Regulation of ATP-sensitive K$^+$ channels by caveolin-enriched microdomains in cardiac myocytes

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Aims ATP-sensitive potassium (K$_{ATP}$) channels in the heart are critical regulators of cellular excitability and action potentials during ischaemia. However, little is known about subcellular localization of these channels and their regulation. The present study was designed to explore the potential role of caveolae in the regulation of K$_{ATP}$ channels in cardiac ventricular myocytes.

Methods and results Both adult and neonatal rat cardiomyocytes were used. Subcellular fractionation by density gradient centrifugation, western blotting, co-immunoprecipitation, and immunofluorescence confocal microscopy were employed in combination with whole-cell voltage clamp recordings and siRNA gene silencing. We detected that the majority of K$_{ATP}$ channels on the plasma membrane of cardiac myocytes were localized in caveolin-3-enriched microdomains by cell fractionation and ultracentrifugation followed by western blotting. Immunofluorescence confocal microscopy revealed extensive colocalization of K$_{ATP}$ channel pore-forming subunit Kir6.2 and caveolin-3 along the plasma membrane. Co-immunoprecipitation of cardiac myocytes showed significant association of Kir6.2, adenosine A$_1$ receptors, and caveolin-3. Furthermore, whole-cell voltage clamp studies suggested that adenosine A$_1$ receptor-mediated activation of K$_{ATP}$ channels was largely eliminated by disrupting caveolae with methyl-$\beta$-cyclodextrin or by small interfering RNA, whereas pinacidil-induced K$_{ATP}$ activation was not altered.

Conclusion We demonstrate that K$_{ATP}$ channels are localized to caveolin-enriched microdomains. This microdomain association is essential for adenosine receptor-mediated regulation of K$_{ATP}$ channels in cardiac myocytes.

KEYWORDS
K$_{ATP}$ Channel; Caveolin-3; Adenosine receptor

1. Introduction

The ATP-sensitive potassium (K$_{ATP}$) channel is a highly abundant plasma membrane protein responsible for linking the cellular energy levels to membrane potentials.$^{1,2}$ In the heart, K$_{ATP}$ channels on the plasma membrane are critical regulators of cellular excitability and action potentials during ischaemia. They are closed under the basal conditions due to inhibition by intracellular ATP, but open under metabolic stress such as ischaemia or hypoxia. Cardiac K$_{ATP}$ channels have been shown to protect against the metabolic insult of ischaemia and contribute to adaptive responses to metabolic stress.$^{3-5}$

It’s been known that cardiac K$_{ATP}$ channels open at micromolar range of intracellular ATP during ischaemia while ATP required to inhibit this channel is in micromolar range.$^{1,6}$ This discrepancy is probably due to the fact that some signalling molecules, which are released during ischaemia, regulate ATP-dependent gating of the K$_{ATP}$ channels by decreasing ATP-sensitivity and thus increasing open probability.$^{7-10}$ Since many signalling molecules and their downstream mediators/effectors concentrate in the microdomains of the plasma membrane, the localization of K$_{ATP}$ channels to these sites may have important implications for channel function and regulation. For rapid and efficient activation of K$_{ATP}$ channels during metabolic stress, the spatial localization of K$_{ATP}$ channels should be of immense importance. It is likely that the K$_{ATP}$ channel signalling is compartmentalized.

Caveolae are specialized plasma membrane microdomains involved in numerous signalling transduction events. Caveolins are the main structural components of caveolae that can bind cholesterol and coat the cytoplasmic surface of these organelles. They comprise a family of three distinct 21–24 kDa isoforms; caveolin-1 and -2 are almost ubiquitously expressed, whereas caveolin-3 is a muscle-specific isoform.$^{11,12}$ In the present study, we tested the hypothesis that subcellular localization of K$_{ATP}$ channels to caveolin-enriched microdomains on the plasma membrane of cardiac myocytes is essential for the regulation of K$_{ATP}$ channels by adenosine receptors.

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2. Methods

2.1 Cell isolation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996). Adult rat ventricular myocytes were isolated from Sprague-Dawley (SD) rats (250–300 g) by enzymatic dissociation. In brief, hearts were excised and retrogradely perfused via aorta with Tyrode’s solution containing (in mM) NaCl 136, KCl 5.4, CaCl2 1.0, MgCl2 1.0, NaH2PO4 0.33, HEPES 10, and glucose 10 at 37 °C. The perfusate was then changed to a Tyrode’s solution that was nominally Ca2+ free but otherwise having the same composition. The hearts were perfused with the same solution containing collagenase for 20 min. 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 a storage solution containing (mM) KCl 20, KH2PO4 10, glucose 10, potassium glutamate 70, β-hydroxybutyric acid 10, taurine 10, mannitol 5, and EGTA 5, along with 1% albumin.

Neonatal myocytes were isolated from 1- or 2-day-old SD rats by collagenase digestion as described previously. Hearts were removed and collected in ice-cold Hank’s balanced salt solution (HBSS) and rinsed free of excess blood. The tissue was minced and collected in 1 mL of fresh HBSS to which 1 mL of 2 mg/mL collagenase type 2 was added. A total of seven rounds of 5 min digestions were performed at 30 °C on a rocking platform. After each digestion, the tissue was gently aspirated up and down three times through a 10 mL pipette and allowed to settle for 1 min. The supernatants of the first three digestions were discarded. The supernatants collected from digestions 4–7 were collected, adjusted to 20% FCS, and centrifuged at 400 g for 5 min. The cell pellets were immediately resuspended in 1 mL DMEM containing 10% FCS. After preplating for 1 h in a tissue culture grade petri dish at 37 °C and 5% CO2 to allow for the attachment of fibroblasts, cell suspension was plated onto collagen-coated coverslips. On second day, cytosine-β-D-arabinofuranoside (10 μg/mL) was added to the medium to inhibit fibroblast growth. Adherent viable myocytes started to contract spontaneously 24 h after plating. Non-myocyte cells were <10% of total cells.

2.2 Small interfering RNA and transfection

The small interfering RNA (siRNA) oligonucleotide targeting caveolin-3 was purchased from Ambion Inc. (Austin, TX, USA). A negative control siRNA (scrambled) was included to monitor non-specific effects. Neonatal ventricular myocytes were transfected with siRNA and pEGFP using Amaxa Kit (Amaxa, Gaithersburg, MD) immediately after preplating step. Forty-eight to 72 h after transfection, western blot was carried out to examine the knockdown of targeted proteins.

2.3 Fractionation of caveolin-enriched membrane

Caveolin-rich fractions from cardiomyocytes were prepared by using a previously described detergent-free method with some modification. Briefly, cell pellet was resuspended in 0.5 M sodium carbonate (pH 11.0; 2 mL) and homogenized sequentially by using a loose-fitting Dounce homogenizer, a Polytron tissue grinder, and a sonicator. The homogenate was adjusted to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM Mes, pH 6.5/0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (in MBS containing 250 mM sodium carbonate) was formed above, by overlaying with 4 mL of 35% sucrose (prepared in MBS with 250 mM sodium carbonate) and then 4 mL of 5% sucrose (again prepared in MBS with 250 mM sodium carbonate). Tubes were centrifuged at 39,000 r.p.m. for 18–20 h in a SW41 rotor. Twelve 1 mL fractions were collected from the top to the bottom of the gradient for subsequent analysis by western blot.

2.4 Western blotting

Immunoblot analysis was carried out as described previously. The density gradient fractions or cell lysates were denatured in a sample buffer, electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were blocked with 5% non-fat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4) and incubated for 1 h 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.

2.5 Co-immunoprecipitation

Immunoprecipitation experiments were performed as described previously. Adult rat ventricular myocytes or neonatal rat hearts were homogenized in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, pH 7.6) containing 1% Triton X-100 and 1 mM sodium orthovanadate with protease inhibitor tablets (Roche). Insoluble materials were pelletted by centrifugation at 10,000 g for 15 min and the lysates were precleared by incubation with r-protein-G agarose (Invitrogen; 1 h, 4 °C). Five hundred microlitres of cleared lysate (1 μg/μL) was incubated with 5 μg of primary antibodies or 5 μg of appropriate control IgG (or without primary in some cases) overnight at 4 °C. Antigen-antibody complexes were captured with r-protein-G agarose (4 °C, 2 h). Agarose beads were washed four-times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. In some experiments, co-immunoprecipitation was performed in caveolin-rich fractions. The fraction 4, 5, and 6 from sucrose-gradients centrifugation were combined and centrifuged at 40,000 g for 2 h to pellet caveolae, which was then suspended in lysis buffer and sonicated prior to immunoprecipitation. Samples were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and analysed by probing with various antibodies.

2.6 Immunofluorescence

As described previously, the cells were fixed with 4% formaldehyde in PBS for 30 min, blocked, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labelled with primary antibody for 2 h. Cells were then washed three times and labelled with fluorescence-conjugated secondary antibody for 1 h. Immunofluorescence was visualized with confocal laser scanning microscopy. All images were analysed using a background subtraction method offline.

2.7 Electrophysiological recordings

Membrane currents were recorded from adult and neonatal rat myocytes using whole-cell perforated patch and conventional whole-cell configurations, respectively. In the siRNA transfected cells, only GFP-positive neonatal myocytes were used for recording. Cells were perfused with the bath solution containing (mM) NaCl 135, KCl 5.4, MgCl2 1.0, CaCl2 1.0, NaH2PO4 0.33, HEPES 10, and glucose 10 at pH 7.4. The pipette solution contained (mM) KCl 140, MgCl2 1.0, HEPES 10, EGTA 5, and GTP 0.1 at pH 7.3. For conventional whole-cell recordings, 0.25 mM ATP was added to pipette solutions for recordings. For perforated whole-cell configuration, amphotericin B stock solution (30 mg/mL in DMSO) was diluted with the pipette solution to reach the final concentration (300 μg/mL) with the aid of sonication. All experiments were conducted at room temperature (22–25 °C). Borosilicate glass electrodes (outer diameter, 1.5 mm) were used with resistances in range of 3–5 MΩ when filled and connected to a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union...
For the time course of $K_{ATP}$ currents, the cell membrane was held at $-40 \text{mV}$. $K_{ATP}$ currents were calculated as glibenclamide-sensitive currents.

### 2.8 Data analysis

Group data were presented as means $\pm SE$. 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.

### 3. Results

#### 3.1 Localization of $K_{ATP}$ channels in caveolin-enriched microdomains

To determine whether $K_{ATP}$ channels are present in caveolin-rich membrane fractions in cardiac myocytes, we used a detergent-free sucrose gradient extraction-procedure for adult rat ventricular myocytes. Figure 1A shows western blot analysis of 10 mL fractions collected from top to bottom of the sucrose density gradient. The marker proteins for caveolae, caveolin-1, and caveolin-3 were found predominantly in the lower-density fractions 4 and 5 (Figure 1A, upper panel). In the same caveolar-enriched fractions, we detected immunoreactivity for a significant amount of $K_{ATP}$ channel pore-forming subunit Kir6.2 ($\sim80\%$). As reported previously,$^{17}$ caveolin-3 was also detected in heavier samples (fractions 9–12) though enrichment for caveolin-3 occurred only in samples 4 and 5. Clathrin heavy-chain was detected with antibody against clathrin heavy chain across a broad range of the gradient fractions similar to that reported by Sampson et al.$^{18}$ Although there was some overlap between caveolins and clathrin in fraction 5, it should be noted that the distribution of Kir6.2 did not follow that of clathrin but only in caveolin-enriched fraction. Coomassie blue staining of the gel showed that little protein was found in the earlier fractions$^4$–$^5$ that contain substantial amounts of caveolin-3 and caveolin-1 but excludes most of the cellular protein (Figure 1A, lower panel). Measurement of cholesterol levels within each fraction showed cholesterol to be enriched in fractions 4 and 5 (Figure 1B), consistent with the observation that these two fractions represent caveolae-containing membrane fraction of the density gradient. Further, the whole blots from the samples of rat cardiac myocytes probed with antibodies against Kir6.2, caveolin-1, or caveolin-3 are shown in Supplementary material online, Figure S1. A single band of Kir6.2, caveolin-1, or caveolin-3 at the correct molecular mass was detected in the light fractions (fractions 4 and 5). The high molecular mass oligomers of caveolin-1 at 75 kDa were also detected in caveolin-rich fractions. Therefore, $K_{ATP}$ channel pore-forming subunit Kir6.2 is present in the caveolin-rich membrane fractions of cardiac ventricular myocytes.

#### 3.2 Co-immunoprecipitation of $K_{ATP}$ channels and caveolin-3 from adult rat cardiac myocytes

To determine whether muscle-specific caveolin isoform caveolin-3 and Kir6.2 are associated with each other, we performed immunoprecipitation experiments from the cell lysates of adult rat cardiac myocytes. The cell homogenate was incubated with rabbit anti-Kir6.2 antibody to precipitate caveolin-3. The immune complexes were collected with r-protein G beads and analysed by immunoblotting against mouse anti-caveolin-3 and anti-Kir6.2 antibodies. Figure 2 shows that anti-Kir6.2 antibody immunoprecipitated caveolin-3. Conversely, anti-caveolin-3 antibody immunoprecipitated Kir6.2. Neither protein was immunoprecipitated with control IgGs (mouse IgG and rabbit IgG). These results suggest that the Kir6.2 associates with caveolin-3 in adult rat ventricular myocytes.

#### 3.3 Colocalization of $K_{ATP}$ channel pore-forming subunits and caveolin-3

To investigate whether $K_{ATP}$ channel pore-forming subunit Kir6.2 colocalizes with caveolin-3, we employed immunofluorescence confocal microscopy. Figure 3 shows fluorescence images from adult rat ventricular myocytes. The antibody against Kir6.2 (red) or caveolin-3 (green) demonstrated a prominent surface sarcolemmal punctate staining area. Merged images showed significant punctuate areas of colocalization for Kir6.2 and caveolin-3 along the plasma membrane. In agreement with previous observations,$^{19}$ Kir6.2 was clearly localized in peripheral sarcolemma and T tubules. Caveolae vesicles have been noted before by electron microscopic examination of the myocardium on both peripheral plasma membrane and T tubules.$^{20,21}$ Our immunostaining also showed the presence of caveolin-3 in
A1-receptors and Gi are localized in caveolar-enriched fractions.23,24 To determine whether cholesterol-enriched microdomains are essential for adenosine receptor-mediated activation of KATP channels, we tested the effect of disrupting caveolae with a cholesterol depleting agent methyl-β-cyclodextrin (MβCD) on KATP channel activity in adult rat ventricular myocytes. Non-selective adenosine receptor antagonist 8-phenyltheophylline (8-PT) was used to ensure that adenosine effect involves activation of adenosine receptors. Due to strong inhibition of KATP channels by intracellular ATP, most of modulators including adenosine receptors are shown to activate KATP channels only at reduced intracellular ATP level or in the presence of a potassium channel opener. Since we employed the whole-cell perforated patch-clamp technique where physiological ATP level was preserved, we studied the effect of adenosine on KATP channels in the presence of pinacidil (50 μM). The effect of MβCD on KATP channels evoked by pinacidil alone (100 μM) was used as a negative control since pinacidil activates KATP channel by interacting with SUR subunit and its effect should be independent of lipid rafts/caveolae microdomains. As previously reported, adenosine alone did not activate KATP channels due to stronger inhibition by intracellular ATP (data not shown). Pinacidil alone at 50 μM elicited a small K+ current that was completely blocked by selective KATP channel blocker glibenclamide (10 μM, Figure 4A). However, when pinacidil (50 μM) and adenosine (100 μM) were superfused together in the bath solution, they activated a large K+ conductance channel that was completely inhibited by glibenclamide (Figure 4B). When cells were superfused with 10 mM MβCD for 10 min, which was previously shown to disrupt the function of caveolar associated proteins,23 prior to application of adenosine plus pinacidil, we found that MβCD pre-treatment almost completely eliminated the response of KATP channels to adenosine receptors (Figure 4C, 13.19 ± 2.28 vs. 2.37 ± 1.21 pA/pF, Adenosine + pinacidil vs. adenosine + pinacidil + MβCD, P < 0.05, n = 3–4). In contrast, MβCD did not reduce KATP currents activated by 100 μM pinacidil (15.37 ± 3.41 vs. 19.67 ± 4.15 pA/pF, pinacidil vs. pinacidil + MβCD, P = NS), indicating that KATP channels evoked by pinacidil is independent of cholesterol-rich plasma membrane microdomains. These observations indicate that disrupting cholesterol-rich microdomains by MβCD significantly reduced effect of adenosine receptors on KATP channels.

3.5 Effect of disrupting caveolae with small interfering RNA targeting caveolin-3 on adenosine A1 receptor-mediated activation of KATP channels

Although acute MβCD treatment of adult rat myocytes caused caveolar disruption and resulted in the loss of regulation of KATP channels by adenosine receptors, interpretation of results could be complicated by MβCD-induced cholesterol depletion that may influence the regulation of KATP channels outside of caveolae. To determine whether specifically destroying caveolae by siRNA knockdown of caveolin-3 expression alters the regulation of KATP channels by adenosine A1 receptors, we investigated the effect of caveolin-3 siRNA on KATP channel regulation. We first examined whether a pre-designed siRNA oligonucleotide for rat caveolin-3 can reduce endogenous caveolin-3 protein level.
in neonatal rat cardiomyocytes. We found that caveolin-3 siRNA significantly suppressed caveolin-3 but not caveolin-1 protein expression 48 h after transfection with siRNA. In contrast, control siRNA had no effect (Figure 5B). The whole blots for Figure 5B are shown in Supplementary material online, Figure S2. Cells exhibiting GFP that was co-transfected with siRNA were used for whole-cell voltage clamp studies. As shown in Figure 5A, adenosine alone can activate KATP channels after dialysis of pipette solution with a low ATP (0.25 mM). Transfection of neonatal cardiomyocytes with caveolin-3 siRNA largely eliminated the stimulatory effect of adenosine A1 receptors on KATP channels, whereas control siRNA showed no significant changes (1.5 ± 0.6 vs. 24.1 ± 4.0 pA/pF, caveolin-3 siRNA vs. control siRNA, P < 0.01, n = 4–5). In contrast, KATP channels activated by pinacidil (100 μM) were not altered by siRNA targeting caveolin-3 (38.21 ± 13.02 vs. 45.85 ± 10.40 pA/pF, control vs. siRNA, P = NS). Pinacidil followed by glibenclamide was applied to confirm there were functional KATP channels in the cells transfected with caveolin-3 siRNA. Further, the effect of adenosine on KATP channels was almost completely blocked by a selective adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 μM, Figure 5C). Taken together, these findings suggest that adenosine receptor-mediated activation of KATP channels in the cardiac myocytes requires intact caveolae where KATP channels and adenosine receptors associate.

### 3.6 Co-immunoprecipitation of KATP channels and caveolin-3 from neonatal rat hearts

To determine that caveolin-3 and KATP channels reside in the same membrane compartment in neonatal rat hearts as those in adult rat hearts, we subjected the caveolin-rich fractions (fractions 4–6) to immunoprecipitation with anti-caveolin-3 or anti-Kir6.2 antibodies. The immunoprecipitates were analysed by immunoblotting with antibodies against Kir6.2 or caveolin-3. As shown in Figure 6, Kir6.2 co-precipitated caveolin-3 and caveolin-3 co-precipitated Kir6.2. The control samples without primary antibody did not immunoprecipitate the proteins. We also examined the presence of adenosine A1 receptors in the samples obtained by immunoprecipitation with anti-caveolin-3 antibodies. Adenosine A1 receptors were detected in these samples.

Figure 3 Colocalization of Kir6.2 and caveolin-3 (Cav-3). Double labelling confocal images of adult rat ventricular myocytes with anti-Kir6.2 and anti-Cav-3 antibodies. Punctate areas of colocalization (represented by yellow) are apparent along the plasma membrane. The white boxes represent enlarged areas shown in the lower panels.

Figure 4 Caveolar disruption with methy-b-cyclodextrin eliminates adenosine receptor-mediated stimulation of KATP channels. Time course of KATP currents in perforated whole-cell configuration was recorded in the presence of pinacidil 50 μM alone (Pina, A), pinacidil plus Adenosine 100 μM (Pina + Ade, B) at a holding potential of −40 mV in control cells or cells pre-treated with methy-b-cyclodextrin for 10 min (C). Glibenclamide (Glib) was added subsequently to ensure KATP channel activation. (D) Averaged current density in the presence of various agents as indicated, which was calculated by dividing glibenclamide-sensitive currents by cell capacitance. *P < 0.05 vs. Pina. n = 3–4.
These data suggest that K<sub>ATP</sub> channels and adenosine A<sub>1</sub> receptors associate with caveolin-3-rich membrane compartments in the neonatal rat heart.

3.7 Association of adenosine A<sub>1</sub> receptors and K<sub>ATP</sub> channels in caveolin-3-rich fractions of cardiomyocytes

We have shown that Kir6.2 associates with caveolin-3 in the total cell lysates of adult rat cardiomyocytes. In order to determine whether Kir6.2 and adenosine A<sub>1</sub> receptors reside in the same caveolin-enrich microdomains and further elucidate the mechanism involved in adenosine-mediated K<sub>ATP</sub> activation, we performed co-immunoprecipitation experiments in the caveolin-3-enriched fractions of adult rat ventricular myocytes. Cells were pre-treated with MβCD (10 mM) for 30 min prior to sucrose gradient fractionation. Caveolin-rich fractions (fractions 4–6) were immunoprecipitated with antibodies against caveolin-3 (Cav-3) and no change in caveolin-1 (Cav-1). Averaged current density induced by adenosine (100 μM) was shown in cells transfected with nothing, control siRNA, or Cav-3 siRNA. DPCPX was used to ensure that K<sub>ATP</sub> channel activation by adenosine was through the stimulation of adenosine A<sub>1</sub> receptors. **P < 0.01 vs. control for DPCPX group, vs. control siRNA for Cav-3 siRNA group. n = 4–5.

The antibody against adenosine A<sub>1</sub> receptors. There was no detection of Kir6.2, adenosine A<sub>1</sub> receptors, and caveolin-3 in the homogenates without the treatment of primary antibodies. MβCD diminished caveolin-3 that co-precipitated with Kir6.2 and adenosine A<sub>1</sub> receptors, while the levels of Kir6.2 and adenosine A<sub>1</sub> receptors were also reduced. The whole blots from the co-immunoprecipitates probed with
antibodies against Kir6.2, caveolin-3 or adenosine A1 receptors or caveolin-3 are shown in Supplementary material online, Figure S3. These data indicate that KATP channels and adenosine A1 receptors may associate with each other in caveolin-rich membrane microdomains and this association depends on the level of cholesterol.

4. Discussion

In the present study, we demonstrate that localization of KATP channels to caveolin-enriched microdomains in the plasma membrane of cardiac myocytes is essential for adenosine receptor-mediated activation of KATP channels. Specifically, we show that KATP channel pore-forming subunit Kir6.2 is localized in caveolin-enriched membrane fractions, and is associated and colocalized with caveolin-3. Patch-clamp studies reveal that this microdomain association is necessary for KATP channel regulation by activation of adenosine receptors.

KATP channels are regulated by a number of endogenous activators, most of which are released or increased during ischaemia such as noradrenaline,25,26 adenosine,7,27 protein kinase C,8,28 nitric oxide,9 and membrane lipids especially phosphatidylidylinositol-4,5-bisphosphate.10 All of these regulators activate KATP channels by decreasing ATP-sensitivity and thus increasing open probability. It has been proposed that activation of KATP channels by these endogenous regulators may be one of the reasons why KATP channels can be activated at millimolar range of intracellular ATP.29 It is likely that those molecules decrease the ATP-sensitivity of KATP channels within caveolae and thus activates the channels even though intracellular ATP level is relatively high. A recent study shows that KATP channel subunit Kir6.2 is mandatory for optimal adaptation capacity under stress, further indicating that cardiac KATP channels may not be as silent as originally thought and the mechanism may involve regulation of the channel micro-environment.30 The present study revealed a novel mechanism by which KATP channels in the plasma membrane of cardiac myocytes are regulated by caveolin-rich microdomains. This finding indicates that the regulation of KATP channels is more complex than that can be explained by the simple hypothesis of opening by bulk decrease in intracellular ATP.29

In the heart, several ion channels and exchangers are localized in caveolae and have shown functional modulation by caveolae-associated mechanism including the voltage-dependent Na+ channel, voltage-dependent Ca2+ channel, a voltage-dependent K+ channel (Kv1.5), the Na+/Ca2+ exchangers and Na+/K+-ATPase.23,31–35 In vascular smooth muscles KATP channels (Kir6.1 and SUR2B) are also present in caveolae.18,36 It is not known whether cardiac KATP channels, which are composed of Kir6.2 and SUR2A, are localized in caveolin-rich microdomains. While the adenosine A1 receptor is well known to couple to cardiac KATP channels via inhibitory G proteins, and inhibitory G proteins often couple to their effectors by Protein kinase C,27,37,38 it is also not clear whether cholesterol-rich microdomains on the plasma membrane of cardiac myocytes impacts the regulation of KATP channels by adenosine A1-receptor signalling. The present studies provide the first evidence that intact caveolin-rich microdomains are essential for adenosine A1-receptor-mediated KATP channel signalling. This is consistent with the previous finding that adenosine A1-receptors and their downstream signalling molecules including Gi protein and protein kinase C are localized in caveolae.23,24,39,40

In the present study, disruption of caveolae may affect other modulators of KATP channels within caveolae. If adenosine-induced activation of KATP channels is obscured by disrupting other caveolar-associated signalling molecules that are active under basal conditions, pinacidil-evoked KATP currents should also be reduced. Our data showed that disrupting caveolae by either MiCD or caveolin-3 siRNA did not alter the amplitude of pinacidil-evoked KATP currents, indicating that basal KATP activity may not be significantly affected by the net effect of disrupting caveolae.

It is known that caveolin-3 is essential for the formation of caveolae in cardiac myocytes, whereas caveolin-1 is expressed and important in cardiac myocytes. We noticed that we detected significant amount of caveolin-1 in addition to caveolin-3 in our cardiomyocyte preparations. This could raise a question whether caveolin-1 is involved in adenosine-mediated regulation of KATP channels. Our data from neonatal rat cardiomyocytes show that suppression of caveolin-3 by specific siRNA did not alter the expression of caveolin-1 but significantly affected the regulation of KATP channels by adenosine receptors. This observation indicates that caveolin-3 not caveolin-1 play an important role in KATP channel regulation by adenosine receptors in cardiomyocytes.

We understand that there are significant developmental changes in ion-channel expression and/or distribution. The subsarcolemmal localization of KATP channels in adult rat heart may not be the same as that in neonatal rat heart. However, our immunoprecipitation experiments from caveolin-3-enriched fractions of neonatal rat heart demonstrate that Kir6.2 indeed associates with caveolin-3, which is consistent with the observation that KATP channels are functionally regulated by intact caveolae or caveolin-3.

We have previously shown that acute stimulation (<5 min) of adenosine receptors activates KATP channels27,37 and prolonged stimulation (15–30 min) causes internalization of KATP channels in cardiac myocytes.13 In the present study, the KATP currents started to increase significantly around 5 min after superfusion with adenosine, and reached the steady state within 5–10 min. The time course for adenosine to induce a detectable increase in KATP currents and then reach a steady state depends on the rate of superfusion, the level of intracellular ATP, metabolic state of the cell, and the concentration of pinacidil. Although adenosine A1 receptor has been shown to translocate out of caveolae after agonist stimulation, this translocation occurs after prolonged stimulation which lasts for 15 min.24 It is thus likely that the acute effect of adenosine A1 receptors on KATP channels in cardiac myocytes is caveolae-dependent.

In recent years, it has become apparent that the regulation of ion-channel function is not the only means of controlling cell excitability. The trafficking and localization of ion channels with signalling molecules also play an important role. The precise subcellular localization of ion channels is often necessary to ensure efficient integration of both intracellular and extracellular events. The present study has identified a novel mechanism for regulation of cardiac
K\textsubscript{ATP} channel. Giving that caveolae are emerging as important surface-associated organelles, our elucidation of caveolar microdomain-dependent regulation of K\textsubscript{ATP} channels in cardiac myocytes will significantly enhance our knowledge of the complexity of regulation of this macromolecular channel and provide mechanistic insight into how K\textsubscript{ATP} channels control cellular functions.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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