of transfected cells immunoprecipitated with control IgG or in non-transfected cells. When Kir6.2Δ36 was transfected alone into COS-7 cells, Kir6.2Δ36 was detected only in the immunoprecipitate with anti-HA antibody but not in the caveolin-3 immunopreciptates. Similar to Kir6.2C4A, the amount of Kir6.2Δ36 co-immunoprecipitated with caveolin-3 was significantly less than that of Kir6.2 co-immunoprecipitated with caveolin-3 (23.7±0.09%, n=3, p<0.05). These results further confirm that tetrameric Kir6.2 channels can associate with caveolin-3 and the distal portion of the c-terminus of Kir6.2 is not responsible for the interaction.

We have previously shown that the caveolin-3 scaffolding domain plays an important role in K_{ATP} channel association with caveolin-3 [25]. In order to determine whether this interaction is via Kir6.2 or SUR2A subunit, we evaluated the effect of the caveolin-3 scaffolding domain peptide on Kir6.2/SUR2A or Kir6.2 channel coupling to caveolin-3. As shown in Fig. 3, in the absence of the caveolin-3 SDP or presence of the control peptide, caveolin-3 co-immunoprecipitated Kir6.2 or Kir6.2C4A in COS-7 cells transfected with either Kir6.2/SUR2A/caveolin-3 or Kir6.2C4A/caveolin-3. When cell lyates were incubated with the caveolin-3 SDP prior to immunoprecipitation with anti-caveolin-3 antibody, the band intensity of Kir6.2 was substantially reduced in the caveolin-3 immunoprecipitates from cells transfected with Kir6.2/SUR2A/caveolin-3 whereas Kir6.2C4A was not significantly altered in cells transfected with Kir6.2C4A/caveolin-3. The caveolin-3 SDP did not alter recovery of caveolin-3. Densitometric analysis revealed that the caveolin-3 SDP reduced the Kir6.2 co-precipitation by 91% (91.7±3.8%, n=3) while the same peptide decreased the Kir6.2C4A co-precipitation by about 6% (6.3±0.76%, n=3). These data indicate that unlike tetrameric Kir6.2 channels, octameric K_{ATP} channels associate with caveolin-3 via the caveolin-3 scaffolding domain, possibly through interaction with SUR2A subunit.

To determine whether association of octameric Kir6.2/SUR2A or tetrameric Kir6.2 channels with caveolin-3 depends on the level of cholesterol, we examined the effect of disrupting cholesterol with MβCD on co-immunoprecipitation of caveolin-3 with K_{ATP} channels in COS-7 cells transfected with either Kir6.2/

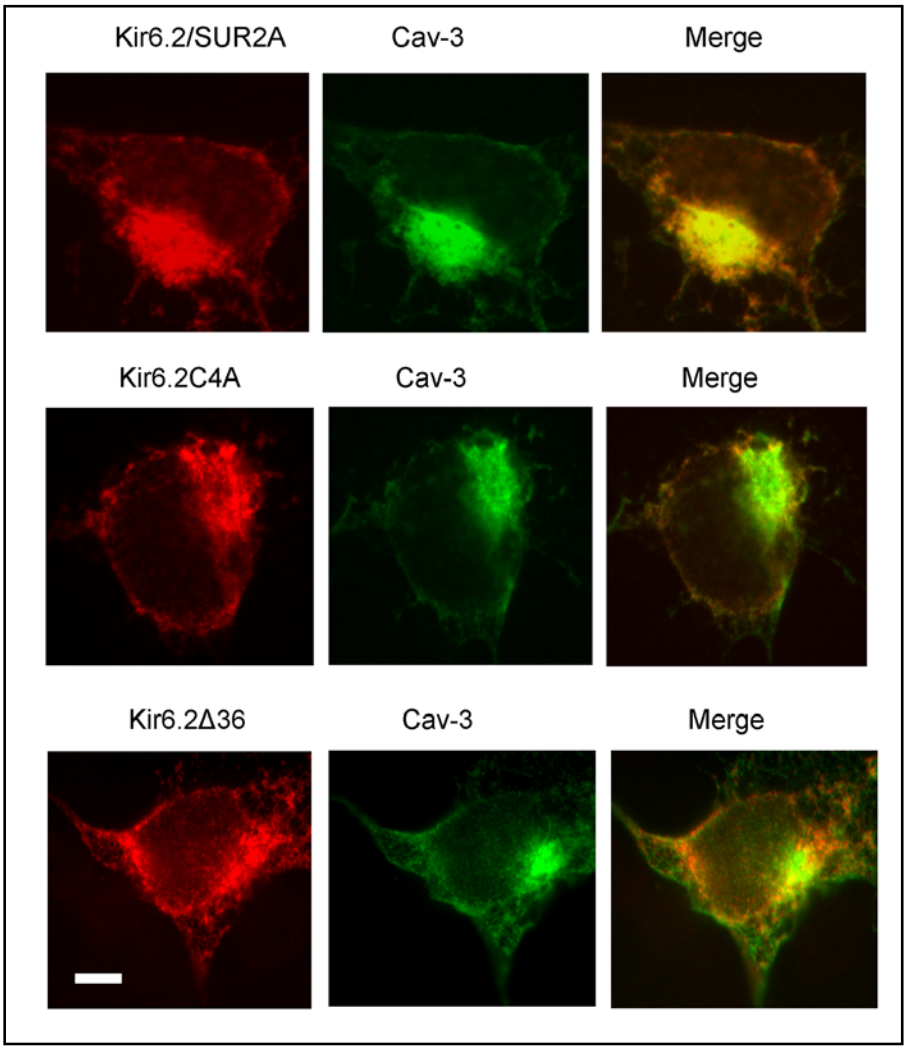

Fig. 5. Caveolin-3 colocalizes with Kir6.2C4A or Kir6.2Δ36 in COS-7 cells. Cells were transfected with HA-Kir6.2/SUR2A/caveolin-3-CFP, Kir6.2C4A/caveolin-3-CFP or Kir6.2Δ36/caveolin-3-CFP. Immunofluorescence staining was performed with anti-HA antibody. HA-tagged Kir6.2 and its mutants (Kir6.2C4A or Kir6.2Δ36) were illustrated by red and caveolin-3-CFP was indicated by Green. The images are representative of three independent experiments. Scale bar: 10 μM.

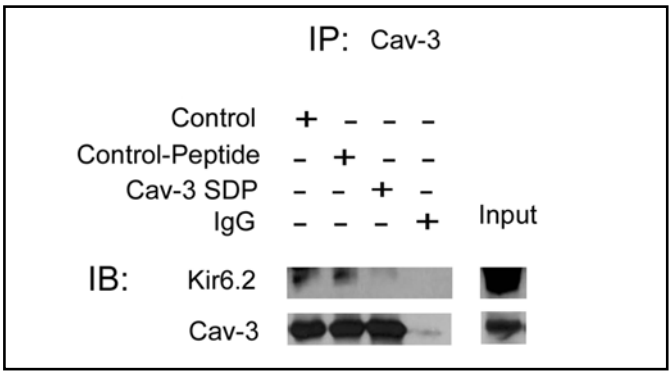

Fig. 6. The Cav-3 SDP prevents the co-immunoprecipitation of caveolin-3 with endogenous K_{ATP} channels in cardiac ventricular myocytes. The Cav-3 SDP (10 μM) or control scrambled peptide was added to the protein lysates before being subjected to immuniprecipitation with anti-caveolin-3 antibody. Control sample was incubated without control IgG. Results are representative of three independent experiments.

SUR2A/ caveolin-3 or Kir6.2C4A/caveolin-3. Cells were pretreated with or without MβCD (10 mM) for 30 minutes prior to homogenization. As shown in Fig. 4, MβCD pretreatment almost completely diminished the level of Kir6.2C4A that was co-precipitated with caveolin-3 in cells co-transfected with Kir6.2C4A/caveolin-3. The Kir6.2 co-precipitation from cells transfected with Kir6.2/SUR2A/caveolin-3 was altered at less degree by MβCD pretreatment (Fig. 4A). When Kir6.2 or Kir6.2C4A bands were normalized to the corresponding controls without MβCD, the percent reduction in Kir6.2 co-precipitation was significantly higher than that in Kir6.2C4A co-precipitaiton (30.0±2.4% vs. 80.0±5.7%, Kir6.2/SUR2A vs. Kir6.2C4A, n=3-4, $p<0.01$). The total caveolin-3 level was not altered by MβCD treatment (Fig. 4B). These results suggest that cholesterol is important for coupling of both octameric and tetrameric K_{ATP} channels with caveolin-3. The coupling of tetrameric Kir6.2 channels with caveolin-3 appears to be more dependent on cholesterol level on the cell membrane,

indicating less possibility of direct interaction between Kir6.2 and caveolin-3.

To confirm whether octameric Kir6.2/SUR2A or tetrameric Kir6.2 channels associate with caveolin-3, COS-7 cells transfected with HA-Kir6.2/SUR2A/caveolin-3-ECFP, HA-Kir6.2C4A/caveolin-3-ECFP or HA-Kir6.2Δ36/caveolin-3-ECFP were immunostained with anti-HA antibody. Immunofluorescence microscopy revealed that Kir6.2 in cells transfected with HA-Kir6.2/SUR2A/caveolin-3-ECFP significantly colocalized with caveolin-3, as indicated by yellow punctate staining along cell peripheral and cell surface (Fig. 5). Cells transfected with HA-Kir6.2C4A/caveolin-3-ECFP or HA-Kir6.2Δ36/caveolin-3-ECFP showed less colocalization of Kir6.2C4A or Kir6.2Δ36 with caveolin-3. Quantitative analysis indicated that 78% of cells transfected with HA-Kir6.2/SUR2A/caveolin-3-ECFP showed significant colocalization of Kir6.2 with caveolin-3 whereas only 27% or 26% of cells transfected with Kir6.2C4A/caveolin-3-ECFP or HA-Kir6.2Δ36/caveolin-3-ECFP, respectively, showed significant colocalization of Kir6.2C4A or Kir6.2Δ36 with caveolin-3. A total of 60 cells were analyzed with 20 cells in each group.

To further elucidate the role of the caveolin-3 scaffolding domain in coupling cardiac K_{ATP} channel to caveolin-3, we examined the effect of the caveolin-3 SDP on co-immunoprecipitation of endogenous cardiac K_{ATP} channels and caveolin-3 in cardiac ventricular myocytes. The cardiac myocytes were isolated by enzymatic dissociation and then homogenized in lysis buffer. The samples were immunoprecipitated with anti-caveolin-3 with or without prior treatment with the caveolin-3 SDP or control peptide. As shown in Fig. 6, Kir6.2 was detected with anti-Kir6.2 antibody in the caveolin-3 immunoprecipitates in the samples treated with control peptide but not in the samples treated with the caveolin-3 SDP. When samples were treated with control IgG for immunoprecipitation, neither Kir6.2 nor caveolin-3 was detected. These observations suggest that endogenous octameric cardiac K_{ATP} channels couple to caveolin-3 via the caveolin-3 scaffolding domain.

Discussion

In the present study, we demonstrate that SUR2A is critical for caveolar targeting of cardiac K_{ATP} channels. We showed that caveolin-3 can associatde with both octameric Kir6.2/SUR2A and tetrameric Kir6.2 channels expressed in COS-7 cells. We found that only octameric K_{ATP} channels couple to caveolin-3 via the caveolin-3 scaffolding domain, as evidenced by the significant reduction in the Kir6.2 co-immunoprecipitation by the caveolin-3 SDP. Association of caveolin-3 with tetrameric Kir6.2 channels was not reduced by the caveolin-3 SDP. Similar results were observed for endogenous octameric K_{ATP} channels in cardiac ventricular myocytes. Immunofluorecence microscopy indicated that octameric K_{ATP} channels colocalized with caveolin-3 at a greater extent than tetrameric K_{ATP} channels. We further demonstrated that the cholesterol depleting agent MβCD substantially reduced the Kir6.2C4A co-precipitation and only moderately decreased the Kir6.2 co-precipitation with caveolin-3. Our data suggest that octameric K_{ATP} channels couple to caveolin-3 by a mechanism that involves the caveolin-3 scaffolding domain, possibly through the caveolin binding motif on SUR2A subunit.

In the present study, we used two types of cells: adult rat cardiomyocytes and COS-7 cells. The advantage of using COS-7 cells in this study is that these cells lack endogenous K_{ATP} channels and caveolin-3, and provide an appropriate heterologous expression system for studying the proteins of interest, especially when mutagenesis is involved. Further, studies have shown that recombinant K_{ATP} channels containing Kir6.2/SUR2A in COS-7 cells have electrophysiological and biochemical characteristics quite similar to native channels [26, 27]. Studies have also shown that recombinant Kir6.2/SUR2A channels in COS-7 cells protect cells against hypoxia-reoxygenation injury [28].

It has been shown that the pancreatic K_{ATP} channels (Kir6.2 and SUR1) are absent from caveolae whereas the vascular smooth muscle K_{ATP} channels (Kir6.1 and SUR2B) are present in caveolae [29, 30]. Further, we have recently shown that the cardiac K_{ATP} channels (Kir6.2/SUR2A) are localized to the caveolin-rich microdomains of cardiac myocytes. These results suggest that SUR2 subunit may be responsible for caveolar targeting of K_{ATP} channels since SUR2A and SUR2B differ only in the last 42 amino acids. Interestingly, we found that by alignment of conserved region of SUR2 (SUR2A and 2B) containing consensus caveolin binding domain, both SUR2A and SUR2B have the same consensus caveolin binding domains in their conserved region assembled near the cytoplasmic side of sarcolemmal membrane. The corresponding region in SUR1 does not have complete and required consensus sequence for binding to caveolin. We did not find any consensus caveolin binding domains in Kir6.2 or Kir6.1. Aromatic-rich sequences within the caveolin binding motif

for caveolin or caveloae-associated proteins are critical for the interaction of caveolin and caveolin binding partners [7]. However, our data do not exclude the possibility that Kir6.2 may interact with caveolin-3 when expressed as tetrameric Kir6.2 channels without SUR subunit. The lack of a caveolin binding domain in Kir6.2 does not necessarily rule out direct interaction with caveolin-3 as caveolins are known to have other binding domains [31]. In the present study, we demonstrate that the caveolin-3 SDP blocked the association of caveolin-3 with Kir6.2/SUR2A octameric channels but not Kir6.2 tetrameric channels, indicating that the caveolin-3 scaffolding domain is important for caveolar targeting of octameric K_{ATP} channels, possibly via the caveolin binding motif in SUR2A. We also found that tetrameric Kir6.2 channels can associate with caveolin-3 and this association depends largely on the cholesterol level of the cell membrane. Although the mechanism by which tetrameric Kir6.2 channels associate with caveolin-3 is not clear, it is less likely that Kir6.2 channels would directly interact with caveolin-3.

Caveolin-3 is muscle-specific and is found rich in both cardiac and skeletal muscle. Caveolin-3 SDP encodes membrane proximal regions of caveolin-3 and is known to functionally interact with some caveolin-associated proteins [32, 33]. Our observations suggest there is an interaction between K_{ATP} channels and caveolin-3. Whether these interactions are direct or through other proteins within a detergent resistant lipid rafts cannot be determined by immunoprecipitation experiments. Studies have suggested that in caveolar microdomains, caveolins exist as large oligmers in association with other proteins. It is likely that K_{ATP} channels exist as a large signaling complex consisting of caveolins and related signaling proteins. Our observation that the caveolin-3 SDP blocked co-immunoprecipitation of Kir6.2/SUR2A and caveolin-3 but not Kir6.2 C4A (or Kir6.2Δ36) and caveolin-3 suggests that coupling octameric K_{ATP} channel to caveolin-3 may be mediated by the caveolin scaffolding domain, possibly through SUR2A either directly or indirectly.

In our co-immunoprecipitaiton experiments, we noticed that the Kir6.2 co-precipitation was significantly less from cells transfected with Kir6.2C4A or Kir6.2Δ36 when compared with that in cells transfected with Kir6.2/SUR2A. The cell surface expression of tetrameric Kir6.2 channels with these mutants has been shown to be lower than that of octameric channels [18]. Therefore, it is possible that the decreased in the Kir6.2 co-immunoprecipitation was in part due to decreased cell surface expression of Kir6.2 channels. Although the mechanism underlying the decreased cell surface expression of tetrameric Kir6.2 channels is not clear, it is suggested that lack of SUR subunit may contribute to the low cell surface expression. Our finding that SUR2A play an important role in coupling of octameric K_{ATP} channels to cavoelin-3 indicates that caveolins may serve as important proteins for anchoring K_{ATP} channels to the cell surface. In support with this notion, recent studies suggest that caveolin-3 on the sarcolemmal membrane is critical for Na^+ current amplitude in cardiac myocytes [16].

The direct interaction between caveolins and interacting proteins may serve to either anchor proteins to caveolae or modulate protein function. Our data suggest that coupling K_{ATP} channels to caveolin-3 is important for caveolar targeting and caveolin association of K_{ATP} channels. K_{ATP} channel microenvironment has been considered to be different from "bulk" cytosol as these channels exist in multimolecular complexes with [34-38] and are functionally regulated by many metabolic enzymes [39, 40]. Glycolytic enzymes are specifically localized to caveolae [41]. It's been suggested that subsarcolemmal ATP may play an important role in activation of K_{ATP} channels at relative high intracellular ATP [42]. The proximity of the subsarcolemmal ATP to the cell surface raises the possibility that caveolae may serve as microdomains for efficient modulation of K_{ATP} channel activity and signaling where nucleotide concentration can be tightly controlled by metabolic enzymes in the subsarcolemmal space. While K_{ATP} channel activation may confer cardioprotection against ischemic injury, excessive K_{ATP} opening has been associated with cardiac arrhythmia. Caveolin-3 appears to have dual functional roles: promote K_{ATP} channel activation by adenosine receptors and inhibit K_{ATP} channel function. A tight regulation of caveolin-3 expression level may play a critical role not only in the caveolar targeting of K_{ATP} channels but also in controlling K_{ATP} channel function and regulation. Different mechanisms of K_{ATP} channel regulation may serve differently under various physiological conditions.

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