ADENOSINE RECEPTOR MEDIATED PROTEIN KINASE C ACTIVATION

IN THE HEART

DISSERTATION

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ABSTRACT

Protein Kinase C (PKC) is a group of enzymes that control the function of other proteins through the phosphorylation of serine/threonine on these proteins. Numerous studies have shown that PKC isoforms differ in their primary structure, subcellular localization, tissue distribution and biological function. It is localized in cytosol in an inactive conformation and is translocated to different subcelluar organelles including plasma membrane, Golgi complex and endoplasmic reticulum (ER) upon activation. In ischemic preconditioning, it is the key mediator since different signaling pathways converge at PKC and it mediates distinct cellular functions by phosphorylating specific downstream target proteins. However, little is known about adenosine- mediated PKC activation in the heart though adenosine is one of the three triggers in ischemic preconditioning. Therefore, understanding the PKC translocation by adenosine receptor can provide useful insights into the protective mechanism of PKC in ischemic preconditioned hearts. Accordingly, the present investigation was performed to determine adenosine receptor-mediated PKC translocation to different subcellular locations in the heart.

We demonstrated that activation of adenosine A1 receptors induced a rapid
association of PKCɛ or PKCδ with caveolin-3 in adult rat cardiac myocytes. Specifically, we found that activation of adenosine A1 receptors with CCPA induced the selective translocation of PKCɛ and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. Notably, we showed that activated PKCɛ and PKCδ were targeted to the caveolin-rich plasma membrane microdomains. We have also shown that PKCɛ and PKCδ colocalize to and associate with caveolin-3. Taken together, we demonstrated that activation of adenosine A1 receptors promotes targeting of novel PKC isoforms, PKCɛ and PKCδ to caveolin-rich plasma membrane microdomains (Chapter 2).

Identification of downstream effectors on the plasma membrane will lead to a better understanding of cardiac protection in the adenosine receptor-mediated PKC signaling in the heart. Using the patch clamp technique, we provide the first evidence that Kir2.1 channels are negatively regulated by caveolin-3, both in HEK293T cells and in rat cardiac myocytes. Specifically, we show that Kir2.1 channel activity is significantly inhibited by expression of caveolin-3 while its expression on the cell plasma membrane is not changed. Further, we demonstrate that caveolin-3 scaffolding domain peptide blocks the caveolin-3 mediated suppression of Kir2.1 current. Moreover, our data indicate that caveolin-3 is critical in PKC induced IK1 inhibition in the heart (Chapter 3).

There is ample evidence that PKCɛ resides in cardiac mitochondria. However, the signals that promote translocation of PKCɛ are largely unknown. We found
that adenosine receptor activation induced a rapid association of PKCε with TOM70, which is dependent on HSP90. Specifically, we showed that activation of adenosine receptors induced a selective translocation of PKCε (but not PKCδ) from the cytosol to mitochondria. We also showed that adenosine-mediated PKCε translocation to mitochondria was significantly reduced by inhibiting HSP90 function or suppressing HSP90 expression. Further, our data indicate that HSP90 is critical for association of PKCε and TOM70. We demonstrate for the first time that adenosine receptor activation induces selective translocation of PKCε to mitochondria. This translocation process is associated with the mitochondrial import machinery TOM70 and is dependent on HSP90 function (Chapter 4).

Collectively, our data demonstrate the molecular mechanism underlying adenosine-mediated targeting of PKC isoforms to different subcellular locations, which consequently regulates the downstream of signaling such as Kir2.1 channel on the plasma membrane. Thus, the work presented in this thesis is not only important for a better understanding of ischemic preconditioning, but also for designing a strategy for clinical application of preconditioning or postconditioning biology.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

AC: Adenylate cyclase
AF: Atrial fibrillation
ATP: Adenosine-5'-triphosphate
ATS: Andersen-Tawil syndrome
BK: Big potassium
BSA: Bovine Serum Albumin
CCPA: 2-chloro-N6-cyclopentyl-adenosine
CSD: Caveolin scaffolding domain
DAG: Diacylglycerol
DAPC: Dystrophin-associated protein complex
DPCPX: 8-Cyclopentyl-1, 3-dipropylxanthine
ECG: Electrocardiography
eNOS: Endothelial NO synthase
GABA: Gamma-aminobutyric acid
GLU-4: Glucose transporter-4
GPCRs: G-protein coupled receptors
HSP: Heat shock protein
IP3: Inositol triphosphate
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<td>IPC</td>
<td>Ischemic preconditioning</td>
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<td>Kir</td>
<td>Inwardly rectifying potassium channel</td>
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<td>M-β-CD</td>
<td>Methyl-beta-cyclodextrin</td>
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<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>PDK</td>
<td>Phospholipid-dependent kinases</td>
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<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIP2</td>
<td>Phosphatidylinositol 4, 5-bisphosphate</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKC</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
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<tr>
<td>ROCs</td>
<td>Receptor-Operated Channels</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDP</td>
<td>Scaffolding Domain Peptide</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SK</td>
<td>Small conductance calcium-activated potassium</td>
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<td>SMOCs</td>
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<td>TOM</td>
<td>Translocase of outer mitochondrial membrane</td>
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CHAPTER 1
INTRODUCTION

1. PROTEIN KINASE C

1.1 Biochemical features of PKC

Protein Kinase C (PKC) is a group of enzymes that control the function of other proteins through the phosphorylation of hydroxyl groups of serine/threonine residues on these proteins. It was first discovered in 1977 by Yasutomi Nishizuka’s group in rat brain and in bovine cerebellum (M. Inoue 1977; Y. Takai 1977). They purified and then characterized this cyclic nucleotide-independent protein kinase and finally termed it as protein kinase C. In general, the PKC isoforms contain a regulatory domain which is near the N-terminal and a catalytic domain close to the C-terminal. These two domains are connected by a flexible hinge region. The N-terminal regulatory domain contains a pseudo substrate region as well as a second messengers (Ca2+ and Diacylglycerol) binding site. The C-terminal catalytic domain is the kinase core which contains the substrate binding domain (C4) and the ATP binding domain (C3).
Till now, at least 12 PKC isoforms have been characterized and according to their primary structures and distinct activators. They are divided into three groups: classical PKC, novel PKC and atypical PKC. Classical PKC, also named as conventional PKC, includes PKC\(\alpha\), \(\beta1\), \(\beta2\) and \(\gamma\). This group of PKCs is activated in the presence of both DAG and \(\text{Ca}^{2+}\) since their cysteine-enriched C1 domain has the DAG sensor and C2 domain acts as the \(\text{Ca}^{2+}\) binding domain. Similar to classical PKCs, novel PKCs also contain C1 and C2 domain in their regulatory domain though the primary structures are inverted(D.R. Dries 2006). However, different from classical PKCs, their C2
domain does not contain Ca\(^{2+}\) sensor in novel PKCs. They are activated by DAG in absence of Ca\(^{2+}\). This family includes PKCδ, ε, η and θ. Atypical PKCs family do not have C1 or C2 domain and are only stimulated by other lipid-derived second messengers.

Over the past few decades, numerous studies have shown that PKC isoforms differ in their primary structure, subcellular localization, tissue distribution and biological function (D.R. Dries 2006; X.Q. Wu 2006; R.A. Fenton 2009). It is known that PKC is localized in cytosol in an inactive conformation with its substrate binding domain occupied with its pseudo-substrate sequence. The exposure of PKC to Ca and DAG (or other signaling molecules) changes PKC into active conformation and recruits PKC to their subcellular membrane location by binding to it RACK protein (Receptors for Activated C Kinase). The binding of PKC to RACK on membrane releases the pseudo-substrate from its binding domain, allowing the binding and then phosphorylation of the substrate on membrane (Y. Haberman 2011). To date, different subcellular relocalization of PKCs has been studied though the downstream of PKC activation remains to be understood.

1.2 PKC in the heart

PKC isoforms have been found in almost all types of cells and tissues. In adult rat cardiac myocytes, multiple PKC isoforms(PKCα, β, and ζ) have been found while the major PKC isoforms detected are PKCδ and ε(K. Naruse
2000). In human heart, Western blot analyses showed that the PKC isoforms found in rat heart are also expressed (G. Simonis 2007). Besides, more PKC isoforms are characterized including PKCλ, θ, and μ (G. Simonis 2007). The PKC distribution between ventricles and atria is isoform-dependent. The major isoforms in ventricle are PKCα and β while PKCζ and δ are the main isoforms in atria. PKCε, a novel PKC, is evenly distributed in these two parts. Also, during postnatal developmental stages, different PKC isoforms expression changes and the total PKC expression level is decreased in human and rat hearts. In both neonatal and adult rat cardiomyocytes, the PKCδ, PKCε, and PKCα isoforms are found (M. Puceat 1994; M.H. Disatnik 1994). The presence of PKCβ1 and β2 in the heart is controversial. These two isoforms were detected in both neonatal and adult rat ventricular myocytes by immunoblotting and immunofluorescence while some researchers have not detected it in rat heart.

It is well accepted that PKCs reside in cytosol at an inactive conformation under basal condition. Upon stimulation, PKCs translocate from cytosol to different membrane structures of the cell. However, studies show that each PKC isoform translocates to different subcellular location upon activation. For example, in adult rat cardiomyocytes, PKCε is present in cytosol and perinucleus (M.H. Disatnik 1994). On activation, it redistribute to plasma membrane, cross-striated structure and mitochondria. The translocation of PKCδ from nucleus to the perinuclear and myofibrils were found by Mochly (D.
Mochly-Rosen 1998). Taken together, all these results suggest that different PKC isoforms may mediate unique downstream pathways by redistribution to various subcellular locations.

Among different PKC target protein in cardiac myocytes, the ion channels have been extensively studied. In rat ventricular myocytes, the activation of PKC by PMA inhibits the voltage-activated potassium channels (M. Apkon 1988). The PKC-dependent modulation of calcium channels were also observed in the heart by PMA activation although the results are controversial. In neonatal rat ventricular myocytes, Lacerda et al observed that the activation of PKC by PMA causes the strong inhibition of transient Ca-current (A. E. Lacerda 1988). Bourinet et al revealed that cardiac L-type Ca-current was increased by PKC (E. Bourinet 1992). Similarly, the regulation of Na channel and Mg channels by PKC is observed in different animal hearts (C.R. Valdivia 2009; M. Guillouet 2011). However, because of the limited availability of specific PKC isoform antagonist, the detailed mechanism underlying channel regulation by PKC is still not clear. More experiments using more specific tools and inhibitors need to be performed to fully understand the role of different PKC isoforms in cardiac electrophysiology.

1.3 PKC in adenosine signaling

The mechanisms underlying adenosine mediated protection in ischemic preconditioning are not fully clear but seem to involve PKC (K. Ytrehus 1994;
P. Ping 1997). It has come to be recognized as the major mediator of ischemic preconditioning. Signals from different receptors (adenosine, opioids and bradykinin) converge at PKC, reaching a threshold activation of kinase necessary to induce protection. In ischemic preconditioning, PKC is activated by translocation from the cytosol to the sarcolemma, providing protection by phosphorylation of end-effectors (P. Ping 2002). It was found that PKC activators mimic the protection effect of ischemic preconditioning and PKC inhibitors abolish the protection by pretreatment with adenosine or by ischemic preconditioning.

Although a large majority of studies support a critical role for PKC in cardioprotection, the way this protection is achieved remains unclear. Studies have shown that PKC activation may regulate several of the key players in ischemic preconditioning, including ROS, GSK-3β, PDH, and PDK. Activation of these signaling pathways leads to the opening of both sarcKATP and mitoKATP channels, the MPTP and Connexin 43. Recent study(A. Kuno 2007) showed that adenosine A2B receptor might be the target downstream from PKC. Activation of PKC with phorbol ester at reperfusion mimics the protection of ischemic preconditioning and this effect could be blocked by an adenosine A2B selective receptor blocker. Conversely, The PKC inhibitors cannot block the protection of an adenosine A2B selective agonist at reperfusion.
Moreover, recently studies support the phospholipase C (PLC) - protein kinase C (PKC) system in adenosine-signaling in the heart. Liang and his colleagues showed that activation of both adenosine A1 and A3 receptors induces the accumulation of diacylglycerol (DAG) in the avian heart (B.T. Liang 1995). The PLC inhibitor, U-73122, abolishes the adenosine-A1 receptor mediated cardioprotection by inhibiting the DAG production. In aortic vascular smooth muscle, the PLC-PKC is also involved in adenosine-A1 receptor mediated vasoconstriction. The adenosine A1 selective agonist, 2-
chloro-N6-cyclopentyl-adenosine (CCPA) induced the contraction of aortic rings while this effect was blocked by PLC inhibitor. Moreover, Marala and others discovered the upregulation of PKC expression level by adenosine A1 receptor activation (R.B. Marala 1995; Wang 2007; H.R. Ansari 2009). All these observations therefore indicate that PKC signaling has a critical role in adenosine receptor activation pathway.

Mitochondria are the “cellular power plants” that produce the most important intracellular energy source-ATP via electron transport and oxidative phosphorylation in the cell. In the heart, it makes up 50% of the cell volume and generates more than 90% of the ATP. Over the last 20 years, numerous studies have shown that PKC activation in mitochondria plays the key role in adenosine-mediated ischemic preconditioning, and mitochondrial dysfunction is the major cause of cell death in ischemia. During ischemic preconditioning, the integrity of mitochondrial membrane and oxidative phosphorylation is preserved while mitochondrial swelling is prevented. Consequently, the ATP production by mitochondria is increased compared to ischemia, and the ATP consumption is reduced. So the protection of mitochondria in ischemic preconditioning prevents the cells from further damage. Studies have already shown that PKC activation is the key step in this mitochondrial protection. Increased PKC activity was found in preconditioned mitochondria, while inhibition of PKC blocks the preconditioning effect in mitochondria. The activated PKC in mitochondria inhibits the formation of mitochondrial
permeability transition pore (MPTP) and regulates the downstream of the signaling such as mito-KATP activation. In spite of its essential role in mediating the protection in adenosine-mediated ischemic preconditioning, the detailed molecular mechanisms underlying PKC protection in mitochondria remains to be fully elucidated.

2. CAVEOLAE

2.1 Biochemical features of caveolae
The cell surface organization of receptors and their signaling partners has been a topic of long-standing interest. Recent data have emphasized the importance of colocalization of receptors with their signaling partners in discrete microdomains so as to facilitate the activation of cellular events. The existence of such domain was initially inferred from the compartmental organization of various cell types. One such subcellular compartment, found in numerous cell types, was termed caveolae (U. Smith 1972).

Caveolae are small (50 to 100 nm in diameter), flask-like invaginations rich in cholesterol and glycosphingolipid on the plasma membrane. These specialized lipid microdomains contain a variety of signaling and transport protein, thereby providing spatial and temporal organization for cellular signal transduction (V. Garg 2009). Caveolins, structural proteins of caveolae, serves as the scaffold and regulator of signaling proteins (K.G. Rothberg
1992). It has been estimated that 144 caveolin molecules are present in each caveolar invagination in the form of homo and hetero-oligomers. They comprise a family of three distinct 21-24 kDa isoforms based on their patterns of expression in different cell types. Caveolin-1(α and β) and -2 are almost ubiquitously expressed whereas caveolin-3 is found in striated (cardiac and skeletal) muscle and certain smooth muscle cells (Anderson 1998).

**Figure 1.3.** Schematic representation of the protein organization of a caveola. (R.S. Ostrom 2004)
Caveolin possesses a domain called the caveolin scaffolding domain (CSD) to which a variety of cellular signaling molecules bind in an inactive state (S.F. Steinberg 2001; T.M. Williams 2004; O. Feron 2006). One mechanism proposed that activation of caveolin leads to conformational changes that release and activate the signaling protein. These signaling molecules include G-protein coupled receptors (GPCRs, e.g., adenosine, opioid, adrenergic), ion channels (e.g., K\textsubscript{ATP}, Voltage-gated K\textsuperscript{+}, and L-type Ca\textsuperscript{2+}), Kinases (e.g., PKA, PKC, Src-family) and other postreceptor components (e.g., Ras, AC, eNOS). Recent studies show that caveolin-1 and caveolin-3 are essential proteins in the signaling cascade involved in cardiac protection (H.H. Patel 2006; Y.M. Tsutsumi 2008). Moreover, the recruitment of adenosine A\textsubscript{2B} receptor to plasma membrane caveolae fraction was observed upon adenosine A\textsubscript{2B} agonist stimulation in T84 cell line (S.V. Sitaraman 2002). Since caveolin scaffolding domain facilitates the interaction and organization of signaling molecules so as to help provide coordinated and efficient signal transduction, it might be possible that caveolin scaffolding domain is the binding site for other protein to form such molecular complex.

2.2 Caveolin/caveolae in the heart

As stated before, caveolae have been reported to regulate many cell functions including signaling and channel activation. In the heart, caveolin regulates the endothelial nitric oxide synthase (eNOS) activity and determines the eNOS signalsome localization. Previous studies have already
demonstrated that caveolin is the negative regulator of eNOS and the caveolae-localization of eNOS is critical in eNOS signaling. This caveolin-mediated eNOS inhibition in the heart is modulated by β-adrenoceptor. Application of caveolin-3 antibody which disrupts the caveolae structure increases the eNOS activity, implicating the role of caveolae in eNOS signaling. Studies further verified that this caveolin-eNOS inhibitory interaction is through the caveolin scaffolding domain since the application of putative CSD also inhibits eNOS activity and NO release in the cells. In transgenic caveolin-1 knockout mice, the caveolae inhibitory effect on eNOS activity was abolished. Therefore, all these observation in cells and caveolin knockout mice underline the importance of caveolin/caveolae in eNOS localization and activity regulation.

Recently, more and more studies showed the essential role of caveolin/caveolae in ischemic preconditioning. Koneru et al found that the ischemic preconditioning effect is mediated by activation of caveolins and their association with eNOS(S. Koneru 2007). In this process, the glucose-4 transporter (GLU-4) is also involved. Das and his colleagues revealed that the cardioprotection in ischemic preconditioning is associated with the generation of the survival signal by interaction of MAP kinase and caveolin in the heart(M. Das 2007). The overexpression of caveolin-3 can induce the endogenous cardioprotection which mimics the ischemic preconditioning effect while knockdown of caveolin expression in transgenic mice completely abolish the
ischemic preconditioning (H.H. Patel 2007; Y.M. Tsutsumi 2008; J.F. Jasmin 2011). The detailed mechanism underlying the caveolin regulation of ischemic preconditioning is still under investigation; however, phosphorylation is reported to be involved in caveolin signaling. Cardiac protection induced by ischemic preconditioning increases the phosphorylation and relocalization of caveolin-1 and Src to the caveolae fractions in rat heart (B.P. Head 2008). PP2, the Src kinase inhibitor, blocks the caveolin-1 phosphorylation and preconditioning induced cardiac protection. All these observations make it very likely that ischemic preconditioning is regulated by caveolae microdomain and this cardiac protection is mediated by phosphorylation of the caveolin protein.

2.3 Caveolae and adenosine receptors

Over the past decades, numerous studies have shown that adenosine receptor mediated signaling is regulated by specific microdomain- caveolae (M. Escriche 2003; R.S. Lam 2009; Z. Yang 2009). It is known that caveolin usually interacts with other signaling molecules through the caveolin scaffolding domain (CSD) which is located near the N terminus of the caveolin sequence. The proteins containing specific amino acid sequences can bind to this domain. These sequences include ΦXXXXΦXXΦ, ΦXΦXXXXΦ and ΦXΦXXXXΦXXΦ where Φ is an aromatic amino (W, F, Y) acid and X represents any amino acid (J. Couet 1997). In all 4 adenosine receptor subtypes, at least one such sequence is found and conserved
across different species, indicating the possible localization of adenosine receptors in caveolae.

In 2000, Lasley first reported that adenosine A1 receptor subtype is localized in caveolae fraction in adult rat cardiac myocytes (R.D. Lasley 2000; R.D. Lasley 2001). Activation of adenosine A1 receptor by A1 specific agonist CCPA caused redistribution of adenosine A1 receptor from caveolae to non-caveolae fraction while this translocation was blocked by A1 specific inhibitor 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX). Our group also showed that adenosine A1 receptor co-immunoprecipitated with caveolin-3 in adult rat cardiac myocytes. Murthy et al found that the activation of adenosine A1 receptor causes the redistribution of Gi to caveolae fraction (K.S. Murthy 2000). Over-expression of adenosine A1 receptors in mice results in cardiac hypertrophy, and this pathology is associated with decreased expression and altered distribution of caveolin-3 (S. Perlini 2007). In addition, Escriche et al showed that caveolae regulate the adenosine A1 receptor internalization and recycling in DDT1MF2 cells (M. Escriche 2003). All these results indicate that caveolae is critical in adenosine A1 receptor localization on cell plasma membrane.

In contrast to the significant evidence that caveolae plays an important role in adenosine A1 receptor localization and signaling, the function of caveolae on the other 3 adenosine receptor subtypes is still under investigation. Few
studies have directly shown the possible localization of these 3 subtypes in caveolae (S. Genedani 2010). However, numerous studies have revealed that caveolae might be involved in these receptor regulations. The major component of the caveolae, cholesterol, was intensively studied. Lam et al showed that depletion of cholesterol level by M-β-CD affects the adenosine A2a receptor activation in mouse (R.S. Lam 2009). In rat embryonic cortical neurons, the application of M-β-CD blocks both the adenosine A2a agonist and antagonist effect in cell signaling. Further, study showed that cholesterol can stabilize the human adenosine A2a receptor configuration. Also, other studies revealed that caveolae or lipid rafts may participate in adenosine A2b and A3 receptor regulation (S.V. Sitaraman 2002; Y. Cordeaux 2008). Taken together, all these observations indicate caveolae plays important role in adenosine receptor localization and regulation.

3. ION CHANNELS

3.1 Potassium channels

All living cells are enveloped by a bilayer of phospholipids termed as plasma membrane which works as a barrier between the cytoplasm and the external environment. The plasma membrane is a dynamic bilayer where other membrane constituents are embedded. Most of them are proteins including receptors, ion channels, cytoskeletal anchors and enzymes, and they are responsible for cell structure, cellular communication and homeostasis. Since
the plasma membrane is differentially permeable to charged molecules including ions, the media possess a concentration gradient of ions which is almost constant over a broad range of cells types and animal species. The distribution of the main ions found in animal cells in the intracellular and extracellular medium is listed in Table 1.1. Because of the ion-concentration differences across the plasma membrane, the charges on either side are different, and this difference causes an electrical potential which is termed as membrane potential. Among different proteins that regulate the relative permeability of the plasma membrane, ion channels are the most effective in controlling membrane permeability to small water-soluble molecules.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Extracellular conc. (mM)</th>
<th>Intracellular conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>130-160</td>
<td>5-20</td>
</tr>
<tr>
<td>K⁺</td>
<td>4-8</td>
<td>130-160</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.2-4</td>
<td>50-1000 (nM)</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1-5</td>
<td>10-20</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>100-140</td>
<td>1-60</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>20-30</td>
<td>1-3</td>
</tr>
</tbody>
</table>

Ion channels are a subset of intrinsic membrane proteins that control the movement of ions down their electrochemical gradient by forming an aqueous pore across the membrane. To this date, more than 300 types of ion channels
have been characterized in living cells. According to the mechanism that “gate” the channels, they are classified into three main groups: voltage-dependent channels, ligand-gated channels and second messenger operated channels. Voltage-dependent channels have the voltage sensors that open or close the channels depending on the membrane potential. Examples of such channels include voltage-gated sodium channels, voltage-gated calcium channels, voltage-gated potassium channels, hyperpolarization-activated cyclic nucleotide-gated channels, and voltage-gated proton channels. The ligand-gated ion channels, which are also known as ionotrophic receptors, are channels that open or close depending on the specific binding of a neurotransmitter or a hormone to the extracellular domain of the receptor protein. This group includes acetylcholine receptors, GABA receptors, ionotropic glutamate-gated receptors and ATP-gated P2X receptors. The second messenger operated channels respond to intracellular second messengers, such as calcium ions or activated G protein subunits. Examples of this type include inward-rectifier potassium channels, calcium-activated potassium channels, two-pore-domain potassium channels, light-gated channels and mechanosensitive ion channels. These three groups are sometimes referred to as VOCs (Voltage-Operated Channels), ROCs (Receptor-Operated Channels), and SMOCs (Second-Messenger-Operated Channels). Although they are classified into different types according to their activation mechanism, they are not mutually exclusive. Some channels may be activated by different manners under different conditions.
In addition to their gating mechanism, channels can also be influenced by some modulatory mechanisms. Phosphorylation and differential expression are the two most studied factors that can modulate the channels’ function (K.L. Sheldon 2011; Y. Aita 2011; M.Y. Song 2012). The channels can be phosphorylated by specific kinases and dephosphorylated by specific phosphatases, and depending on the channel’s type and its phosphorylation site (serine, threonine, and tyrosine residues), the activity of the channels can be either increased or reduced. Another factor that can affect the channel’s activity is their expression level. The varying level of channel expression on plasma membrane is regulated by its synthesis and its degradation.

Potassium channels are the most widely distributed ion channel which is found in virtually all types of cells in all organisms. They are the key regulators of the membrane resting potential, action potential repolarization, and diastolic conductance (W.R. Giles 1985; Z. Zhu 2011; C.G. Ponte 2012). Potassium channels have a tetrameric structure in which four subunits associate to form trans-membrane aqueous pores through which potassium ions specifically permeate. More than 80 mammalian genes have been characterized that can encode potassium channel subunits. However, all the potassium channel subunits have a distinctive pore-loop structure that is responsible for the potassium selectivity. Near the center of the trans-membrane span, there is a 10 Å wide water-filled pore which reduces the
energetic barrier for the potassium. Though found in different cells and different organisms, all the potassium channels share a single basic function: formation of a potassium ion specific trans-membrane permeation. According to their locations, they are involved in a variety of cellular processes, such as shaping the action potentials, setting the resting membrane potentials, and regulating the secretion of hormones. Malfunction of the potassium channels can cause atrial fibrillation and arrhythmias since they regulate the action potential duration in the heart (C.M. Jackson 2007; S. Makary 2011).

According to their functions and gating mechanisms, potassium channels are divided into four major types: Calcium-activated potassium channels, voltage-gated potassium channels, tandem pore domain potassium channels and inwardly rectifying potassium channels. The calcium-activated potassium channels are activated in response to the elevation of calcium concentration in the cell. Examples of this type include big potassium (BK) channel and small conductance calcium-activated potassium (SK) channel. Voltage-gated potassium channels belong to the voltage-gated ion channel family which is controlled by the trans-membrane voltage. They include Kv11.1 (hERG) and Kv7.1 (KvLQT1) potassium channels and are involved in repolarization of action potential and regulation of action potential frequency. The tandem pore domain potassium channel is a family of 15 members which possess Goldman-Hodgkin-Katz (open) rectification. Their activities are regulated by oxygen tension, G-proteins and pH, and their function is to maintain the
resting potential. Inwardly rectifying potassium channel (Kir, IRK) is a family of seven members named as Kir1 – Kir7. These channels differ from the “typical potassium channels” which are responsible for passing outward rather than inward potassium currents at repolarizing stage of an action potential. An inward rectifying potassium channel moves potassium ion into the cell more easily than outward direction. The inward rectification by these channels is regulated by the endogenous polyamines and magnesium in the cell (Y. Fujiwara 2002; Y.V. Kucheryavykh 2007). At positive potential, these polyamines, together with magnesium, insert into the channel pore and block the outward potassium currents. This blockage is both voltage dependent and direction dependent. To date, Kir channels have been characterized in various cell types and they play different roles in cell biology. For example, Kir channel in cardiac myocytes maintains a more prolonged cardiac action potential (R.B. Sekar 2007). In endothelial cells, Kir controls the nitric oxide (NO) level by regulating the activity of nitric oxide synthase. Insulin release is mediated by these channels in pancreatic beta cells (J. Ferrer 1995). Mutation or dysfunction of these channels can cause severe diseases such as Andersen’s syndrome, atherosclerosis, and thyrotoxic hypokalaemic periodic paralysis (A. Puwanant 2010; B.C. Lim 2010; C.J. Cheng 2011).
3.2 *Kir2.x in the heart*

The mammalian Kir2.x family is composed of five subtypes: Kir2.1, 2.2, 2.3, 2.4 and 2.6. Except for Kir2.6, all the other four subtypes are found in the mammalian heart, although the existence of Kir2.4 in the heart is still under debate (E. Zitron 2008; A. Liu 2010). The Kir channel family is encoded by KCNJ genes, and the amino acid sequence of Kir2.1 share more than 60% identity with other Kir2.x subtypes. Initially, it was thought that Kir2.x subunits can only form homotetramers. However, recent studies have shown that in some cases heterotetramers are formed both in vitro and in vivo (R. Preisig-Muller 2002; V. Munoz 2007; B.K. Panama 2010). For example, both heteromeric complex of Kir2.1/2.2 and Kir2.1/2.3 channels have been characterized in mouse cardiomyocytes (R. Preisig-Muller 2002). Depending on the species, the expression level of different Kir2.x subunits changes. While Dhamoon and his colleagues (A.S. Dhamoon 2004) found that Kir2.3 isoform is the major contributor of IK1 in mouse hearts, others found Kir2.2 subtype plays important role in guinea pig ventricle. In spite of this, it is well accepted that Kir2.1 is the major subtype contributing to IK1 in the heart in different species (G.R. Li 2009; R. Gomez 2009; A. Liu 2010).

During rat embryonic development, Kir2.1 subunit expression is first found on day 12 (T.Y. Nakamura 1999; M.D. Harrell 2007). Although its expression may be down-regulated or even disappeared in some organs such as the thalamus and hippocampus, the Kir2.1 expression level in the heart is
relatively high during embryogenesis and even increases till adulthood. The Kir2.2 expression is also found at day 12 during embryonic development, and its level is relatively low in the heart compared to Kir2.1. In the heart, only little Kir2.3 is found during embryonic development, and its expression level does not change after birth. The Kir2.4 expression is similar to Kir2.3, and its expression does not increase after birth.

During the period of cardiac myocyte development, the intensity of IK1 current is increased. Using Kir2.1 and Kir2.2 knockout animal models, it has been concluded that IK1 in the mouse cardiomyocytes is mainly carried by Kir2.1 (R. Gomez 2009). The Kir2.1 knockout mouse died within 12 h after birth because of the respiratory problems. No IK1 current was detected in myocytes isolated from neonatal mice with Kir2.1 knockout (J.J. Zaritsky 2001). Notably, Kir2.1 knockouts have the broader action potentials and slower heart rates compared with wild-type mice. However, the Kir2.2 knockout mice can survive to adulthood without any developmental abnormalities, although the IK1 current is decreased by 50% (J.J. Zaritsky 2001). Using the dominant negative form of Kir2.1 or Kir2.2 in ventricular myocytes from rabbit, the IK1 current was reduced by around 70% (C. Zobel 2003). All these results supported that Kir2.1 plays an essential role in the genesis of IK1 in the heart and Kir2.2 subunits are likely to assemble with Kir2.1 to form the IK1 current.
3.3 PKC/caveolin in Kir2.1 regulation

Although the regulation of the Kir2.1 channels by protein kinases has been intensively studied, the results obtained from different groups are inconsistent. In 1994, Fakler et al found that the Kir2.1 current was decreased by more than 70% by PKC stimulator 12-O-tetradecanoylphorbol-13-acetate (TPA) in Xenopus oocytes and this down-regulation of Kir2.1 current might be caused by PKC phosphorylation (B. Fakler 1994). The PKC-mediated Kir2.1 inhibition was blocked by general PKC inhibitor. In contrast, Scherer et al found that Kir2.1 currents are modulated by adrenoceptors via PKC-dependent pathways and application of PKC inhibitor chelerythrine suppresses this modulation (D. Scherer 2007). In Kir2.1/Kir2.2 and Kir2.1/Kir2.3 heteromeric channels, similar results were obtained (C. Kiesecker 2006), indicating the direct regulation of Kir2.1 subunit by PKC. In cerebral arterial smooth muscle cells, Wu et al found that hyposmotic challenge inhibits the Kir current (B.N. Wu 2007). The PKC agonist, PMA, mimics the hyposmotic-induced inhibition while the PKC antagonist blocks this inhibition. In rabbit coronary arterial smooth muscle cells, endothelin-1 inhibits the Kir current through PKC and PKC inhibitor blocks this inhibition while the PKC activator reduced Kir2.1 current (W.S. Park 2005).

Atrial fibrillation is the most common arrhythmia among patients with cardiac arrhythmia. Studies have shown that up-regulation of IK1 in atrium plays a very important role in atrial fibrillation while others found that the
overexpression of Kir2.1 channels in mouse can cause atrial fibrillation (S. Kharche 2008; Z. Girmatsion 2009). Also, the gain-of-function mutation of Kir2.1 channels was found in patients with atrial fibrillation, indicating the link between Kir2.1 dysfunction and atrial fibrillation (M. Xia 2005; S. Kharche 2008). Interestingly, recently studies showed that the significantly decreased caveolin-3 expression in atrial fibrillation in canine heart (Y.Y. Chen 2009). Although there is no direct evidence that Kir2.1 is regulated by caveolin-3 in atrial fibrillation in the heart, it is possible that these two proteins are both important in atrial fibrillation disease development. Given that Kir2.1 contains the caveolin-3 scaffolding domain motif, we can not rule out the possibility that caveolin-3 might regulate the Kir2.1 channel through CSD. In Andersen-Tawil syndrome (ATS), which is also named as long QT syndrome, more than 60% of the ATS patients are caused by mutation in the KCNJ gene which encodes the inward rectifying Kir2.1 channel (M.N. Obeyesekere 2011; A. Modoni 2012). Recently, more and more studies showed that mutation in caveolin-3 gene can induce and is associated with long-QT syndrome (M. Vatta 2006; A. Vega 2009). This further indicates the possible role of caveolin-3/caveolae in Kir2.1 current regulation.

4. OBJECTIVES

It is well-accepted that PKC is the key mediator in ischemic preconditioning, but little is known about the PKC activation in adenosine receptor signaling.
The objectives of the present investigations were two-fold. First, to examine the PKC isoform(s) translocated to caveolae microdomain by activation of adenosine receptor and how this process may regulate the IK1 current in cardiomyocytes. Second is to understand the PKC translocation to mitochondria stimulated by adenosine receptor.

As mentioned above, PKC plays an important role in ischemic preconditioning and it is translocated to different subcellular organelles upon activation. However, little is known about adenosine mediated PKC activation in the heart although adenosine is one of the three triggers in ischemic preconditioning. Therefore, understanding the PKC translocation by adenosine receptor can provide useful insights into the protective mechanism of PKC in ischemic preconditioned heart. Consequently, we identified the PKC isoform(s) translocated to caveolae on plasma membrane by adenosine A1 receptor activation in rat cardiac myocytes (Chapter 2). Besides, we also studied the possible role of caveolin-3 in IK1 current regulation by PKC (Chapter 3). Additionally, we explored the role of heat shock protein (HSP) and translocase of outer mitochondrial membrane (TOM) in PKC translocation to mitochondria.

Our investigation into the specific membrane microdomain will reveal the importance of certain structures in PKC signaling regulation. It will also provide a detailed understanding of the molecular mechanism underlying
adenosine-mediated mitochondrial targeting of PKC isoforms. The answers to these questions are not only important for a better understanding of ischemic preconditioning, but also for designing a strategy of clinical application of preconditioning or postconditioning biology.
CHAPTER 2

ADENOSINE A1 RECEPTORS SELECTIVELY TARGET PROTEIN KINASE C ISOFORMS TO THE CAVEOLIN-RICH PLASMA MEMBRANE IN CARDIAC MYOCYTES

Abstract

Adenosine is a naturally occurring nucleoside that has been shown to regulate a variety of functions in the cardiovascular system. However, the mechanisms in adenosine receptor signaling are not completely understood. Given that adenosine receptors have been linked to protein kinase C (PKC) in cardioprotection and caveolae is critical for receptor signaling, we sought to determine whether activation of adenosine A1 receptors induces selective translocation of PKC isoforms to the membrane from the cytosol and whether activated PKC is targeted to the caveolin-rich plasma membrane microdomains. The freshly isolated adult rat cardiac myocytes were used to examine PKC isoforms including PKCα, PKCβ, PKCε, PKCδ and PKCζ. Immunoblot analysis revealed that the immunoreactivity for PKCε or PKCδ but not for PKCα, PKCβ or PKCζ increased significantly in the membrane
fractions from cells pretreated with the selective adenosine A1 receptor agonist 2-chloro-N (6)-cyclopentyladenosine (CCPA, 100 nM) when compared with non-stimulated cells. The effect of CCPA on PKCε or PKCδ translocation was blocked by adenosine A1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX, 100 nM). When Western blot was performed from the caveolin-enriched plasma membrane fractions, the immunoreactivity for PKCε or PKCδ but not PKCα, PKCβ or PKCζ was enhanced significantly by CCPA. Furthermore, PKCε and PKCδ were detected in the anti-caveolin-3 immunoprecipitates but not in the samples without primary antibody. Immunofluorescence staining further indicates increased colocalization of PKCε or PKCδ with caveolin-3 at cell peripheral region and T-tubularlike structures in response to adenosine A1 receptor activation. In conclusion, we demonstrate that activation of adenosine A1 receptors promotes the selective translocation of PKCε and PKCδ to the caveolin-enriched plasma membrane microdomains in cardiac myocytes.
Adenosine is a natural nucleoside produced from the cardiovascular system and exerts a variety of functions including bradycardia, hypotension and coronary vasodilation (K. Mubagwa 2001). Adenosine recognizes specific cell surface receptors including four adenosine receptor subtypes (A1, A2A, A2B and A3). All these receptor subtypes are coupled to guanine nucleotide binding proteins (G proteins). The most characterized mechanism is the effect on adenylate cyclase. In addition, another important pathway involving protein kinase C (PKC) has been postulated to play a key role in adenosine receptor signaling (P. Henry 1996; Y. Liu 1996; K. Hu 1999).

PKC exists as a family of at least 12 isoforms. PKC isoforms mediate distinct cellular functions by phosphorylating specific downstream target proteins. Substrate specificity is most likely associated with subcellular localization of activated PKC isoforms. In the heart, a variety of signaling molecules have been localized in caveolae (R.G. Parton 2007; S. Calaghan 2008). Caveolae are small (50 to 100 nm) cholesterol and sphingolipid enriched “cave”-like invaginations of the surface membrane, very rich in many of the signaling molecules (V.O. Rybin 1999; C. Dessy 2000; R.D. Lasley 2001; P.A. Insel 2005; J.B. Morris 2006). These microdomains may act to generate subcellular signaling compartments by recruiting interacting signaling molecules. Indeed, cardiac myocyte caveolae are the focal points for activated PKC isoforms and
PKC can be activated by either exogenous PMA or endogenous receptor-mediated signaling. While PMA activates both classical and novel PKC isoforms, translocation of PKC by distinct receptor signaling is isoform selective. Adenosine A1 receptor is well known to couple to inhibitory G proteins, and inhibitory G protein often couple to their effectors by PKC (G.E. Kirsch 1990; E. Kim 1997; K. Hu 1999). It has been shown that PKCε translocation to cardiac sarcolemma or T-tubular structures is involved in adenosine receptor-mediated responses (J.W. Lester 2000; K. Miyazaki 2004; R.A. Fenton 2009). Recent evidence suggests that adenosine A1 receptor activation can selectively modify phosphatase and mitogen-activated protein kinases (MAPK) activities in caveolin-rich cardiac membrane fractions (C. Ballard-Croft 2008). However, it is not known whether adenosine receptor activation targets translocated PKC isoforms to the caveolin-rich plasma membrane microdomains. To determine whether activation of adenosine A1 receptors promotes translocation and caveolar targeting of specific PKC isoforms, we employed the freshly isolated adult cardiac myocytes and examined whether activation of adenosine A1 receptors by CCPA induces translocation of PKC isoforms to the cell membrane and whether translocated PKC isoforms by CCPA are targeted to caveolin-rich plasma membrane microdomains. We focused on examining six major isoforms of PKC that are known to be expressed in adult rat
cardiomyocytes (V.O. Rybin 1994; S. Kawamura 1998). Our results demonstrate that activation of adenosine A1 receptors increases immunoreactive membrane PKCε and PKCδ but not PKCα, PKCβ and PKCζ in the caveolin-rich microdomains.

**Materials and Methods**

**Materials**

Mouse IgG directed against caveolin-3 and rabbit IgG directed against PKCε, PKCβ1, PKCβ2 or PKCδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG directed against PKCα was purchased from Cell Signaling Technology (Danvers, MA). Rabbit IgG directed against PKCζ was purchased from Santa Cruz Biotechnology or Sigma (St. Louis, MO). The phorbol ester phorbol-12-myristate-13-acetate (PMA), its inactive congener 4α-PMA, adenosine A1 receptor agonist 2-chloro-N (6)-cyclopentyladenosine (CCPA) and adenosine A1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) were supplied from Sigma. PMA, 4α-PMA, CCPA and DPCPX were all used at a final concentration of 100 nM. DPCPX was applied 5 min before and during addition of CCPA to cell suspension. All drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.
Isolation of cardiomyocytes

Adult rat ventricular myocytes were isolated from adult Sprague–Dawley rats (250 to 300 g) by enzymatic dissociation (K. Hu 1996; K. Hu 2000). In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O2) Tyrode’s solution containing (in mM) NaCl 126, 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 is nominally Ca2+ free but otherwise has 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 cells were initially stored in a solution containing (in 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, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with or without PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min at 37 °C prior to homogenization for subsequent biochemical and immunofluorescence experiments.

Purification of caveolin-enriched membrane fractions

Caveolin-rich fractions from adult rat cardiomyocytes were prepared by using
a previously described detergent-free method with some modification (V. Garg 2009). Briefly, freshly isolated cardiomyocytes were pretreated without or with PMA or CCPA for 5 min at 37 °C. Cells were then spin down and resuspended in 0.5M sodium carbonate and homogenized. 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 rpm for 18–20 h in an SW41 rotor. Twelve 1-ml fractions were collected from the top to the bottom of the gradient for subsequent analysis by Western blot. Caveolin-rich fractions (fractions 4–6) which contain caveolin but exclude most other cellular proteins were centrifuged at 40,000×g for 2 h to pellet caveolae, which was then suspended in lysis buffer and sonicated. Samples were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane and analyzed by probing with various antibodies.

Membrane fractionation and western blotting

Freshly isolated cardiac myocytes were incubated in the presence and absence of PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min prior to
homogenization. Cell lysates were first centrifuged at 1000×g to get rid of unbroken cells and nucleus. Particulate and cytosolic fractions were prepared by centrifugation at 27,000×g for 1 h or 45,000×g for 30 min. Immunoblot analysis was carried out as described previously (V. Garg 2007; J. Jiao 2008). Briefly, the cytosolic and particulate fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and 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 2 h at room temperature or overnight at 4 °C 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.

**Co-immunoprecipitation**

Immunoprecipitation experiments were performed as reported previously (J.D. Jiao 2008; V. Garg 2009). Cells were pretreated with or without CCPA prior to homogenization. The cell lysate was incubated with or without antibody against caveolin-3 for 2 h at 4 °C. Antigen–antibody complexes were captured with r-protein-A agarose (4 °C, 30 min). Agarose beads were washed 4 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS–PAGE, transferred onto a
nitrocellulose membrane and analyzed by probing with antibodies against PKCε, PKCδ or caveolin-3.

Immunofluorescence confocal microscopy

As described previously (V. Garg 2009), after pretreated cells with or without CCPA, 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 labeled with primary antibody for 2 h. Cells were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 h. Immunofluorescence was visualized with confocal laser scanning microscopy. All images were analyzed using a background subtraction method offline.

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

Effect of PMA on translocation of PKC isoforms from the cytosol to the particulate fraction
To investigate whether activation of adenosine receptors induces translocation of PKC isoforms from the cytosol to the particulate (membrane) fraction, we first examined the subcellular localization of a representative isoform from each PKC subgroup, i.e. PKC-α (the classic PKC), -ɛ (the novel PKC) and -ζ (the atypical PKC) following PMA (100 nM) treatment. It is known that PMA activates the classical and novel but not the atypical PKC isoforms. As expected, Western blot analysis of cytosolic and membrane fractions separated by high speed centrifugation from rat cardiomyocytes detected all three PKC isoforms: PKCα, PKCɛ and PKCζ. Immunoblots with antibody against PKCα revealed a prominent protein band, which migrated with an apparent molecular mass of ~80 kDa. Antibody directed against PKCɛ detected a 90-kDa band while PKCζ was detected as a protein with a molecular mass of ~78 kDa and appears as doublets. Our results revealed that PMA pretreatment (37 ºC, 5 min) induced a significant redistribution of PKCα and PKCɛ from the cytosol to the membrane but not PMA-insensitive isoform PKCζ (Figure 2.1A). The membrane to cytosol ratios of both PKCα and ε increased approximately threefold in PMA-treated cells when compared with control group (Figure 2.1B). The inactive congener 4α-PMA did not cause translocation of these PKC isoforms. Thus, we are able to demonstrate that PMA induces translocation of PKCα and PKCɛ but not PKCζ under our experimental condition.
Figure 2.1. Subcellular redistribution of PKC isoforms in response to PMA.

(A) Representative Western blots of three to four independent experiments (three to four hearts). (B) Membrane to cytosol ratio as indexes of PKC isoform translocation. They were calculated by relative densitometry and normalized to 100% of control. Soluble and particulate fractions were prepared from cells incubated at 37°C for 5 minutes in the absence or presence of PMA (100 nM) or 4a-PMA (100 nM). Equal amounts of total proteins were loaded in each lane, separated by SDSPAGE and transferred onto nitrocellulose membranes. Membranes were probed with antibodies directed against PKCα, PKCε, or PKCζ and incubated with horseradish peroxidase (HRP)-linked secondary antibodies. Immunoblots were detected with enhanced chemiluminescence and multiple exposures to film. *p<0.05 vs. control.
Figure 2.1

A

PKCα
PKCe
PKCζ

Cytosol | Membrane

B

Membrane/Cytosol Ratio (% of control)

PKCα

**

PKCe

**

PKCζ
**Figure 2.2. Subcellular redistribution of PKC isoforms in response to CCPA.** (A) Purity of the membrane or cytosol fraction. (B) Representative Western blots of three independent experiments (three hearts). (C) Membrane to cytosol ratio calculated by relative densitometry and normalized to 100% of control as indexes of PKC isoform translocation. Cells were incubated without or with CCPA (100 nM) or CCPA plus DPCPX (100 nM) for 5 minutes at 37°C and then fractionated into soluble and particulate fractions. The adenosine A1 selective agonist CCPA induced significant translocation of PKCε and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. The adenosine A1 receptor antagonist DPCPX prevented CCPA-induced translocation of PKCε and PKCδ. Equal amounts of total proteins were loaded in each lane. *p<0.05 vs. control.
Figure 2.2

A

α-tublin
Na/K ATPase

B

PKCα
PKCβ1
PKCβ2
PKCδ
PKCε
PKCζ

Cytosol Membrane

C

PKCα
PKCβ1
PKCβ2
PKCδ
PKCε
PKCζ

Membrane/Cytosol Ratio (% of Control)

Control CCPA DPCPX
Control CCPA DPCPX
Control CCPA DPCPX
Control CCPA DPCPX
Control CCPA DPCPX
**Effect of adenosine A1 receptors on translocation of PKC isoforms from the cytosol to the particulate fraction**

We then determined the effect of adenosine A1 receptors on subcellular distribution of PKC isoforms. We examined six PKC isoforms that are present in the adult rat cardiomyocytes, including PKCα, PKCβ1, PKCβ2, PKCε, PKCδ and PKCζ. Cells were incubated with or without the selective A1 receptor agonist CCPA (100 nM) or CCPA plus the selective A1 receptor antagonist DPCPX (100 nM) for 5 min at 37 °C prior to homogenization and fractionation. The purity of the membrane or cytosolic fractions was examined with α-tubulin and Na/KATPase (Figure 2.2A). Western blot from cell homogenates revealed significant translocation of PKCε and PKCδ from the cytosol to the membrane in CCPA-treated group (Figure 2.2B). It was noted that the CCPA-induced translocation of PKCε or PKCδ was moderate when compared with PMA-treated group. Densitometric analysis indicated that the membrane to cytosol ratio was increased by 79% (179.16 ± 21.52%, n=3, p<0.05 vs. control) for PKCε and 81% (181.26 ± 14.82%, n=3, p<0.05 vs. control) for PKCδ in response to CCPA (Figure 2.2C). When data were normalized to control within the cytosol or membrane fraction, the CCPA treatment significantly increased membrane-associated immunoreactivity by 49% (149.43 ± 10.78%, n=3, p<0.05 vs. control) for PKCε and 66% (166.46 ± 23.18%, n=3, p<0.05 vs. control) for PKCδ. The signal from the cytosolic fraction was decreased by 16% for PKCε (84.12 ± 7.43%, n=3, p=NS vs. control) and 10% for PKCδ.
(90.10 ± 1.40%, n=3, p=NS vs. control). Thus, CCPA produce a decreased but not statistically different signal in the cytosolic PKCε and PKCδ. Treatment with CCPA plus adenosine A1 receptor antagonist DPCPX (100 nM) significantly prevented CCPA-induced translocation of PKCε and PKCδ. The membrane to cytosol ratio for PKCε was 116.80 ± 8.33% (n=3, p=NS vs. control) and for PKCδ was 129.48 ± 11.07% (n=3, p=NS vs. control) in the presence of CCPA and DPCPX. Thus, our data show that CCPA promoted the selective translocation of PKCε and PKCδ, the effect was blocked by DPCPX. Further, the subcellular distribution of PKCα, PKCβ1 or PKCβ2 was not altered significantly by CCPA treatment, suggesting that these PKC isoforms may not be as important as PKCε and PKCδ in adenosine receptor signaling in adult rat cardiomyocytes. As expected, our data also showed that CCPA had no apparent effect on the redistribution of diacylglycerol-insensitive PKCζ. These results suggest that activation of adenosine A1 receptors promotes selective translocation of PKCε and PKCδ but not PKCα, PKCβ or PKCζ to the membrane from the cytosol in the adult rat cardiomyocytes.

*Effect of adenosine A1 receptors on targeting PKC isoforms to the caveolin-rich plasma membrane*

Translocation to different cellular compartment enables PKC to colocalize with both activators and substrate. Since adenosine A1 receptors have been shown to reside in the caveolae of cardiac myocytes (R.D. Lasley 2001), we would
predict that their downstream signaling molecules such as PKC isoforms would specifically target these microdomains of plasma membrane. We have previously shown that using a detergent-free sucrose gradient extraction procedure, the muscle-specific caveolin-3 isoform is enriched in fractions 4–6 from adult rat cardiomyocytes (V. Garg 2009). Thus, in the present study caveolin-rich fractions 4–6 were collected to determine alterations in PKC isoform expression from cardiomyocytes with and without PMA or CCPA stimulation. The blots were probed with anticaveolin-3 antibody to confirm the caveolin-rich fractions. Freshly isolated adult cardiomyocytes were incubated with PMA (100 nM) or CCPA (100 nM) for 5 min at 37 °C prior to fractionation. As shown in Fig. 2.3A, the antibody against caveolin-3 detected abundant caveolin-3, a signature protein for the caveolae of cardiomyocytes, indicating that isolated fractions were caveolin-enriched fractions. Under control condition, only small amounts of immunoreactivity for all six PKC isoforms were detected in the caveolin-rich fractions. However, the band intensity for PKCα, PKCβ, PKCδ or PKCε increased significantly in response to the stimulation with PMA. Activation of adenosine A1 receptors with CCPA showed less but significant increase for PKCε and PKCδ. Densitometric analysis revealed that CCPA treatment enhanced the expression of PKCε in the caveolin-rich fraction by 105% (205.59 ± 51.79%, n=3, p<0.05 vs. control) and PKCδ by 53% (152.50 ± 16.86%, n=3, p<0.05 vs. control), while the levels of immunoreactivity for PKCα, PKCβ1 or PKCβ2 were not altered significantly (Figure 2.3B). As predicted, incubation with either PMA or CCPA did not lead
**Figure 2.3. PKC isoforms translocated to caveolin-rich microdomains in response to stimulation with CCPA.** (A) Representative Western blots of three independent experiments from caveolinenriched fractions. (B) Expression of PKC isoforms in caveolin-rich fractions calculated by relative densitometry and normalized to 100% of control. Cells were incubated in the absence or presence of PMA (100 nM) or CCPA (100 mM) at 37°C for 5 minutes before processed for purification of caveolin-rich fractions. The caveolin-rich fractions 4-6 by sucrose gradient centrifugation were centrifuged at 40,000g for 2 hr to pellet caveolae, which was then suspended in lysis buffer and sonicated. Equal amounts of caveolae proteins were loaded and resolved by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and analyzed by probing with various antibodies. Similar to PMA, CCPA caused significant increase in the expression of PKCε and PKCδ in the caveolin-rich fractions although the degree of targeting PKC isoforms to caveolae by CCPA was less than that by PMA. Caveolin-rich fractions were verified by the heavy bands detected by anti-caveolin-3 antibody. *p<0.05 vs. control.
Figure 2.4. Effect of CCPA on co-precipitation of caveolin-3 with PKCε or PKCδ. After pretreatment with or without CCPA (100 nM), cells were homogenized. Cell lysates containing equal amounts of total proteins were incubated with or without anti-caveolin-3 (Cav-3) antibody 2 hours at 4°C. Antigen-antibody complexes were captured with r-protein-A agarose for 30 min at 4°C. Agarose beads were washed 4-times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and analyzed by probing with antibody against PKCε, PKCδ or caveolin-3. A smaller amount of PKCε and PKCδ remained constitutively associated with caveolin-3 under the basal condition. CCPA increased the translocation and association of PKCε and PKCδ with caveolin-3.
to the recruitment of diacylglycerol/PMA-insensitive atypical PKCζ to the caveolin-rich domains. These results indicate that activation of adenosine A1 receptors promotes rapid association of PKCε and PKCδ with caveolin-rich plasma membrane microdomains.

*Effect of adenosine A1 receptors on co-immunoprecipitation of PKCε and PKCδ with caveolin-3*

PKC isoforms have been shown to localize to caveolae and interact with caveolin in a caveolin-subtype- and PKC isoform-dependent manner (N. Oka 1997; P. Liu 2002). To further determine whether activated PKCε or PKCδ associates with caveolin-3 in cardiac myocytes and whether this association can be up-regulated by activation of adenosine A1 receptors, we performed co-immunoprecipitation in the cell lysates from cardiac myocytes pretreated with or without CCPA (100 nM). Cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-caveolin-3 antibody. The immune complex was collected with protein A agarose beads. Samples were resolved, transferred and analyzed by immunoblotting with antibody against PKCε, PKCδ or caveolin-3. As shown in Fig. 4, caveolin-3 coprecipitated with PKCε and PKCδ under basal condition. Both proteins were not detected in the lysates without immunoprecipitation with anti-caveolin-3 antibody. Interestingly, pretreatment with CCPA significantly increased PKCε level by 67% (167.31 ± 8.65%, n=7, p<0.05 vs. control) and PKCδ by 53% (153.67 ± 7.34%, n=6,
p<0.05 vs. control) in the caveolin-3 immunoprecipitates. CCPA did not alter the recovery of caveolin-3. These data demonstrate that activation of adenosine A1 receptors with CCPA promotes the translocation and association of PKCɛ and PKCδ with caveolin-3-rich membrane fraction in adult rat cardiomyocytes.

Effect of adenosine A1 receptors on colocalization of PKCɛ and PKCδ with caveolin-3

To further confirm PKCɛ and PKCδ localize to caveolin-rich microdomains, we assessed whether both isoforms colocalize with the marker protein of cardiomyocyte caveolae, caveolin-3, after stimulation of adenosine A1 receptors with CCPA at 100 nM. Fig. 5 shows confocal immunofluorescence images for PKCɛ, PKCδ and caveolin-3 from adult rat ventricular myocytes. These images revealed that the limited basal levels of PKCɛ and PKCδ were present in the caveolin-3-associated membrane in the cells without CCPA pretreatment, as indicated by a few yellow punctate staining along the cell peripherals. The staining pattern with anti-PKCɛ or anti-PKCδ antibody demonstrated that both PKCɛ and PKCδ are mostly diffused throughout of cells and also located at cross-striated structures. Following CCPA stimulation, the antibody against PKCɛ demonstrated a prominent surface sarcolemmal punctate staining area. The merged images showed significant areas of colocalization for PKCɛ and caveolin-3 along the cell peripherals, T-tubular-like
Figure 2.5. Confocal laser microscopic imaging of adult rat cardiomyocytes in response to CCPA. Cells were incubated with or without CCPA (100 nM) for 5 minutes at 37°C before they were subject to permeabilization and immunostaining with antibody against PKCε, PKCδ or caveolin-3 (Cav-3). Both PKCε and PKCδ was predominantly associated with cytosol in unstimulated cells but translocated to cell peripherals and T tubular like structures on stimulation with CCPA. Results are representative of three independent experiments.
Figure 2.5

A

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B

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structures and cell-to-cell contact. A similar staining pattern of PKCδ by CCPA was observed except that PKCδ has more pronounced T-tubular staining when compared with PKCε. A total of 40 cells was imaged and analyzed. While 25% of cells for PKCε and 30% for PKCδ showed significant colocalization in the control group, the number of cells showing colocalization with caveolin-3 in CCPA-treated groups was increased to 83% for PKCε and 73% for PKCδ. These results indicate that activation of adenosine A1 receptors induces recruitment of PKCε and PKCδ to the caveolin-3-associated plasma membrane domains.

**Discussion**

The present study demonstrated that activation of adenosine A1 receptors promotes targeting of novel PKC isoforms, PKCε and PKCδ to caveolin-rich plasma membrane microdomains. We demonstrated that activation of adenosine A1 receptors induced a rapid association of PKCε or PKCδ with caveolin-3 in adult rat cardiac myocytes. Specifically, we found that activation of adenosine A1 receptors with CCPA induced the selective translocation of PKCε and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. Importantly, we showed that activated PKCε and PKCδ were targeted to the caveolin-rich plasma membrane microdomains. We have also showed that PKCε and PKCδ colocalize to and associate with caveolin-3. This observation is consistent with the notion that activated PKC isoforms are
recruited to caveolae via signaling mechanisms that are distinct among different PKC isoforms.

It is known that membrane-bound translocation of PKC from cytosol is a hallmark of PKC activation (M. Csukai 1999). Translocation to different cellular compartments enables PKC to colocalize with both activators and substrates. Individual PKC isoforms are believed to mediate distinct cell functions. Upon stimulation, they are directed to distinct subcellular membrane regions by binding to their specific activated C kinase. The activated isoforms are anchored close to their particular substrates. Although pharmacological evidence suggests that adenosine receptors are linked to PKC activation (Y. Liu 1996; K. Hu 1999), limited study has directly addressed translocation of PKC isoforms by adenosine receptors. Importantly, no study has ever explored the caveolar targeting of specific PKC isoform(s) induced by adenosine receptors. In the present study, we examined the effect of adenosine A1 receptors on the selective translocation and caveolar targeting of PKC isoforms. We found that under basal conditions, the level of immunoreactivity for PKCε and PKCδ in cardiomyocyte caveolae was limited but enhanced significantly following stimulation of adenosine A1 receptors with CCPA. Other PKC isoforms tested including PKCα, PKCβ and PKCζ were not significantly translocated to the cell membrane from the cytosol by stimulating adenosine A1 receptors. These observations provide the novel evidence that activation of adenosine A1 receptors induces the selective translocation of PKCε and PKCδ but not PKCα.
PKCβ and PKCζ in adult rat cardiomyocytes and the translocated PKCɛ and PKCδ are targeted to the caveolin-rich plasma membrane. We chose a 5-min treatment with CCPA because our preliminary data showed that 5-min incubation with CCPA induced the maximum translocation of PKC isoforms.

Although our data demonstrate that activation of adenosine A1 receptors promotes translocation of PKCɛ and PKCδ but not PKCα, PKCβ and PKCζ, these results do not exclude the possibility that other adenosine receptors may cause translocation of one or more of these PKC isoforms. For example, A3 receptors are linked to activation of PKCδ (T.C. Zhao 2003). It is likely that the same PKC isoforms activated by different adenosine receptors may associate with distinct downstream signaling pathways and serve distinct roles. It is also possible that activation of the same adenosine receptors stimulates more than one PKC isoforms, each serving a different role under certain physiological conditions (T.R. Xu 2009; Z. Zhang 2009). In addition to translocation of PKC isoforms, our finding indicates the importance of caveolae microdomains in the adenosine receptor-mediated translocation of PKC. A1 receptor activation has been shown to increase PKCδ in the membrane fraction (P. Henry 1996) and PKCɛ in the membrane and T-tubules of cardiac myocytes (J.W. Lester 2000; R.A. Fenton 2009). These observations are consistent with our finding that activation of adenosine A1 receptors promotes translocation of PKCδ or PKCɛ to the caveolin-rich membrane fractions given that caveolae are also localized to the T-tubules (R.C. Balijepalli 2006). However, Lester and Hofmann report
that adenosine receptor activation causes translocation of PKCε but not PKCδ (J.W. Lester 2000). The discrepancy between this study and the present study may be due to different adenosine receptor agonist used. We used CCPA (100 nM) which is a selective adenosine A1 receptor agonist while Lester et al. employed N6-(2-phenylisopropyl)-adenosine R-(−) isomer (R-PIA) at 100 μM that could activate both A1 and A3 receptors as authors claimed. Even though different PKC isoforms may be targeted to caveolae upon activation, activated PKC isoforms within caveolae may associate with distinct cellular functions due to differential targeting of separate populations of caveolae which may contain different sets of signaling proteins. PKCε and PKCδ are the major Ca2+-independent PKC isoforms and the most abundant PKC isoform found in the adult rat cardiomyocytes. The adenosine receptor-induced caveolar targeting of PKCε and PKCδ may have important implications in adenosine-mediated cardioprotection (S. Kawamura 1998).

It is known that upon activation, PKC isoforms translocate to multiple subcellular sites including plasma membrane, mitochondria or nucleus. Differential targeting of activated PKC isoforms to distinct subcellular localization determines their substrate specificity. In the present study, we focused on examining PKC translocation to the caveolar plasma membrane since the intact caveolae structure in cardiac myocytes has been shown to be required for the cardioprotection of ischemic preconditioning (H.H. Patel 2007; Y.T. Horikawa 2008). Our observation that adenosine A1 receptors promote
the selective translocation of PKCε and PKCδ to caveolar plasma membrane microdomain implies that caveolae may serve as a focal point for efficient signaling transduction involving adenosine A1 receptors. Further, both PKCε and PKCδ have generated considerable interest in cardiac signaling because of their association with ischemic preconditioning (P. Ping 2001). Of particular interest is the suggestion that a delay in the onset of cardioprotection is due to the time taken for PKC isoform translocation and that the memory of preconditioning is due to PKC dwelling in a translocated state (M.V. Cohen 2000). Nevertheless, our results do not exclude the possibility that activation of adenosine A1 receptors may promote translocation of PKCε or/and PKCδ to other subcellular sites such as mitochondria or nucleus. In fact, mitochondrial translocation of PKCε has been linked to cardioprotection although molecular mechanism by which PKCε targets to mitochondria remains elusive (G.S. Liu 1999; G.W. Dorn II 1999).

In conclusion, our data demonstrate that activation of adenosine A1 receptor promotes selective translocation and caveolar targeting of PKCε and PKCδ but not PKCα, PKCβ and PKCζ. This finding provides new mechanistic insight into our understanding the role of caveolae in adenosine receptor-mediated PKC signaling and may have important implication in cardioprotection.
CHAPTER 3

PROTEIN KINASE C REGULATES THE KIR2.1 ACTIVATION THROUGH CAVEOLIN-3

Abstract

Inwardly rectifying potassium channels, Kir2.x channels in particular, are crucial in shaping the action potential and stabilizing the resting potential in cardiac myocytes. However, little is known about the subcellular localization of the Kir2.1 channel, an important component of IK1. Besides, the detailed mechanism underlying Kir2.1 regulation by PKC remains to be investigated. The present study was designed to determine whether Kir2.1 channels are localized to the caveolar plasma membrane and regulated by PKC through caveolin-3. We found that about 40% of the cellular content of Kir2.1 was retained in the caveolin-enriched fractions in adult rat cardiomyocytes. In contrast, clathrin heavy chain was detected across a broad range of the gradient fractions. Measurement of cholesterol levels within each fraction showed cholesterol to be enriched in the caveolin-fractions. Co-
immunoprecipitation data from cardiac myocytes indicated that Kir2.1 was associated with caveolin-3 and this interaction was increased by PMA treatment. In HEK293T cells transfected with Kir2.1 and caveolin-3, a significant proportion of Kir2.1 protein was localized to the caveolin-3 enriched fractions when compared to that in cells transfected with Kir2.1 alone. Immunofluorescent staining showed that a significant portion of Kir2.1 channels colocalized with caveolin-3 in both transfected HEK293T cells and rat cardiomyocytes. Furthermore, the whole-cell voltage clamp data revealed that in rat cardiac myocytes, the basal IK1 currents at -120 mV from a holding potential of -50mV were enhanced by caveolin-3 scaffolding domain peptide. Application of PMA inhibits the IK1 current significantly while caveolin-3 scaffolding domain peptide blocks PMA inhibition on IK1 current. In HEK293T cells, over-expression of caveolin-3 significantly reduced Kir2.1 currents. Application of caveolin-3 scaffolding domain peptide in the pipette disrupted the caveolin-3 inhibitory effect on Kir2.1 current in cells co-transfected with Kir2.1 and caveolin-3. In addition, our data showed that caveolin-3 P104L mutant also inhibited the effect of caveolin-3 on Kir2.1 while mutant itself has no effect on Kir2.1 currents in cells transfected with Kir2.1. In conclusion, our results suggest that a significant portion of Kir2.1 channels targets to the caveolin-enrich plasma membrane microdomains of rat cardiac myocytes, and are negatively regulated by caveolin-3 in PKC signaling. Caveolin-3 scaffolding domain may be involved in this Kir2.1 regulation by caveolin-3.
**Introduction**

In cardiomyocytes, the classical inwardly rectifying potassium channels Kir2.1 is abundantly expressed (B.K. Panama 2007). It is critical in shaping the cardiac action potential and in maintaining the membrane resting potential. Numerous studies have found that Kir2.1 plays an important role in some cardiac diseases including Anderson syndrome, Short QT syndrome, and even atrial fibrillation (H.A. El 2009; J.B. Kim 2009; T. Hattori 2012). These cardiac channelopathy are considered to be a consequence of loss-of-function or gain-of-function in Kir2.1 channels.

It is well known that Kir2.x channels are regulated by several intra- and extracellular signaling molecules including polyamines, Mg2+, and protein kinases. Recent evidence suggests that phosphorylation of the Kir2.1 channels by protein kinases determines the channels activity. Protein kinase C (PKC) has been found to mediate the inhibitory effect of ET1 receptors on Kir channels (W.S. Park 2005). Application of PKC activator PMA or PKC inhibitor staurosporine also regulates the Kir currents in different cell types (W.S. Park 2005; C. Kiesecker 2006; D. Scherer 2007).

Caveolae, rich in cholesterol, sphingolipid and the structure protein caveolin, are small invaginations on the cell plasma membrane. It plays as a cell
signaling center by concentrating the signaling molecules to the microdomain. Indeed, cardiac myocyte caveolae are essential in protein trafficking, signal transduction, and channel activation. Our previous study shows that PKC(s) is translocated into caveolae upon activation by adenosine A1 receptor in adult rat cardiac myocytes (Z. Yang 2009). However, whether caveolae plays an important role in Kir2.1 channel regulation by PKC is not known. In the present study, we tested the hypothesis that caveolin-3 negatively regulates the Kir2.1 current, and this process is important in PKC regulation of Kir2.1 channel activity. We examined the effect of caveolin-3 on Kir2.1 channel current in HEK293T cells, and the effect of caveolin-3 scaffolding domain on Kir2.1 current both in HEK293T and adult rat cardiac myocytes. Further, we investigated the role of caveolin-3 in PKC induced Kir2.1 channel inhibition in cardiac myocytes. Our results demonstrate that caveolin-3 negatively regulated the Kir2.1 channel activity, and caveolin-3 scaffolding domain is involved in this regulation. This regulatory mechanism is also important in PKC regulation of Kir2.1 current.

MATERIALS AND METHODS

Materials.

Goat IgG directed against caveolin-3 and rabbit IgG directed against Kir2.1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary
antibody directed against goat conjugated with Cy3 or mouse conjugated with Cy2 was purchased from Jackson Immuno Research Laboratories (West Grove, PA). The caveolin-3 scaffolding domain peptide containing the putative scaffolding domain of caveolin-3 (amino acids 55-74, DGVWRVSYTTFTVSKYWCYR) was obtained from AnaSpec Inc. (San Jose, CA, USA). Cadmium chloride, barium chloride and 4-aminopyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.

*Isolation of cardiomyocytes.*

Adult rat ventricular myocytes were isolated from adult Sprague Dawley rats (250 to 300 g) by enzymatic dissociation (K. Hu 1996; K. Hu 2000). In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O2) Tyrode's solution containing (mM) NaCl 126, 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 is nominally Ca2+ 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 cells were initially stored in a solution containing (mM) KCl 20, KH2PO4 10, glucose 10, potassium glutamate 70, ß-hydroxybutyric
acid 10, taurine 10, mannitol 5, and EGTA 5, along with 1% albumin, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with various agents for 5 minutes at 37°C prior to subsequent biochemical and immunofluorescence experiments.

*Cell culture and transfection.*

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100IU/ml), and streptomycin (100μg/ml). Cells were plated onto 10-mm microscope cover glass and transfected with HA-tagged Kir2.1, with or without caveolin-3-ECFP, by using FuGENE6 (Roche). Cells were also transfected with GFP as the control to ensure transfection. Experiments were conducted 1-2 days after transfection.

*Fractionation of caveolin-enriched membrane*

Caveolin-rich fractions were prepared by a standard detergent-free method with some modification. Briefly, cell pellet suspended in 0.5M Na2CO3 (pH 11.0, 2 ml) was homogenized. After the homogenate was adjusted to 45% sucrose in an ultracentrifuge tube, a 5-35% discontinuous sucrose step gradient was formed above, by overlaying with 4 mL of 35% sucrose and then 4 mL of 5% sucrose. Tubes were centrifuged at 39,000 rpm for 18-20 hours 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. The cholesterol concentration in each fraction was determined by colorimetric method using a cholesterol assay kit (Roche).

**Western blotting.**

Immunoblot analysis was carried out as described previously (V. Garg 2007; J. Jiao 2008). Briefly, the cytosolic and particulate fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and 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 2 h at room temperature or overnight at 4 °C 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.

**Co-immunoprecipitation.**

Immunoprecipitation experiments were performed as reported previously (V. Garg 2009; Z. Yang 2009; W. Sun 2010). Cells were pretreated with or without adenosine prior to homogenization. The total cell fraction was incubated with
primary antibody for 2 hours at 4°C. Antigen-antibody complexes were captured with r-protein-G agarose (4°C, 2 hours). Agarose beads were washed 4-times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies.

*Immunofluorescence microscopy.*

As described previously (Z. Yang 2011), 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 for 30 min, and labeled with primary antibody for 2h. Cells were than washed three times and labeled with fluorescence-conjugated secondary antibody for 1h. Immunofluorescence was visualized with Nikon fluorescence microscopy or with confocal laser scanning microscopy. All images were analyzed using a background subtraction method offline.

*Electrophysiology studies*

The membrane currents were recorded from adult rat cardiac myocytes using the conventional whole cell configuration. In the Kir2.1 transfected HEK293T cells, only GFP or CFP-positive cells were chosen for recording. In brief, cells were superfused with the bath solution containing (mM) NaCl 135, KCl 5.4,
MgCl₂ 1.0, CaCl₂ 1.0, NaH₂PO₄ 0.33, CdCl₂ 1, 4-AP 2, HEPES 10, and glucose 10 at pH 7.4 (pH adjusted with NaOH). The pipette solution contained (mM) KCl 140, MgCl₂ 1.0, HEPES 10, EGTA 5, and GTP 0.1 at pH 7.3 (pH adjusted with KOH). Data were sampled with an A/D converter (Digidata 1322A, Axon Instruments) and stored on the hard drive of a computer for subsequent analysis. The recording was filtered with a low-pass corner frequency of 2 kHz. Borosilicate glass electrodes were used with resistances in range of 3-5 MΩ when filled and connected to a patch-clamp amplifier (Axopatch 200B, Axon Instruments). All the recording were carried out at room temperature (22-25°C).

Kir2.1 or IK1 currents were calculated as Ba-sensitive currents.

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

Localization of Kir2.1 channels in caveolin-enriched microdomains.

To investigate whether the Kir2.1 channels are present in caveolin-enriched plasma membrane fractions in cardiomyocytes, we used a detergent-free
sucrose gradient extraction–procedure for adult rat ventricular myocytes. Twelve 1 mL fractions were collected from the top to the bottom of the gradient for subsequent analysis by western blot. As shown in Figure 3.1, the marker proteins caveolin-3 was found predominantly in the lower–density fractions (Fraction 4-7). Clathrin heavy chain was detected with antibody against clathrin heavy chain across a broad range of the gradient similar to that reported by Vivek et al. The Kir2.1 showed the two-peak pattern distribution in the twelve fractions. More than 40% (41.8 ± 7.2 %) of the total Kir2.1 are concentrated in fraction 4-7 while the other half are distributed in non-caveolae fractions. Measurement of cholesterol levels in each fraction showed cholesterol to be enriched in fraction 4 and 5, which are in consistent with the result that these two fractions represent caveolae-containing membrane fraction of the density gradient.

_Effect of PMA on co-immunoprecipitation of Kir2.1 channels and caveolin-3 adult rat cardiac myocytes._

To determine whether muscle-specific caveolin isoform caveolin-3 and Kir2.1 are associated with each other and whether this interaction is affected by PKC activation, we performed immunoprecipitation experiments from the cell lysates of adult rat cardiac myocytes. The cells were incubated with different drugs for 5 mins, and the cell homogenate was incubated with rabbit anti-Kir2.1 antibody or mouse anti-caveolin-3 respectively. The immune complexes were collected
Figure 3.1. Kir2.1 is partially enriched in caveolar plasma membrane in adult rat cardiomyocytes. (A) One milliliter fractions were collected from the top of the gradient and analyzed with antibody against Kir2.1, caveolin-3, clathrin heavy chain and calreticulin. Representative Western blots of three to four independent experiments (B) Relative cholesterol levels of each fraction.
Figure 3.2. Effect of PMA on co-immunoprecipitation of Kir2.1, caveolin-3 and PKCε from adult rat cardiac myocytes. Adult rat cardiac myocytes were pre-treated without or with PMA, 4α-PMA, or PMA plus chelerythrine (Che) for 10 min prior to homogenation. Cell lysates containing equal amounts of total proteins were incubated with anti-caveolin-3 or anti-Kir2.1 antibody 2 h at 4 degree. Agarose beads were washed 4 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were analyzed by 10% SDS-PAGE. (A) Immunoprecipitation of anti-caveolin-3 in cardiac myocytes without or with PMA, 4α-PMA, or PMA plus Che. (B) Quantification of co-precipitated Kir2.1 and PKCε with caveolin-3. (C) Immunoprecipitation of anti-Kir2.1 in cardiac myocytes without or with PMA, 4α-PMA, or PMA plus Che. (D) Quantification of co-precipitated caveolin-3 and PKCε with Kir2.1. Results are data from 3 independent experiments. *P< 0.05 vs. control.
Figure 3.2

A
IP: Cav-3

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<th>4α-PMA</th>
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B

Relative Content (% of Control)

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C
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D

Relative Content (% of Control)

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with r-protein G beads and analyzed by immunoblotting against mouse anti-
caveolin-3, rabbit anti-Kir2.1, and rabbit anti-PKCε. Figure 3.2 showed that
anti-Kir2.1 antibody immunoprecipitated with caveolin-3, and this association
was increased by PMA treatment (215.6 ± 23.2%, n=3, P<0.05 vs. control).
PKCε was also immunoprecipitated with caveolin-3 and the amount found in
the complex was also increased by PMA treatment (147.1 ± 7.8%, n=3, P<0.05
vs. control), which is in consistent with our former finding that activation of
PKCε increases its association with caveolin-3. Here, we also used 4α-PMA as
a negative control and found that 4α-PMA has no effect on this interaction.
When the general PKC inhibitor chelerythrine was applied in PMA group, the
PMA effect was blocked and the association returned to the basal level.
Similarly, anti-caveolin-3 antibody immunoprecipitated with Kir2.1 and PKCε,
and this association was enhanced by PKCε activation with PMA treatment.
Neither protein was immunoprecipitated with control IgGs. These results
suggest the Kir2.1 associates with caveolin-3 and PKCε in adult rat ventricular
myocytes and this association is increased with the PKCε activation.

Effect of caveolin-3 scaffolding domain peptide on IK1 current in adult rat
cardiac myocytes

The caveolin-3 scaffolding domain peptide, which corresponds to the putative
scaffolding domain of caveolin-3, has been shown to directly modulate some
proteins function. It has also been previously shown to participate in protein-
protein interaction with caveolin-3. Here, we used the caveolin-3 scaffolding domain peptide (50 μM) to determine whether there is a direct functional interaction between caveolin-3 and Kir2.1 channels and whether this caveolin-3 scaffolding domain is involved in this interaction. The whole cell patch-clamp technique was explored, and the caveolin-3 SDP was introduced into the cell interior from the patch pipette. Due to the lack of specific Kir2.1 channel blocker, we recorded the total barium-sensitive current as the IK1 current in adult rat cardiac myocytes where the major component of IK1 is Kir2.1 current. Figure 3.3 summarizes the current density for control, caveolin-3 SDP and scramble peptide. Application of caveolin-3 SDP 20 mins before recording significantly increased the IK1 current by 71.7% compared to control group (171.7 ± 18.7 %, P<0.05, vs. control) while the scramble peptide has no effect on Kir2.1 current (106.6 ± 7.3 %, P>0.05, vs. control). The above data suggest that caveolin-3 regulates the function of Kir2.1 channels and caveolin-3 scaffolding domain is involved in this regulation.

Effect of caveolin-3 on Kir2.1 current in HEK293T cells.

To further characterize whether the caveolin-3 regulates the Kir2.1 current directly, we took the advantage of HEK293T cells which lack the endogenous caveolin-3. We investigated the Kir2.1 current in HEK293T cells co-transfected with GFP or caveolin-3-CFP, and only cells that express the GFP or CFP were selected for recording. Results showed that expression of Kir2.1 in HEK293T
Figure 3.3. Effect of caveolin-3 scaffolding domain peptide on IK1 current in cardiac myocytes. (A) Original IK1 current recording obtained by conventional whole-cell currents clamp recordings at a holding potential of -50 mV in rat cardiac myocytes. Cells were applied without or with caveolin-3 scaffolding domain peptide (SDP) or scramble peptide (Sc Peptide) 20 min before recording. (B) Mean ± S.E. of current-voltage relations in different groups. (C) Mean percentage of IK1 currents compared with control group in cells with different treatments. N=5. Data are normalized to control group. **P<0.01 vs. control.
Figure 3.3

A

Control  SDP  Sc. Peptide

B

mV

-120  -90  -60  -30  0

-30  -20  -10  0  10  20

pA/pF

Control  SDP  Sc. peptide

C

Current (% of control)

Control  SDP  Sc. Peptide
Figure 3.4. Effect of caveolin-3 on Kir2.1 current in HEK293T cells. Cells were transfected with Kir2.1/GFP or Kir2.1/Caveolin-3 48 h before experiment. Cells only have the GFP or CFP were chosen for recording. (A) Original Kir2.1 current recording obtained by conventional whole-cell currents clamp recordings at a holding potential of -40 mV in cells with or without caveolin-3 transfection. (B) Mean ± S.E. of current-voltage relations in control cells without or with Ba\(^{2+}\) or in cells with caveolin-3 co-transfection. (C) Mean percentage of Kir2.1 currents compared with control group in cells transfected with caveolin-3. N=5. Data are normalized to control group. **P< 0.01 vs. control.
Figure 3.4

A

Control

BaCl$_2$

Cav-3

150 pA/pF

50 ms

B

mV

-120 -60 -30 0

Pa/pF

Control

Cav-3

BaCl$_2$

C

Current (% of control)

Control

BaCl$_2$

Cav-3

**
cells produced strong inwardly rectifying currents that could be blocked by 500 μM Ba2+. The mean current amplitude at -120 mV was -299.9 ± 37.5 pA/pF (n=5) (Figure 3.4). Overexpression of caveolin-3 significantly reduced Kir2.1 currents by 74% (25.8 ± 6.6%, n=5, P<0.01 vs. control) (Figure 3.4). These observations further indicate that caveolin-3 has the direct inhibitory effect on Kir2.1 channels.

Effect of caveolin-3 scaffolding domain peptide on Kir2.1 current in HEK293T cells

In adult rat cardiac myocytes, we found that application of caveolin-3 scaffolding domain peptide significantly increased the IK1 current. Here, we further study this interaction in transfection system. Figure 3.5 summarizes the current density for control, caveolin-3, caveolin-3 SDP, caveolin-3 plus caveolin-3 SDP and caveolin-3 plus scramble peptide groups. Caveolin-3 SDP significantly blocked the effects of caveolin-3 on Kir2.1 currents (80.5 ± 10.3%, n=5, P>0.05 vs. control). However, in the cells without caveolin-3 expression, the caveolin-3 SDP had no effect on Kir2.1 currents. The scramble peptide has no effect on Kir2.1 current. The above data further verified that caveolin-3 negatively regulates the function of Kir2.1 channels and caveolin-3 scaffolding domain is involved in this regulation.

Effect of caveolin-3 dominant negative mutant on Kir2.1 current

To further investigate the caveolin-3 effect on Kir2.1 channels, we took the advantage of caveolin-3 P104L, a dominant negative mutant of caveolin-3 (Figure
Figure 3.5. Effect of caveolin-3 scaffolding domain peptide on Kir2.1 current in HEK293T cells. Cells were transfected with Kir2.1/GFP or Kir2.1/Caveolin-3 48 h before experiment. (A) Original Kir2.1 current recording obtained by conventional whole-cell currents clamp recordings at a holding potential of -40 mV in cells with different transfections. (B) Mean ± S.E. of current-voltage relations in different groups. (C) Mean percentage of Kir2.1 currents compared with control group in cells with different treatments. N=5. Data are normalized to control group. **P< 0.01 vs. control.
Figure 3.6. Effect of caveolin-3 dominant negative mutant on Kir2.1 current in HEK293T cells. Cells were transfected with Kir2.1/GFP, Kir2.1/Caveolin-3, Kir2.1/Caveolin-3-P104L, or Kir2.1/Caveolin-3/Caveolin-3-P104L 48 h before experiment. (A) Original Kir2.1 current recording obtained by conventional whole-cell currents clamp recordings at a holding potential of −40 mV in cells with different transfections. (B) Mean ± S.E. of current-voltage relations in different groups. (C) Mean percentage of Kir2.1 currents compared with control group in cells with different transfections. N=5. Data are normalized to control group. **P< 0.01 vs. control.
Figure 3.6

A

Cav-3 P104L

Cav-3 + Cav-3 P104L

0

50 ms

100 pA/pF

B

-120

-60

-30

0

-100

-200

-300

-400

pA/pF

Control

Cav-3

Cav-3 P104L

Cav-3 + Cav-3 P104L

C

Current (% of control)

Control

Cav-3

Cav-3 P104L

Cav-3 + Cav-3 P104L

**
Coexpression of the caveolin-3 P104L mutant with Kir2.1 did not significantly change the Kir2.1 current compared to the control group which is transfected with Kir2.1 and GFP (84.3 ± 5.2%, n=5, P>0.05 vs. control). In contrast, coexpression of caveolin-3 with Kir2.1 reduced the Kir2.1 currents robustly when compared to control (25.8 ± 6.6%, n=5, P<0.01 vs. control). Interestingly, when caveolin-3 P104L mutant was coexpressed with caveolin-3, the Kir2.1 currents was not reduced strongly compared to caveolin-3 group (60.2 ± 5.3%, n=5, P<0.05 vs. control), indicating that the caveolin-3 effect was partially blocked by caveolin-3 P104L mutant. Since this caveolin-3 P104L has been shown to block the exportation of newly synthesized caveolin-3 from ER to cell membrane, this Kir2.1 current increase is possibly due to the decreased expression of caveolin-3 on cell plasma membrane. Taken together, these findings further suggest that caveolin-3 can inhibit the Kir2.1 channel activity.

Effect of caveolin-3 on Kir2.1 plasma membrane expression

To further investigate the mechanism underlying the Kir2.1 current reduction by caveolin-3, we employed immunofluorescence microscopy to study the effect of caveolin-3 on Kir2.1 expression on the cell plasma membrane. The reduction of Kir2.1 may either caused by the decreased Kir2.1 expression on cell surface or by direct inhibition by caveolin-3 without changing the Kir2.1 level on membrane. As shown in Figure 3.7A, the staining pattern of Kir2.1 (red) and caveolin-3 (green) showed the partially colocalization of Kir2.1 and caveolin-3 along the plasma.
membrane. The merged images showed significant area of colocalization for Kir2.1 and caveolin-3 on cell surface. By measuring the fluorescence intensity, the amount of Kir2.1 expression on cell surface was calculated (Figure 3.7B). A total of 40 cells was imaged and analyzed. There was no great difference in Kir2.1 cell surface expression level between groups without or with caveolin-3 (105.4 ± 11.0%, n=3, P>0.05 vs. control) transfection. These data suggest that caveolin-3 inhibits the Kir2.1 current directly without changing its cell membrane expression.

Effect of caveolin-3 on IK1 regulation by PKC

PKC has been found to mediate the Kir2.1 current in different cells. Here, we used the PMA to induce the PKC activation to determine whether caveolin-3 is important in PKC-induced Kir2.1 channel regulation. As shown in Figure 3.8, PKC activation by PMA cause a strong inhibition of IK1 current in cardiac myocytes (59.9 ± 14.1%, n=5, P<0.05 vs. control) while 4α-PMA, the negative control of PMA, has no effect on IK1 current (96.2 ± 5.5%, n=5, P>0.05 vs. control). This PMA inhibitory effect on IK1 is blocked by PKC inhibitor chelerythrine (94.5 ± 9.8%, n=5, P>0.05 vs. control). Interestingly, the PMA inhibitory effect on IK1 current was completely blocked by application of caveolin-3 SDP 20 mins before recording(124.3 ± 7.5%, n=5, P>0.05 vs. control) while the scramble peptide has no effect on PMA-induced inhibitory of IK1 current (57.4 ± 8.1%, n=5, P>0.05 vs. PMA). These results suggest that
Figure 3.7. Effect of caveolin-3 on Kir2.1 plasma membrane expression. Cells were transfected with Kir2.1/GFP or Kir2.1/Caveolin-3 48 h before staining. Immunofluorescence staining was performed with anti-HA antibody (A) Representative image of the immunofluorescence staining with different transfection. HA-tagged Kir2.1 was illustrated in red and caveolin-3-CFP, GFP were indicated in green. Scale bar: 10 μM (B) Quantification of the Kir2.1 plasma membrane expression in different groups. Results are data from 3 independent experiments. *P< 0.05 vs. control.
Figure 3.7

A

Kir2.1

Kir2.1 + Cav-3

Cav-3  
Kir 2.1  
Merge

B

Kir2.1 Relative Content (% of Control)

Kir2.1  
Kir2.1 + Cav-3

84
Figure 3.8. Effect of caveolin-3 on IK1 regulation by PKC. (A) Original IK1 current recording obtained by conventional whole-cell currents clamp recordings at a holding potential of -50 mV in rat cardiac myocytes. (B) Mean ± S.E. of current-voltage relations in different groups. (C) Mean percentage of IK1 currents compared with control group in cells with different treatments. N=5. Data are normalized to control group. **P< 0.01 vs. control.
Figure 3.8

A

Control  PMA  4α-PMA

PMA + Che  SDP + PMA  Sc. peptide + PMA

10 pA/pF
50 ms

B

mV

-120 -60 -30 0 5

Current (% of control)

Control  PMA  4α-PMA  PMA + Che  SDP + PMA  Sc. peptide + PMA

#  *  #  #  *
PKC inhibits the IK1 current through caveolin-3 in cardiac myocytes and caveolin-3 scaffolding domain is involved in this regulation.

**Discussion**

In the present study, we provide the first evidence that Kir2.1 channels are negatively regulated by caveolin-3, both in HEK293T cells and in rat cardiac myocytes. Specifically, we show that Kir2.1 channel activity was significantly inhibited by expression of caveolin-3 while the Kir2.1 expression on cell plasma membrane was not changed. Further, we demonstrate that caveolin-3 SDP blocked the caveolin-3 mediated suppression of Kir2.1 current. Moreover, our data indicate that caveolin-3 is critical in PKC induced IK1 inhibition in the heart and caveolin-3 scaffolding domain is involved in this regulation.

It is well accepted that the cell membrane participates actively in signaling transduction, protein trafficking, and cellular adaptation to the outer environments. One type of well characterized lipid rafts, caveolae, has revolutionized our notions of protein targeting and regulation on cell membrane. Numerous studies have shown that caveolin, the structure protein of caveolae, regulates the ion channel activity(Y.S. Markandeya 2011). Also, evidence has already shown that phosphorylation of Kir2.1 channels by protein kinases determine the channel activity(I. Alesutan 2011). Our previous studies have already shown the involvement of caveolae in adenosine-mediated PKC
activation (Z. Yang 2009). However, no study has directly addressed the regulation of Kir2.1 by caveolin-3. In the present study, experiments were designed to determine whether Kir2.1 is localized in caveolae and regulated by caveolin-3. We found that under basal condition, about half of the total Kir2.1 channels are localized within caveolae in adult rate cardiac myocytes. The physical association of Kir2.1 and caveolin-3 was confirmed by co-immunoprecipitation in adult rat cardiac myocytes. Functionally, results showed that caveolin-3 negatively regulates the Kir2.1 current and caveolin-3 scaffolding domain is involved in this regulation. PKC activation inhibits the Kir2.1 channel while application of caveolin-3 scaffolding domain peptide abolishes this inhibitory effect. Taken together, our data indicate that caveolin-3 can negatively regulate the Kir2.1 channel function and it is important in PKC mediated Kir2.1 inhibition pathway.

Studies have already shown that Kir2.x channels are associated with the dystrophin-associated protein complex (DAPC) (D. Leonoudakis 2004). Moreover, caveolin-3 has been shown to interact with dystrophin in DAPC, and also regulates the distribution of DAPC (J. Schultz 1998; F. Sotgia 2000; F. Galbiati 2001). In muscle cells, DAPC is abundantly distributed (G.E. Crawford 2000). The overexpression or knockdown of caveolin-3 in cardiac myocytes significantly changes the DAPC expression and distribution in the cells (F. Galbiati 2001; B. Aravamudan 2003). Besides, the dynamin, which is known to mediate the caveolae internalization, controls the Kir2.1 internalization.
However, whether there is a direct protein-protein interaction between Kir2.x and caveolin-3 is not clear. Our data show that caveolin-3 may colocalize with Kir2.1 on plasma membrane and has the negative effect on Kir2.1 channel activation. It is known that caveolin usually interacts with other signaling molecules through the caveolin scaffolding domain (CSD) which is located near the N terminus of the caveolin sequence. The proteins contain the specific amino acid sequences that can bind to this domain. These sequences include ΦXXXXΦXXΦ, ΦXΦXXXXΦ and ΦXΦXXXXΦXXΦ where Φ is an aromatic amino (W, F, Y) acid and X represents any amino acid (J. Couet 1997). In Kir2.1 channel sequences, at least two such sequences (WRWMLVIF and WLFFGCVF) are found and conserved across different species. Our data indicate that the caveolin-3 scaffolding domain is involved in the caveolin-3–Kir2.1 protein-protein interaction although the detailed underlying mechanism is still not clear.

Although it is well accepted that phosphorylation of Ki2.x by different protein kinases modulates the Kir2.x channel activation (W.S. Park 2005; D.Y. Zhang 2011; I. Alesutan 2011), it is still not clear how PKC regulates the Kir2.1 currents. We have recently discovered that activation of adenosine A1 receptor induces the translocation of specific PKC from the cytosol to the caveolar plasma membrane (Z. Yang 2009). Given that the activated PKCs are located in the caveolae and Kir2.1 is partially distributed in this lipid raft, it is possible that caveolin-3 plays an important role in IK1 regulation by PKC. Recently,
more and more studies have shown that caveolin is important in cell signaling and ion channel activation (H. Couchoux 2011; J. Gao 2011; M.A. Riddle 2011; Y.S. Markandeya 2011). Our data showed that general PKC activator PMA can reduce the IK1 current in cardiac myocytes. While caveolin-3 scaffolding domain peptide was applied before PMA, the PMA effect on IK1 current was largely blocked. These results indicate that caveolin-3 is involved in PKC regulation of IK1 in the heart and CSD is involved in this regulation.

In summary, we demonstrate that caveolin-3 negatively regulates the Kir2.1 current, and IK1 current regulation by PKC is dependent on caveolin-3. This finding provides new mechanistic insight into our understanding of the role of caveolae in ion channel activation and may have important implications in ion channel diseases.
CHAPTER 4

MOLECULAR MECHANISM UNDERLYING ADENOSINE RECEPTOR-MEDIATED MITOCHONDRIAL TARGETING OF PROTEIN KINASE C

Abstract

Activation of protein kinase C (PKC) via adenosine receptors is known to be involved in the cardioprotection of ischemic preconditioning (IPC). Specifically, activation of PKCε is critical for cardioprotection. There is ample evidence that PKCε resides in cardiac mitochondria. However, the signals that promote translocation of PKCε are largely unknown. The present study was designed to determine whether and how adenosine receptor activation induces translocation of PKCε to mitochondria. Freshly isolated adult rat cardiac myocytes and rat heart-derived H9c2 were used in the study. Immunofluorescence imaging of isolated mitochondria showed that PKCε but not PKCδ was localized in mitochondria and this mitochondrial localization of PKCε was significantly increased by adenosine treatment. The adenosine-induced increase in PKCε-positive mitochondria was largely prevented not only by PKC inhibitor chelerythrine, but also by the HSP90 inhibitor geldanamycin.
and by siRNA targeting HSP90. Immunoblot analysis from percoll-purified mitochondria further demonstrated that adenosine mediated a significant increase in mitochondrial PKCε but not PKCδ. This effect was blocked by inhibiting PKC activity with chelerythrine and bisindoylmaleimide. Furthermore, co-immunoprecipitation data showed that PKCε but not PKCδ was associated with TOM70 and HSP90, and this association was enhanced by adenosine treatment. Moreover, adenosine-induced association of PKCε with TOM70 was reduced by suppressing HSP90 expression with siRNA. In conclusion, we demonstrate that adenosine induces HSP90-dependent translocation of PKCε to mitochondria, possibly through mitochondrial import machinery TOM70. These results point out a novel mechanism in regulating PKC in mitochondria and suggest an important implication in ischemic preconditioning or postconditioning.
INTRODUCTION

Activation of protein kinase C (PKC) has been shown to be involved in the protective effect of ischemic preconditioning (IPC) and postconditioning (C.E. Murry 1986; Downey 2004; S Dong 2010). Specifically, activation of PKC isoform PKCε is critical for cardioprotection (G.S. Liu 1999; G.W. Dorn II 1999; G.R. Budas 2007). Numerous reports have shown that selective activation of PKCε confers cardioprotection whereas inhibition of PKCε prevents protection.

It has been known that upon activation, PKC translocates to multiple subcellular localizations including mitochondria, nucleus and plasma membrane (C.P. Baines 2002; M. Uecker 2003; G.R. Budas 2007). There is ample evidence that PKC substrates reside in cardiac mitochondria (C.P. Baines 2002; C.P. Baines 2003; M. Jaburek 2006; M. Ogbi 2006; G.R. Budas 2007). While some studies demonstrate that PKCε is present constitutively within cardiac mitochondria, others suggest that mitochondrial translocation of PKCε is required for protective effect (G.W. Dorn II 1999; M. Jaburek 2006; G.R. Budas 2007). Given that most of mitochondrial proteins are nuclear-encoded, synthesized in the cytosol, and transported to mitochondria through the protein-conducting pores formed by translocases of the outer (TOM) and inner (TIM) mitochondrial membrane (N. Pfanner 1997; K. Kaldi 1998; T. Lithgow 2000), PKCε may be translocated to mitochondria via import mechanism in order to interact with its substrates within mitochondria.
Adenosine receptors are G-protein-coupled receptors and consist of 4 subtypes-A1, A2A, A2B and A3. It is generally believed that A1 and A3 receptors are coupled to Gi/Go proteins whereas A2A and A2B are linked to Gs proteins. While different receptor subtypes may be associate with distinct signaling pathways, all of these receptor subtypes are shown to be cardioprotective against ischemia/ reperfusion-induced injury (V.J. McIntosh 2012). Importantly, the adenosine-receptor-mediated cardioprotection has been shown to be associated with the activation and translocation of PKC isoforms from cytosolic to membrane fractions (G.E. Kirsch 1990; E. Kim 1997; K. Hu 1999; Z. Yang 2009). However, whether adenosine receptors induce translocation of PKC isoforms to mitochondria is not known. In recent years, studies have shown that molecular chaperone heat shock protein (HSP)90 and HSP70, which are linked to IPC, can facilitate mitochondrial protein transport by association with import machinery TOM70 in mammalian cells (J.C. Young 2001; K. Boengler 2005). Further, a recent report indicates that some components of the mitochondrial import machinery are down-regulated during ischemia but preserved by IPC (K. Boengler 2006), indicating an important role of TOM70 in cardioprotection. In the present study, we tested the hypothesis that adenosine induces mitochondrial targeting of PKCε, and this process is regulated by HSP90-dependent mitochondrial import mechanism. We examined the effect of adenosine on the dynamic movement of PKCε from cytoplasm to mitochondria and its regulation by TOM70 and HSP in the freshly isolated adult cardiac myocytes and rat heart-derived H9c2 cells. We
demonstrated that adenosine promoted PKCε translocation to mitochondria. This regulatory mechanism largely depends on cellular HSP90, possibly via TOM70. Our data indicates a novel mechanism underlying adenosine-induced PKC targeting to mitochondria.

MATERIALS AND METHODS

Materials.

Rabbit IgG directed against PKCε or PKCδ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Adenosine, adenosine receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), chelerythrine, HSP90 inhibitor geldanamycin and HSP70 inhibitor quercetin were supplied from Sigma-Aldrich (St. Louis, MO). Adenosine was dissolved in deionized water. All other drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.

Isolation of cardiomyocytes.

Adult rat ventricular myocytes were isolated from adult Sprague Dawley rats (250 to 300 g) by enzymatic dissociation(K. Hu 1996; K. Hu 2000). In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O2) Tyrode's solution containing (mM) NaCl 126, KCl 5.4, CaCl2 1.0,
MgCl2 1.0, NaH$_2$PO$_4$ 0.33, HEPES 10, and glucose 10 at 37°C. The perfusate was then changed to a Tyrode's solution that is nominally Ca2+ 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 cells were initially stored in a solution containing (mM) KCl 20, KH$_2$PO$_4$ 10, glucose 10, potassium glutamate 70, β-hydroxybutyric acid 10, taurine 10, mannitol 5, and EGTA 5, along with 1% albumin, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with various agents for 5 minutes at 37°C prior to subsequent biochemical and immunofluorescence experiments.

Small interfering RNA.

The small interfering RNA (siRNA) oligonucleotide targeting rat HSP90α and β was purchased from Ambion Inc. (Austin, TX, USA). A negative control siRNA (scrambled) was included to monitor nonspecific effects.

Cell culture and transfection.

Rat heart-derived H9c2 cells were cultured in Dulbecco’s modified Eagle’s medium DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin (J. Jiao 2008). H9c2 cells were
transfected with siRNA using Hi-perfect siRNA transfection reagent (J. Jiao 2008). Experiments were carried out 48 to 72 hours after transfection.

*Preparation of the cytosolic or mitochondrial fraction or intact mitochondria.*

Intact mitochondria or mitochondrial or cytosolic fractions were prepared from H9c2 cells and adult rat cardiomyocytes by differential centrifugation (V. Garg 2007; J.D. Jiao 2008; Z. Yang 2009). For Western blot experiments, mitochondrial fraction was further purified by 30% percoll ultracentrifugation (V. Garg 2007; J.D. Jiao 2008). For mitochondrial colocalization experiments, cells were stained with a mitochondrial marker MitoTracker (200 nM) for 15 minutes before fractionation. In brief, cells after treatment with various agents at 37°C were collected in an ice-cold homogenizing buffer containing (in mM) 250 mM sucrose, 5 mM HEPES, 5 mM EDTA, proteinase inhibitor cocktail. Two 15 s homogenization cycles were performed on ice. The homogenate was centrifuged at 1000g for 10 minutes at 4°C to remove nuclei and debris. The mitochondrial and cytosolic fractions were prepared by centrifugation at 8500×g for 20 minutes at 4°C. The supernatants were then centrifuged at 27,000g for 1 h at 4°C and the resulting supernatant was used as the soluble cytosolic fraction. The pellet containing the mitochondrial fraction was resuspended in the homogenizing buffer and further centrifuged at 8500g for 20 min at 4°C. The washed mitochondria were then resuspended. For percoll purification, the crude mitochondrial suspension (0.5 ml) was laid on the top of
10 ml of a solution containing 30% percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.4). Self-generating percoll gradient was developed by centrifugation at 95000g for 30 minutes at 4°C. Mitochondrial band was collected by a Pasteur pipette and washed in the homogenizing buffer.

**Western blotting.**

Immunoblot analysis was carried out from the cytosolic and mitochondrial fractions (V. Garg 2007; Z. Yang 2009). The purity of mitochondrial or cytosol fractions was evaluated using antibody against the mitochondrial marker protein prohibitin, the cytosol marker GAPDH and the plasma membrane marker Na+/K+ ATPase to ensure there is no significant contamination in the cytosolic or mitochondrial fraction. Briefly, the percoll-purified mitochondrial and cytosolic fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and 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 2 hours at room temperature or overnight at 4°C with primary antibodies in TBS, 0.05% tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 minutes and developed using the ECL detection system.
Co-immunoprecipitation.

Immunoprecipitation experiments were performed as reported previously (V. Garg 2009; Z. Yang 2009; W. Sun 2010). Cells were pretreated with or without adenosine prior to homogenization. The mitochondrial fraction was incubated with primary antibody for 2 hours at 4°C. Antigen-antibody complexes were captured with r-protein-G agarose (4°C, 2 hours). Agarose beads were washed 4-times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies.

Immunofluorescence microscopy.

As described previously (V. Garg 2009), intact mitochondria were isolated from cells pretreated with MitoTracker and/or other agents. Mitochondria is fixed with 4% formaldehyde in PBS for 30 minutes, blocked, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 minutes), and labeled with primary antibody for 2 hr. Mitochondria were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 hr. Immunofluorescence was visualized with Nikon fluorescence microscope. All images were analyzed using a background subtraction method offline.
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

Effect of adenosine on mitochondrial localization of PKCε.

To investigate role of adenosine on mitochondrial localization of PKCε, we examined whether adenosine promotes mitochondrial translocation of PKCε and whether this translocation is dependent on PKC activation. Given that determining whether proteins are localized in mitochondria is difficult in intact adult cardiomyocytes due to tightly packed myofibrils and sub-organelles, we isolated mitochondria from adult rat cardiac myocytes and analyzed mitochondria for the presence of protein of interest. This method has been demonstrated to be an effective way to localize proteins in mitochondria(K. Boengler 2005; V. Garg 2007). The freshly isolated adult rat cardiac myocytes were pretreated without or with adenosine (10 μM), adenosine plus chelerythrine (10 μM) or adenosine plus DPCPX (10 nM) for 5-10 minutes. The concentration of the PKC inhibitor chelerythrine was chosen based on our previous studies(K. Hu 1995; K. Hu 2003; V. Garg 2007). Cells were also
stained with a mitochondria-selective probe MitoTracker-green before mitochondrial fractionation. Intact mitochondria were obtained by differential centrifugation. As shown in Figure 4.1, a small portion of PKCe was localized in mitochondria that were identified by MitoTracker-green. However, adenosine pretreatment significantly increased mitochondrial localization of PKCe, as evidenced by increased yellow punctate staining. The adenosine-induced increase in mitochondrial PKCe was largely blocked by PKC inhibitor chelerythrine. The quantification analysis (Fig. 4.1B) revealed that the percent of PKCe-positive mitochondria, which was normalized to total mitochondria, was significantly increased by adenosine treatment (72.3 ± 7.6% vs. 21.1 ± 4.5%, adenosine vs. control, p<0.01) and this effect was largely eliminated by DPCPX (31.5 ± 6.4% vs. 72.3 ± 7.6%, DPCPX vs. adenosine, p<0.01). The adenosine effect on PKCe was also prevented by chelerythrine (39.8 ± 6.7% vs. 72.3 ± 7.6%, chelerythrine vs. adenosine, p<0.01). The data were collected from 4 to 6 independent experiments. Fig. 4.1B (right panel) shows that PKCδ was not detected in mitochondria from cells with or without adenosine treatment. We also examined other major PKC isoforms including PKCα, β1, and β2 in the rat cardiac myocytes. We found that none of them was significantly present in mitochondria in response to adenosine stimulation (data not shown). These results suggest that PKCe is present in mitochondria at a relatively low level under control condition, but can be increased by adenosine-induced PKC activation.
Figure 4.1. Effect of adenosine on mitochondrial localization of PKCε in response to adenosine. Isolated mitochondria were prepared from adult rat cardiac myocytes treated without or with adenosine (Ado), Ado + chelerythrine (Che) or Ado + 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Percentage of mitochondria with PKCε-positive or PKCε-positive fluorescence. Results are representative of 4-6 independent experiments. **p<0.01 vs. control, ## P<0.01 vs. adenosine.

Figure 4.2. Effect of adenosine on translocation of PKCε to mitochondria from cytosol. Subcellular fractionation was performed from adult rat cardiac myocytes which were treated without or with adenosine (Ado), or Ado + chelerythrine (Che) or bisindolylmaleimide (Bis). (A) Representative Western blots of three independent experiments with antibody against PKCε or PKCδ. (B) Mitochondria to cytosol ratio as indexes of PKCε or PKCδ translocation to mitochondrial from cytosol. They were calculated by relative densitometry and normalized to control. Results are data from 3 independent experiments. *p<0.05 vs. control, #p<0.05 vs. adenosine.
Figure 4.1

A

Mitochondria  PKCε  Merge

Ctrl

Ade

Ade + Che

B

PKCε-positive Mitochondria (%)  PKCε-positive Mitochondria (%)

0  20  40  60  80  100

Ctrl  Ade  Ade + Che  Ade + DPCPX

0  20  40  60  80  100

Ctrl  Ade

**  ##  ##
Figure 4.2

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</table>

B

PKCε

Mitochondrial/Cytosol (% of Control)

- Ctrl: 100
- Ade: 300 ± 10
- Ade+Che: 150 ± 15

PKCδ

Mitochondrial/Cytosol (% of Control)

- Ctrl: 100
- Ade: 100 ± 10
- Ade+Che: 120 ± 10

* Significant difference from Ctrl
# Significant difference from Ade
Effect of adenosine on translocation of PKC\(\varepsilon\) from the cytosol to mitochondrial fraction.

To further confirm the effect of adenosine on mitochondrial translocation of PKC\(\varepsilon\), immunoblot analysis was carried out for Percoll-purified mitochondrial fraction prepared from adult rat cardiomyocytes pretreated without or with adenosine, adenosine plus chelerythrine, or adenosine plus bisindolylmaleimide (30 nM) for 5-10 minutes. Enrichment of mitochondria was identified by antibody against prohibitin, a molecular marker of mitochondria. Immunoblots with antibody against Na+/K+-ATPase, a plasma membrane protein marker revealed no significant contamination of mitochondrial or cytosolic fraction with plasma membrane. GAPDH and prohibitin also serve as loading control for cytosol and mitochondrial fractions, respectively. Western blot with antibody against PKC\(\varepsilon\) revealed a prominent protein band in mitochondrial fraction whereas antibody against PKC\(\delta\) did not detect a significant protein band in mitochondria (Figure 4.2A). Adenosine treatment induced a significant redistribution of PKC\(\varepsilon\) but not PKC\(\delta\) from the cytosol to mitochondria. The mitochondria to cytosol ratios of PKC\(\varepsilon\) increased about 2.5 folds in adenosine-treated cells when compared to control group (Figure 4.2B, 274.4 \(\pm\) 92.1\% vs. control, \(p<0.05\), \(n=3\)). The PKC inhibitor chelerythrine blocked the effect of adenosine-induced translocation of PKC\(\varepsilon\) to mitochondria (166.7 \(\pm\) 26.5\% vs. 274.4 \(\pm\) 92.1\%, chelerythrine vs. adenosine, \(p<0.05\), \(n=3\)). Similar results were also obtained with highly selective PKC inhibitor
bisindolylmaleimide (115.7 ± 8.1% vs. 202.5 ± 37.4%, bisindolylmaleimide vs. adenosine, p<0.05, n=3). In contrast, no significant amount of PKCδ was redistributed to mitochondria in response to adenosine treatment. These results indicate that adenosine induces translocation of PKCε to mitochondria from cytosol via activation of PKC.

Effect of inhibiting HSP90 activity on adenosine-mediated mitochondrial localization of PKCε

HSP90 has been linked to cardioprotection of ischemic preconditioning and mitochondrial import (J.C. Young 2003). We sought to determine whether HSP90 is involved in adenosine-induced mitochondrial translocation of PKCε. Cardiomyocytes freshly isolated from adult rat hearts were pretreated with or without adenosine or adenosine plus a selective HSP90 inhibitor geldanamycin (1 μM) for 5-10 minutes before mitochondrial fractionation. As shown in Figure 4.3, geldanamycin significantly inhibited adenosine-induced increase in mitochondrial PKCε, as indicated by reduction in PKCε-positive mitochondria. Quantitative data showed that adenosine induced about 3-fold increase in PKCε-positive mitochondria which was significantly eliminated by geldanamycin (38.2 ± 7.4% vs. 72.3 ± 7.6%, geldanamycin vs. adenosine, p<0.01). These data indicate that adenosine increases translocation of PKCε to mitochondria, possibly through enhancing HSP90 activity.
Figure 4.3. Effect of inhibiting HSP90 activity on adenosine-induced mitochondrial localization of PKCε. Isolated mitochondria were prepared from adult rat cardiac myocytes which were treated without or with adenosine (Ade), Ade + chelerythrine (Che), or Ade + geldanamycin (Geld). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Percentage of mitochondria with PKCε-positive fluorescence. Results are data from 4-6 independent experiments. **P< 0.01 vs. control, ## P<0.01 vs. adenosine.

Figure 4.4. Effect of suppressing HSP90 expression with HSP90 siRNA on adenosine-induced increase in mitochondrial PKCε. Mitochondria were prepared from rat heart-derived H9c2 cells without or with the treatment of adenosine (Ade), Ade + chelerythrine (Che), Ade + HSP90-siRNA, or Ade + control siRNA (C-siRNA). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Western blot of total cellular fraction with antibody against HSP90. Cells were treated with HSP90-siRNA or C-siRNA. (C) Percentage of mitochondria with PKCε-positive fluorescence. Results are data from 3 independent experiments. **P< 0.01 vs. control, ## P<0.01 vs. adenosine.
Figure 4.3

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B

![Bar Chart](image4)
Figure 4.4

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**B**

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**C**

Box and whisker plot showing PKCε-positive Mitochondria (%)

- Ctrl
- Ade
- Ade + Che
- Ade + DPCPX
- Ade + HSP90-siRNA
- Ade + C-siRNA

- **Significance:**
  - *p < 0.05
  - **p < 0.01
Effect of suppression of HSP90 with siRNA on adenosine-mediated mitochondrial localization of PKC

In order to confirm the specific role of HSP90 on adenosine-induced mitochondrial translocation of PKCε, we studied the effect of suppressing endogenous HSP90 with siRNA targeting HSP90 (α and β isoforms) in the heart-derived H9c2 cells. While H9c2 cells are myoblast cell line derived from embryonic rat heart, these cells have been used extensively for studies involving cardioprotection, heat shock proteins and protein kinases. We employed H9c2 cells because they display many properties similar to those in adult cardiomyocytes and are easily transfected (G. Ilangoan 2004; J.D. Jiao 2008). As shown in Figure 4.4B, transfection of H9c2 cells with siRNA targeting HSP90 significantly reduced the expression of endogenous HSP90. Similar to that in cardiac myocytes, adenosine pretreatment in H9c2 cells increased the mitochondrial targeting of PKCε (53.6 ± 4.7% vs. 14.5 ± 2.1%, adenosine vs. control, p<0.01), and the effect was largely prevented by pretreatment with chelerythrine (21.6 ± 5.9% vs. 53.6 ± 4.7%, chelerythrine vs. adenosine, p<0.01). Interestingly, suppression of HSP90 with siRNA significantly prevented adenosine-induced translocation of PKCε to mitochondria (23.6 ± 7.2% vs. vs. 53.6 ± 4.7%, HSP90-siRNA vs. adenosine, p<0.01), while the negative scrambled siRNA did not have any effect on adenosine-induced increase in PKCε-positive mitochondria (Figure 4.4A and 4.4C, 47.24 ± 2.40% vs. adenosine, p=NS). The
adenosine-induced increase in mitochondrial PKCε was also inhibited by DPCPX (Figure 4.4C 20.8 ± 4.2% vs. 53.6 ± 4.7%, DPCPX vs. adenosine, p<0.01). Figure 4.4B showed significant suppression of HSP90 expression with siRNA targeting HSP90 (α and β). The data were analyzed from 4 to 6 independent experiments. These results further demonstrated that HSP90 is involved in adenosine-induced translocation of PKCε to mitochondria.

Effect of adenosine on co-immunoprecipitation of PKCε, HSP90 and TOM70.

To determine whether PKCε associates with HSP90, HSP70 or/and TOM70 and whether this association can be up-regulated by adenosine, we performed co-immunoprecipitation in the mitochondrial fraction prepared from cardiomyocytes pretreated with or without adenosine (10 μM). As expected, the total cellular HSP90 or HSP70 was not altered by adenosine treatment (data not shown). When the mitochondrial homogenates were immunoprecipitated with anti-HSP90, anti-PKCε or anti-TOM70 antibody, the immunoblotting with various antibodies revealed that HSP90, PKCε and TOM70 are associated with each other. In contrast, no significant amount of PKCδ or HSP70 was detected in HSP90, PKCε or TOM70 precipitates (Figure 4.5A, upper and lower panels). Pretreatment with adenosine significantly increased PKCε co-precipitated by anti-HSP90 antibody (214.3 ± 56.3% vs. control, p<0.05) or anti-TOM70 antibody (228.3 ± 30.8% vs. control, P<0.05).
Figure 4.5. Co-immunoprecipitation of HSP90, PKCε and TOM70 from adult cardiac myocytes. (A) Immunoprecipitation was performed with anti-HSP90, anti-PKCε or anti-TOM70 antibody from mitochondrial fraction or protein lysate (Input) of cardiac myocytes treated with or without adenosine. (B) Quantification of co-precipitated HSP90, PKCε and TOM70. Results are data from 3 independent experiments. *P< 0.05 vs. control.

Figure 4.6. Effect of HSP90 siRNA on co-precipitation of PKCε and Tom70. (A) Immunoprecipitations of mitochondrial fractions from H9c2 cells treated without or with adenosine (Ade), HSP90-siRNA, or Ade + HSP90-siRNA. (B) Quantification of TOM70 co-precipitated by PKCε. Results are data from 3 independent experiments. *P< 0.05 vs. control, # P<0.05 vs. adenosine.
Figure 4.5

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PKCε

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TOM70

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PKCε

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Figure 4.6

A

IP: PKCε

PKCε

TOM70

B

TOM70 Relative Content (% of Control)

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* Significant difference compared to Ctrl
# Significant difference compared to Ade

Input, Ctrl, Ade, HSP90-siRNA, Ade + HSP90-siRNA, IgG
Similarly, adenosine also increased TOM70 level co-precipitated by anti-HSP90 antibody (195.9 ± 17.8% vs. control, P<0.05) or anti-PKCε antibody (215.8 ± 36.7% vs. control, P<0.05), and increased HSP90 co-precipitated by anti-PKCε antibody (183.3 ± 4.3% vs. control, P<0.05) or anti-TOM70 antibody (187.9 ± 17.6% vs. control, P<0.05). However, HSP70 level co-precipitated by anti-PKCε, anti-HSP90 or anti-TOM70 antibody was not increased significantly by adenosine. Adenosine did not significantly alter the recovery of HSP90, PKCε or TOM70 in the HSP90, PKCε and TOM70 precipitates, respectively. As a control, HSP90, PKCε or TOM70 was not detected in the homogenates treated with control antibody IgG. These results indicate that pretreatment with adenosine for 5-10 minutes did not significantly increase HSP90 expression level but promoted association of HSP90, TOM70 and PKCε.

Effect of suppressing HSP90 expression on association of PKCε and TOM70.

To determine whether adenosine-mediated association of PKCε with TOM70 is dependent on HSP90, we performed co-immunoprecipitation in the mitochondrial fraction prepared from H9c2 cells pretreated with or without adenosine (10 μM). Our data showed that TOM70 was co-precipitated by PKCε under the basal condition and this association was enhanced by adenosine treatment (186.6 ± 16.7% vs. control, p<0.05). However, suppression of HSP90 with siRNA significantly inhibited adenosine-induced association of PKCε with TOM70 (97.6 ± 16.2% vs. 186.6 ± 16.7%, adenosine
+ HSP90 siRNA vs. adenosine, p<0.05). The basal level of association of PKCε and TOM70 was not altered by HSP90 siRNA, indicating the importance of adenosine-induced increase in HSP90 activity for association of PKCε with TOM70. As a control, TOM70 was not detected in the homogenates treated with control antibody IgG. These results indicate that pretreatment with adenosine for 5-10 minutes significantly enhanced association of PKCε with TOM70, the effect was prevented by suppression of HSP90 expression (Figure 4.6).

**DISCUSSION**

Our study demonstrated that adenosine promotes translocation of a novel PKC isoform, PKCε, to mitochondria. We found that adenosine receptor activation induced a rapid association of PKCε with TOM70, which is dependent on HSP90. Specifically, we showed that activation of adenosine receptors induced a selective translocation of PKCε (but not PKCδ) from the cytosol to mitochondria. We also showed that adenosine-mediated PKCε translocation to mitochondria was significantly reduced by inhibiting HSP90 function or suppressing HSP90 expression. Further, our data indicate that HSP90 is critical for association of PKCε and TOM70. We demonstrate for the first time that adenosine receptor activation induces selective translocation of PKCε to mitochondria. This translocation process is associated with the mitochondrial import machinery TOM70 and is dependent on HSP90 function.
It is well known that upon stimulation, PKC isoforms are translocated to their distinct subcellular membrane regions where the activated isoforms are anchored close to their particular substrates (K. Inagaki 2006). Studies have shown that PKCε is present in the mitochondrial inner membrane and interacts with mitochondrial components, such as the pore component of cardiac mitochondrial permeability transition pore (C.P. Baines 2003; G.R. Budas 2007). Phosphorylation by PKCε enhances the activity of cytochrome c oxidase subunit IV (M. Ogbi 2006). Although numerous evidence suggests that adenosine receptors are linked to PKC activation (Y. Liu 1996; K. Hu 1999), no study has directly addressed translocation of PKC isoforms to mitochondria after activation of adenosine receptors. In the present study, experiments were designed to determine whether PKCε is indeed localized to mitochondria under basal condition and whether the mitochondrial localization of PKCε is regulated by adenosine. Specifically, we examined whether adenosine induced translocation of PKCε from cytosol to mitochondria. We found that under basal conditions, the level of PKCε in mitochondria is relatively low but enhanced significantly following adenosine stimulation. In contrast, no significant amount of PKCδ was detected in mitochondria with or without adenosine. These observations provide the novel evidence that adenosine induces selective translocation of PKCε to mitochondria.
Although many studies have reported PKCε localized to mitochondria (C.P. Baines 2003; G.R. Budas 2007), the existence of PKCε in mitochondria is still puzzling, in particular on the mitochondrial inner membrane. It is well known that many protein translocated into mitochondria should possess N-terminal mitochondrial targeting sequence. Translocases in mitochondria recognize the signal sequence and then mediate the import and sorting of proteins. However, no mitochondrial targeting sequence in PKCε and other PKC isoforms has been found. It is therefore not clear how PKCε gets into mitochondria. It is generally believed that mitochondrial import machinery TOM20 recognizes classical N-terminal mitochondrial targeting sequences whereas TOM70 interacts with internal targeting sequences such as those in the multitransmembrane carrier proteins of the inner mitochondrial membrane (A.J. Davis 1998; J. Brix 1999; K.N. Truscott 2003). It is noted that some proteins lack the N-terminal mitochondrial targeting sequence, but are targeted to mitochondrial inner membrane (K. Boengler 2005). The present study shows that adenosine promotes association of PKCε with the mitochondrial import machinery TOM70, indicating the importance of mitochondrial import in PKCε translocation to mitochondria. Furthermore, the translocation process appears to be dependent on HSP90, a protein that has been shown to assist with mitochondrial import (J.C. Young 2003). Our data demonstrate that suppressing HSP90 expression with specific siRNA prevented adenosine-induced association of PKCε and TOM70, indicating that both HSP90 and TOM70 are important for mitochondrial translocation of PKCε. It has been shown that
some components of the mitochondrial import machinery are down-regulated during ischemia but preserved by IPC (K. Boengler 2006), suggesting mitochondrial import could be a novel mechanism by which PKCε confers cardioprotection (J.C. Young 2003). Indeed, a recent study by Budas and the colleagues indicates that HSP90-mediated mitochondrial import of PKCε plays an important role in the protection of the myocardium from ischemia and reperfusion injury (G.R. Budas 2010).

In addition to translocation to mitochondria, PKC has been shown to translocate to the plasma membrane, nucleus and other subcellular organelles upon activation. We have recently discovered that adenosine A1 receptor activation induces PKCε and PKCδ translocation to the caveolar plasma membrane (Z. Yang 2009). Here, we showed that adenosine receptor activation also caused PKCε translocation of mitochondria. Although different receptor subtypes and their signaling may be associated with differential translocation of PKC isoforms, our observations suggest that adenosine receptor signaling can potentially influence various subcellular localizations of different PKC isoforms and may serve distinct roles in a variety of cellular events. There is growing evidence that adenosine contributes significant cardioprotection via adenosine receptor subtypes and their distinct signal transduction pathways, such as PKC isoforms and their down-stream signaling including mitogen-activated protein kinase and PI3 kinase/protein kinase B (M.V. Cohen 2008). While it is well-known that adenosine A1 receptors is associated with PKC activation and
translocation to the plasma membrane activation via PLC/PLD, recent reports indicate that adenosine A1 receptors-induced PKC activation may further facilitate adenosine A2b receptor signaling which then regulates survival kinases PI3Kinase and ERK (S. Philipp 2006; A. Kuno 2007; M.V. Cohen 2008). Because PI3 kinase has also been shown to activate PKC either via direct activation by lipid products or via phosphorylation by PDK1 (H. Tong 2000), it is likely that adenosine receptors may cause PKC activation and targeting to distinct subcellular localizations such as mitochondria via adenosine A2b receptor-dependent signaling pathways. A recent study indicates that A2b receptors are present in or near mitochondria rather than on the sarcolemma, suggesting the possible involvement of mitochondria in adenosine A2b receptor-mediated signaling (K. Grube 2011).

Our finding indicates a novel mechanism involved in the adenosine-mediated PKC signaling that is dependent on mitochondrial import machineries. Adenosine receptor signaling has been associated with cardioprotection against ischemic/reperfusion injury (V.J. McIntosh 2012). Given that most of mitochondrial proteins are imported from cytosol via translocons on the mitochondrial membranes, the dynamic regulation of protein import to mitochondria by adenosine signaling could be a novel mechanism mediating cardioprotection. The number of PKC substrates in mitochondria has been proposed, such as aldehyde dehydrogenase 2, cytochrome c oxidase subunit IV, mitochondrial permeability transition pore, and mitochondrial ATP-sensitive
K+ channels (G.R. Budas 2007). The present study provides a detailed understanding of the molecular mechanism underlying adenosine-mediated mitochondrial targeting of PKC isoforms, which increase our knowledge about adenosine-mediated cardioprotection against ischemic injury, and may lead to identification of potential therapeutic targets.

In summary, we demonstrate that adenosine receptor activation induces translocation of PKCε to mitochondria, and this process is associated with TOM70 and dependent on HSP90. Our observations may have important implication in cardioprotection associated with adenosine receptor signaling.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Protein Kinase C (PKC) is a group of enzymes that control the function of other proteins through the phosphorylation of serine/threonine on these proteins. Numerous studies have shown that PKC isoforms differ in their primary structure, subcellular localization, tissue distribution and biological function. It is localized in cytosol in an inactive conformation and is translocated to different subcellular organelles including plasma membrane, Golgi complex and endoplasmic reticulum (ER) upon activation. In ischemic preconditioning, it is the key mediator since different signaling pathways converge at PKC and it mediates distinct cellular functions by phosphorylating specific downstream target proteins. Therefore, understanding the PKC translocation by adenosine receptor can provide useful insights into the protective mechanism of PKC in ischemic preconditioned heart. Accordingly, the present investigation was performed to determine adenosine receptor mediated PKC translocation to different subcellular locations in the heart.
We demonstrated that activation of adenosine A1 receptors induced a rapid association of PKCε or PKCδ with caveolin-3 in adult rat cardiac myocytes. Specifically, we found that activation of adenosine A1 receptors with CCPA induced the selective translocation of PKCε and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. Importantly, we showed that activated PKCε and PKCδ were targeted to the caveolin-rich plasma membrane microdomains. We have also showed that PKCε and PKCδ colocalize to and associate with caveolin-3. Taken together, we demonstrated that activation of adenosine A1 receptors promotes targeting of novel PKC isoforms, PKCε and PKCδ to caveolin-rich plasma membrane microdomains.

Identification of downstream effectors on plasma membrane will lead to a better understanding of cardiac protection in the adenosine mediated PKC signaling in the heart. Using the patch clamp technique, we provide the first evidence that Kir2.1 channels were negatively regulated by caveolin-3, both in HEK293T cells and in rat cardiac myocytes. Specifically, we show that Kir2.1 channel activity was significantly inhibited by expression of caveolin-3 while its expression on cell plasma membrane was not changed. Further, we demonstrate that caveolin-3 SDP blocked the caveolin-3 mediated suppression of Kir2.1 current. Moreover, our data indicate that caveolin-3 is critical in PKC induced IK1 inhibition in the heart.
There is ample evidence that PKCε resides in cardiac mitochondria. However, the signals that promote translocation of PKCε are largely unknown. We found that adenosine receptor activation induced a rapid association of PKCε with TOM70, which is dependent on HSP90. Specifically, we showed that activation of adenosine receptors induced a selective translocation of PKCε (but not PKCδ) from the cytosol to mitochondria. We also showed that adenosine-mediated PKCε translocation to mitochondria was significantly reduced by inhibiting HSP90 function or suppressing HSP90 expression. Further, our data indicate that HSP90 is critical for association of PKCε and TOM70. We demonstrate for the first time that adenosine receptor activation induces selective translocation of PKCε to mitochondria. This translocation process is associated with the mitochondrial import machinery TOM70 and is dependent on HSP90 function.

Taken together, our data demonstrate the molecular mechanism underlying adenosine-mediated targeting of PKC isoforms to different subcellular locations, which consequently regulates the downstream signaling such as Kir2.1 channel on the plasma membrane. Thus, the work presented in this thesis is not only important for a better understanding of ischemic preconditioning, but also for designing a strategy of clinical application of preconditioning or postconditioning biology.
However, it leaves several questions that need to be answered to better understand the adenosine-mediated cardiac protection through PKC signaling. Adenosine receptors are now classified into at least four different subtypes. All these subtypes are expressed in cardiac myocytes, although controversy exists regarding the presence and function of adenosine A2a receptor in the heart. Our data show the involvement of adenosine A1 receptor in the adenosine-mediated PKC signaling in the cardiac myocytes. However, the functions of other adenosine receptors should not be ruled out. Numerous studies have already shown that adenosine A3 might be involved in cardiac protection in ischemic preconditioned heart, while recently studies have shown that adenosine A2b receptor plays an important role in the PKC signaling in ischemic preconditioning. It is known that the adenosine A1 and A3 receptor subtypes inhibit the adenylate cyclase and decrease the cAMP through the Gi/o proteins. Opposing to A1 and A3 subtypes, the A2 subtypes can activate the adenylate cyclase and increase the cAMP via the stimulatory protein Gs. Although studies have shown that all these four adenosine receptors are important in cardiac protection in ischemic preconditioning, it is still a mystery as to how the opposite effect (inhibition or stimulation) on the same target (adenylate cyclase and cAMP) induces the same cardiac protective effect. The involvement of other signaling pathways by adenosine receptors, such as Src and MAPK pathways might be the explanation. Moreover, more work is needed to determine the extent to which the effects reported for adenosine receptor depend on animal species and specific experimental conditions.
Identification of the downstream pathways involved in adenosine-mediated PKC activation can lead to a better understanding of adenosine-induced cardiac protection in ischemic preconditioning. Although many studies have reported PKCε to be localized in mitochondria, the existence of PKCε in mitochondria is still puzzling, in particular its presence on the mitochondrial inner membrane. Our previous studies showed the involvement of mito-KATP in the protection of hypoxic preconditioning and the regulation of KATP by PKCε in mitochondrial inner membrane. The present study shows that the PKCε level in mitochondria is low under the basal condition, while adenosine treatment significantly increases the translocation of PKCε from cytosol to mitochondria. Based on these findings, it is quite possible that PKCε regulates the mitochondrial protein directly in mitochondria by protein phosphorylation. Indeed, the increase in mitochondrial protein phosphorylation was found in ischemic preconditioning. Additionally, numerous studies recognize the mitochondrial permeability transition pore (MPTP) as the final target in ischemic preconditioning and PKC inhibits the formation of MPTP in cardiac protection. Thus, detailed investigations into the effect of PKC on mitochondria and the underlying mechanism will be useful in understanding the cardiac protective role of adenosine in the ischemic preconditioned heart.

It is clear that PKC translocates to different subcellular locations upon activation. However, the link between different translocations is still under investigation. Our preliminary data show that the knock-down of caveolin-3
blocks the PKC translocation to mitochondria, indicating the possible link
between PKC cell membrane targeting and mitochondrial targeting. It is highly
possible that PKC targeting into mitochondria is caveolin-dependent. One
possible explanation is that PKC translocation into mitochondria is delivered by
caveosome. Caveosome is the caveolin-containing endosome, and it has been
categorized as intermediates in the trafficking of virus and certain proteins
from cell membrane to endoplasmic reticulum and Golgi apparatus. Some
studies also showed that PKC is abundant in caveosome, further indicating the
role of caveosome in PKC trafficking.

Although our data show that PKC regulates the Kir2.1 channel through
caveolin-3, and caveolin-3 scaffolding domain is involved in this regulation, the
detailed mechanism of this regulation is still unclear. Since Kir2.1 channel
activation is regulated by its phosphorylation level, we cannot rule out that
caveolin-3 regulates the Kir2.1 by phosphorylation. Studies have already
shown that the phosphorylation of caveolin-1 can modulate the activation of
caveolin-dependent signaling pathways. Therefore, it is useful to study the
phosphorylation level between caveolin-3 and Kir2.1. In short, detailed
investigation into the effects of phosphorylation of caveolin-3 will be valuable in
understanding its role in Kir2.1 regulation.


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