ION CHANNELS, PROTEIN KINASE C AND CAVEOLAE IN CARDIOPROTECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Chen Kang, M.S.
College of Pharmacy

The Ohio State University

2015

Dissertation Committee:
Professor Keli Hu, Advisor
Professor Lane J. Wallace
Professor Nam Lee
ABSTRACT

Ischemic heart disease is one of the leading causes for death in the whole world. It has been the top one killer. In general, ischemic heart disease results from the blockage of the arteries so the blockage in the arteries reduces the supply of blood to the heart muscle causing severe consequences such as heart attack. Treatment for ischemic heart disease involves improving blood flow to the heart muscle. Treatment may include medications, a procedure to open blocked arteries or bypass surgery. Until now, scientists are still looking for more effective ways to reduce the damage to the heart from ischemic heart disease. Cardiac cells preserve a variety of mechanisms in order to protect them from myocardial ischemia. In 1986, Murry and colleagues found out that repeated short episodes of sub-lethal ischemia protected the myocardium against a subsequent lethal ischemic insult which is defined as ischemic preconditioning (IPC). IPC is a powerful form of endogenous protection against myocardial infarction, which has been demonstrated in several animal species and, recently, in isolated human cardiomyocytes. In addition to IPC and its powerful role in cardioprotection, there are numerous other signalings and effectors contributing to the cardioprotection against ischemia. One of them is TASK1 channel, which stabilizes resting
membrane potential especially during cardiac ischemia/hypoxia because disruption of ion homestasis eventually causes cell death.

Activation of protein kinase C (PKC) via adenosine receptors is known to be involved in the cardioprotection of ischemic preconditioning. Specifically, activated PKCε translocates to mitochondria is thought to be critical of cardioprotection. However, it is still elusive how PKCε translocates to mitochondria. The present study was designed to determine how activation of adenosine receptor induces translocation of PKCε to mitochondria and whether this translocation is age-dependent (Chapter 2). In addition, the two-pore domain potassium channel TASK1 is strongly expressed in the heart and has been shown to regulate the resting membrane potential and action potential. Modulation of the TASK1 background currents provides a mechanism for control of cellular excitability. However, little is known about the localization and regulation of TASK1 in the heart especially whether TASK1 is modulated by caveolar microdomain -- one of the most abundant microstructure in cardiac cells. So the following study was designed to determine whether TASK1 is modulated by caveolin-3 (Cav-3) via association with Cav-3 (Chapter 3). We were further interest in whether TASK1 channels play protective role in myocardial hypoxia. We were trying to investigate the molecular mechanisms by which hypoxia regulates TASK1 channels (Chapter 4).
In conclusion, we demonstrate that adenosine-induced translocation of PKCε to mitochondria is mediated by caveolin-3-dependent PKC signaling and this process is age-related, possibly through regulation of HSP90 and TOM70 expression. Moreover, our study indicates that TASK1 is functionally regulated by Cav-3 probably mainly through association with Cav-3. In the following study, we found out that TASK1-like channels plays a protective role in cardiac hypoxia. TASK1-like channels were blocked by hypoxia mimicked by metabolic inhibition and this inhibition was mainly through the activation of PKC.

These results not only point out a novel mechanism in regulating PKC function in mitochondria which is involved in ischemic preconditioning against ischemia but also illustrate regulation of cardiac TASK1 channels and protective role in cardiac hypoxia. Above all, our data are supportive for possible roles of mitochondrial translocation of PKCε and TASK1 channels in the cardioprotection against hypoxia. **Thus, the work presented in this thesis is not only important for a better understanding of signaling related to preconditioning and modulation of TASK1 in cardiomyocytes, but also provides with potential effectors in cardioprotection against ischemia in the future studies.**
ACKNOWLEDGEMENTS

I feel great pleasure to acknowledge the contribution of those people who helped me in journey through the graduate school at The Ohio State University.

At the outset, I would like to extend my deepest thanks to Dr. Keli Hu who not only served as my supervisor but also challenged and encouraged me throughout my academic program. Her amicable and helpful nature made it very easy to work my way through the graduate school. I appreciate the time effort and guidance from my committee members: Dr. Lane J Wallace and Dr. Nam Lee. I also thank all the members of the Division of Pharmacology for their assistance and advice.

I thank Dr. Zhaogang Yang for teaching me several techniques during the early years of my graduate school and also for his useful inputs throughout my stay here. I appreciate Yang’s help with my experiments, which was instrumental in my research. I am also grateful to all my fellow graduate students in the college of Pharmacy for their support.
I would also like to thank my parents who have always wished the best for me and have supported me through all the odds. Words alone cannot express what I owe them for their unconditional love and encouragement, and their patient love is always a source of motivation for me.
VITA

From Anyue County, Sichuan Province, China

2004-2008 .........................................................B.S. Traditional Chinese Pharmacy
China Pharmaceutical University, China

2010-2012 ................................................................. M.S. Pharmacology
The Ohio State University, Ohio

2012-present ............................................................. Graduate Teaching Associate
The Ohio State University, Ohio
FIELDS OF STUDY

Major Field: Pharmacy
# TABLE OF CONTENTS

**PAGE**

Abstract .................................................................................................................. ii

Acknowledgments.................................................................................................. vii

Vita......................................................................................................................... ix

Fields of study........................................................................................................ x

List of Figures.......................................................................................................... xvii

Abbreviations.......................................................................................................... xx

Chapters

Chapter 1. Introduction .......................................................................................... 1

1.1. Caveolae as central signaling center and its effectors ............................... 1

1.1.1. Features and properties of caveolae ................................................... 1

1.1.2. Caveolae and signaling molecules ....................................................... 4

1.1.2.1. Ion channels ................................................................................. 5

1.1.2.2. Protein Kinases C ....................................................................... 7

1.1.3. Caveolae and caveolins in cardioprotection

ix
against ischemia.................................................................9

1.2. Cardiac ion channels.........................................................12
  1.2.1 Cardiac action potential and major ionic currents.............13
  1.2.2. Leak K\textsuperscript+ currents and two pore domain potassium channels....16
    1.2.2.1 Structural basis of two pore domain potassium channels.........................16
    1.2.2.2. Biophysical properties of two pore domain potassium channels.................17
    1.2.2.3. TWIK-related acid-sensitive K\textsuperscript+ channel (TASK)..............18
    1.2.2.4. Regulations of TASK channels.................................................20
    1.2.2.5. Physiological functions mediated by TASK channels.............................21

1.3. Role of ion channels in cardiac ischemia and cardiac protection........23
  1.3.1. Introduction of cardiac ischemia...........................................23
  1.3.2. Ischemic preconditioning against cardiac ischemia ..............24
  1.3.3. Role of potassium channels in cardioprotection against cardiac ischemia..........................................................26
    1.3.3.1. Inward rectifying K\textsuperscript+ channels in cardiac protection................26
    1.3.3.2. TASK channels in cardiac protection........................................28

1.4. Objectives ..............................................................................28
Chapter 2. Age-dependent alteration in targeting protein kinase C to mitochondria in cardiomyocytes.................................31

2.1. Abstract.........................................................................................................................31

2.2. Introduction ....................................................................................................................33

2.3. Materials and Methods ...............................................................................................35

2.3.1. Materials ....................................................................................................................35

2.3.2. Isolation of cardiomyocytes .....................................................................................36

2.3.3. Preparation of mitochondrial fraction or intact mitochondria ....37

2.3.4. Cell culture and transfection..........................................................38

2.3.5. Western blotting ......................................................................................................38

2.3.6. Immunofluorescence microscopy ............................................................................39

2.3.7. Data analysis ............................................................................................................39

2.4. Results ..........................................................................................................................40

2.4.1. Role of adenosine A2B receptors and PI3K signaling pathway in the translocation of PKCε to mitochondria........40

2.4.2. Effect of suppressing caveolin-3 on adenosine-mediated mitochondrial translocation of PKCε............................44

2.4.3. Effect of adenosine on caveolin-3 expression in cardiomyocytes ................................48

2.4.4. Adenosine induced PKCε translocation to mitochondria in the heart from the aged rats........................................52

2.4.5. Expression of HSP90 and TOM70 in aged rats ........................................53
2.5. Discussion........................................................................................................59

Chapter 3. Distribution and functional modulation of TASK1..................................64

3.1. Abstract.............................................................................................................64
3.2. Introduction ......................................................................................................65
3.3. Materials and Methods.....................................................................................67
  3.3.1. Materials .......................................................................................................67
  3.3.2. Isolation of cardiomyocytes .........................................................................68
  3.3.3. Cell culture and transfection .......................................................................69
  3.3.4. Western blotting .........................................................................................69
  3.3.5. Co-immunoprecipitation .............................................................................70
  3.3.6. Immunofluorescence microscopy .................................................................71
  3.3.7. Electrophysiology studies ............................................................................71
  3.3.8. Data analysis ................................................................................................72
3.4. Results................................................................................................................72
  3.4.1. TASK1 currents are pH-sensitive and methanandamide-sensitive ...............72
  3.4.2. TASK1 currents are negatively regulated by caveolin-3 in HEK293T cells .....76
  3.4.3. Expression of TASK1 channels in HEK293T cells and its association with caveolin-3 ..........................................................77
3.4.5. TASK1 currents are not affected by caveolin-3 mutant in HEK293T cells……………………………………………………………82

3.4.6. TASK1 is affected by caveolin-3 possibly mainly through functional modulation in HEK293T cells.................................86

3.4.7. Expression of TASK1 channels in adult rat cardiomyocytes and its association with caveolin-3.................................88

3.5. Discussion…………………………………………………………………………………………91

Chapter 4. TASK1 is regulated by hypoxia-induced protein kinase C signaling…………………………………………………………97

4.1. Abstract……………………………………………………………………………………..97

4.2. Introduction……………………………………………………………………………99

4.3. Materials and Methods…………………………………………………………………101

4.3.1. Materials………………………………………………………………………………….101

4.3.2. Isolation of cardiomyocytes………………………………………………………….101

4.3.3. Cell culture and transfection………………………………………………………102

4.3.4. Induction of hypoxia………………………………………………………………….103

4.3.5. Cell viability assay……………………………………………………………………103

4.3.6. Electrophysiological studies……………………………………………………….104

4.3.7. Data analysis…………………………………………………………………………105

4.4. Results…………………………………………………………………………………106
4.4.1. pH-sensitive currents are down-regulated by extracellular acidification and up-regulated by alkalinization in cardiomyocytes .........................................................106

4.4.2. Effect of TASK1 channel blocker methanandamide on steady-state outward currents in cardiomyocytes ........109

4.4.3. TASK1-like currents are down-regulated by metabolic inhibitor sodium cyanide in cardiomyocytes .............110

4.4.4. TASK1-like currents were down-regulated by metabolic inhibition mainly through PKC in cardiomyocytes ........114

4.4.5. Protective role of TASK1 in prolonged hypoxia in cardiomyocytes or HEK293T cells ........................................116

4.5. Discussion ........................................................................................................................................120

Chapter 5. Conclusions and future directions .................................................................125

Bibliography ......................................................................................................................................129
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Schematic representation of caveolae and caveolins</td>
</tr>
<tr>
<td>1.2.</td>
<td>Schematic depicting of caveolae, resident structural proteins, caveolin, and certain bindin partners</td>
</tr>
<tr>
<td>1.3.</td>
<td>Domain composition of PKC family members</td>
</tr>
<tr>
<td>1.4.</td>
<td>Simplified adenosine signaling pathways of myocardial preconditioning</td>
</tr>
<tr>
<td>1.5.</td>
<td>Cardiac action potential and the schematic representation of the major ionic currents contributing to its waveform</td>
</tr>
<tr>
<td>1.6.</td>
<td>Schematic transmembrane topology of human two-pore domain potassium channels</td>
</tr>
<tr>
<td>1.7.</td>
<td>Representative current traces of voltage-gated and background potassium channels</td>
</tr>
<tr>
<td>1.8.</td>
<td>Regulation of TASK-1 and TASK-3</td>
</tr>
<tr>
<td>2.1.</td>
<td>Effect of adenosine on mitochondrial localization of PKCε from young adult rat cardiomyocytes</td>
</tr>
<tr>
<td>2.2.</td>
<td>Effect of suppressing caveolin-3 expression with caveolin-3 siRNA on adenosine-induced increase in mitochondrial PKCε from H9c2 cells</td>
</tr>
</tbody>
</table>
2.3. Effect of adenosine on mitochondrial localization of PKCε from H9c2 cells.................................................................50

2.4. Effect of adenosine on the expression of caveolin-3 in young adult rat cardiomyocytes..................................................51

2.5. Effects of adenosine on mitochondrial localization of PKCε from young adult and middle-aged rat cardiomyocytes..............................56

2.6 Expression of HSP90 and TOM70 from young adult or middle-aged rat cardiomyocytes..........................................................58

3.1. Current components sensitive to various pH and methanandamide......75

3.2. Current components decreased by caveolin-3
   (Cav-3) overexpression..........................................................................79

3.3. TASK1 is associated with caveolin-3 (Cav-3) in transfected HEK293T cells..............................................................................80

3.4. Co-immunoprecipitation of TASK1 and caveolin-3 (Cav-3) from transfected HEK293T cells.........................................................81

3.5. Current components unaffected by caveolin-3 mutant (Cav-3 P104L)
   overexpression........................................................................................85

3.6. The effect of caveolin-3 mutant (Cav-3 P104L) on wild-type caveolin-3 (Cav-3) expression level at cell peripheral areas..............87

3.7. TASK1 is associated with caveolin-3 (Cav-3) in adult rat cardiomyocytes.................................................................................89

3.8. Co-immunoprecipitation of TASK1 and caveolin-3 (Cav-3)
from freshly isolated adult rat cardiomyocytes

4.1. Current components sensitive to various pH in cardiomyocytes

4.2. Current components sensitive to methanandamide and sodium cyanide were mainly TASK1-like currents in cardiomyocytes

4.3. Current components sensitive to sodium cyanide and its loss-of-sensitivity after treated with BIM in cardiomyocytes

4.4. Cell viability assays for cardiomyocytes or HEK293T cells under different treatment

4.5. TASK1 expression with or without hypoxia in adult rat cardiomyocytes
ABBREVIATIONS

AC: Adenylate cyclase
AF: Atrial fibrillation
APD: Action potential duration
ATP: Adenosine-5'-triphosphate
Ade: Adenosine
BIM: Bisindolylmaleimide
BK: Big potassium
BSA: Bovine Serum Albumin
CAV: Caveolin
CSD: Caveolin scaffolding domain
DAG: Diacylglycerol
DAPC: Dystrophin-associated protein complex
DPCPX: 8-Cyclopentyl-1, 3-dipropylxanthine
ECG: Electrocardiography
ER: Endoplasmic reticulum
eNOS: Endothelial NO synthase
GABA: Gamma-aminobutyric acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>HSP:</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IP3:</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IPC:</td>
<td>Ischemic preconditioning</td>
</tr>
<tr>
<td>KCO:</td>
<td>Potassium channel opener</td>
</tr>
<tr>
<td>LQTS:</td>
<td>Long QT syndrome</td>
</tr>
<tr>
<td>M-β-CD:</td>
<td>Methyl-beta-cyclodextrin</td>
</tr>
<tr>
<td>MPTP:</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NADPH:</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBD:</td>
<td>Nucleotid binding domain</td>
</tr>
<tr>
<td>NO:</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS:</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PDK:</td>
<td>Phospholipid-dependent kinases</td>
</tr>
<tr>
<td>PDH:</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PI3K:</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2:</td>
<td>Phosphatidylinositol 4, 5-bisphosphate</td>
</tr>
<tr>
<td>PKA:</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC:</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC:</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA:</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>ROCs:</td>
<td>Receptor-Operated Channels</td>
</tr>
<tr>
<td>ROS:</td>
<td>Reactive oxygen species</td>
</tr>
</tbody>
</table>
SID: Self-interacting domain
SDP: Scaffolding Domain Peptide
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SK: Small conductance calcium-activated potassium
SMOCs: Second-Messenger-Operated Channels
TOM: Translocase of outer mitochondrial membrane
TALK: TWIK-related alkaline pH-activated potassium channel
TASK: TWIK-related acid sensitive potassium channel
TEA: Tetraethylammonium
THIK: Tandem pore domain halothane-inhibited potassium channel
TM: Transmembrane
tMD: Transmembrane domain
TREK: TWIK-related potassium channel
TRESK: TWIK-related spinal cored potassium channel
TWIK: Tandem of pore domain in a weak inward rectifying potassium channel
VOCs: Voltage-Operated Channels
4-AP: 4-aminopyridine
CHAPTER 1
INTRODUCTION

1.1 Caveolae as central signaling center and its effectors

1.1.1 Features and properties of caveolae

Small invaginated membrane structures called "caveolae" are the best characterized lipid rafts (Figure 1.1). They were first discovered in the early 1950s by using electron microscopy [1, 2], these invaginated membrane structures are enriched in cholesterol. Caveolae are found in the plasma membrane in almost all of the cell types [3] especially in muscle cells. There is an exception because neurons lack caveolae microstructure [4, 5]. Different cell types possess different amount of caveolae in their plasma membrane [6, 7]. A major defining and essential feature of caveolae is the presence of its marker protein -- caveolin proteins [8], which are one of the most important scaffolding proteins residing in the plasma membrane which interact with cholesterol-rich lipid raft domains. Lipid rafts are defined as sterol- and sphingolipid-enriched domains which compartmentalize cellular processes [9]. Cholesterol is another
major component of caveolae [10] which has been an its indispensable role in the formation of complete caveolae microstructure [11].

![Schematic representation of caveolae and caveolins][1]

**Figure 1.1** Schematic representation of caveolae and caveolins [9].

In addition, depletion of cholesterol from caveolae using cyclodextrin chemicals makes caveolae loses its invagination along the plasma membrane and become flat shaped which leads to the reduction or elimination of caveolae from the cell plasma membrane [11]. Therefore, it is reported that a critical amount of cholesterol is essential for the formation and maintenance of caveolae microstructure [12]. Taken together, the oligomerization of the caveolins during the trafficking process through ER/Golgi apparatus to plasma membrane [13]
and interaction with cholesterol help generate the invaginated shape of caveolae.

In mammalian species, three caveolin-encoding genes (CAV1, CAV2, and CAV3) are thought to be responsible for the caveolin protein expression [9]. These three genes express six subtypes of caveolin proteins: caveolin-1α and -1β, caveolin-2α, -2β, -2γ, and caveolin-3 [9, 14]. Caveolins (18–25 kDa proteins) possess a unique hairpin structure with cytoplasmic N- and C-termini [8]. Both of which have been demonstrated to interact with various proteins. Caveolin-1 (Cav-1) is expressed in most cell types including adipocytes, endothelial, epithelial, fibroblast and smooth muscle cells [11, 15] while caveolin-3 (Cav-3) is specifically expressed in cardiac muscle as well as in skeletal and smooth muscle [16]. Cavs-1 and -3 are expressed independently of each other and are required for the formation of caveolae. On the contrary, caveolin-2 (Cav-2) associates with Cav-1 or -3 but is not involved in caveolae formation [17-19].

1.1.2 Caveolae and signaling molecules
Caveolins possess a unique domain (the caveolin scaffolding domain, CSD) to which signaling molecules bind in an inactive state [8]. When signaling pathways activate effector proteins which bind to the CSD, it can lead to conformational changes of the binding proteins in order to release and activate these effector to continue with the downstream signaling [20, 21] (Figure 1.2).
However, not all the signaling molecules bind to CSD, and it has been reported that the N-terminal of caveolin proteins can also serve as scaffolding domain interacting with different kinds of effectors [22].

**Figure 1.2** Schematic depicting of caveolae, resident structural proteins, caveolin (with its topology in the plasma membrane), and certain binding partners that interact with the caveolin scaffolding domain (CSD, green) [23].

1.1.2.1 Ion channels
The subcellular localization of ion channels to caveolae allows the interaction of these channels with caveolin CSD which are further involved in specific macromolecular signaling complexes in a relative compartmentalized lipid microenvironment [24-26]. Moreover, the macromolecular signaling complexes in which ion channels are involved have high probabilities in participating in critical signaling transduction pathways. Thus, it is very important to investigate the effect of caveolin proteins on various types of ion channels’ physiological properties. Several ion channels and exchangers have been localized to caveolae, and other channels have been associated with noncaveolar lipid rafts in different cells types, including cardiomyocytes [27-30]. In particular, Ca\(^{2+}\), K\(^+\), Na\(^+\), and Cl\(^-\) channels are targeted to caveolae and associate with caveolins [24-26, 31]. In several cell types, transporters and exchanger such as Ca\(^{2+}\)-ATPase, inositol 1, 4, 5-trisphosphate receptors (IP3R), Ca\(^{2+}\) pumps, L-type Ca\(^{2+}\) channels, large-conductance Ca\(^{2+}\)-activated K\(^+\) channels, calmodulin, and transient receptor potential (TRP) channels, localize in cholesterol-rich membrane domains [28, 29, 32, 33]. The localization of these effectors to caveolin/caveolae provides the membrane rafts and/or caveolae having an ability in modulation of Ca\(^{2+}\) current flow through the plasma membrane of cells which further control the excitation-contraction or the excitation-secretion processes [28, 31, 32].
In addition to Ca\textsuperscript{2+} channels, the Na\textsuperscript{+} pump, Na\textsuperscript{+}/K\textsuperscript{+}-ATPase all reside in caveolae microstructure in a number of cells, including smooth muscle and cardiac cells, thereby helping to maintain the Na\textsuperscript{+} gradient which is especially critical to the excitable cells [34-36]. Moreover, K\textsuperscript{+} channels are key regulators of the resting membrane potential, which governs excitability which affects pacemaking function, and of AP repolarization, which determines AP duration and susceptibility to a variety of arrhythmia mechanisms [37-39]. Under pathophysiological conditions, dysregulation of K\textsuperscript{+} channels leads to the dysregulation of cell resting membrane potential causing severe damage to cell viability [40]. Thus, given the fact that different kinds of K\textsuperscript{+} channels target to caveolae/caveolin in excitable cells such as cardiac cells, roles of caveolins on these channels are becoming more and more important fields for research. For example, targeting of voltage-gated K\textsuperscript{+} channels to caveolae appears to be important for cellular excitability. In fibroblasts the Kv1.5 subunit targets to caveolae and colocalizes with caveolin-1 [38], Kv2.1 localizes in membrane rafts including caveolae. Specifically, it has been shown the existence of a relevant association and pathophysiologic interaction between Kir2.1 and caveolin-3 and its mutation types. Mutated caveolin-3 inhibits the activity of Kir2.1 significantly compared with wild-type caveolin-3 [41]. Given the importance of Kir2.1 in maintaining normal cardiac cell resting membrane potential and other cardiac physiological functions, loss of Kir2.1 function in the presence of caveolin-3 mutations results in LQTS [42-45]. In smooth muscle
cells, a subunit of ATP-sensitive potassium channels (K\textsubscript{ATP}), which are important for the modulation of vascular tone, forms a complex with adenylyl cyclase (AC) in caveolae which allows for sustained activation by protein kinase A [39]. Although some channels directly interact with caveolins through their caveolin-binding motifs, there are still some ion channels which lack of caveolin-binding motifs can interact with caveolae/caveolin proteins possibly via other chaperone or scaffolding proteins. This expands the scope of caveolin-associated ion channels. For example, Kv1.5, which lacks of caveolin-binding motif, can associate with caveolae mediated by other proteins [37]. Above all, the findings related to regulation of ion channels by caveolae/caveolin suggest that alterations or mutations in caveolae/caveolin may shift the localization of channels and change their normal physiological activities including cellular excitability. In this dissertation, we’ve identified whether TASK1, one of the members of a newly discovered potassium channel family -- two pore domain potassium channel family [46], is modulated by caveolin-3 via association.

1.1.2.2 Protein Kinases C

There are 10 members of PKC found in mammalian species [47, 48]. They are grouped into three classes based on their domain composition (Figure 1.3) [49]. They first contains the four conventional isozymes: PKC\textalpha [50], the alternatively spliced PKC\textbeta I and PKC\textbeta II (which differ in the last 43 amino acids) and PKC\textgamma. Besides, there are four novel isozymes, PKC\textdelta, PKC\textepsilon,
PKC\(\eta\), and PKC\(\theta\). The last class are the atypical isozymes PKC\(\zeta\) and PKC\(\iota\) (human). The corresponding isozyme of PKC\(i\) in murine is PKC\(\lambda\). All family members share the same architecture: a carboxyl-terminal kinase domain linked by a flexible hinge segment to an amino-terminal region.

![Domain composition of PKC family members](image)

*Figure 1.3* Domain composition of PKC family members [51].

containing regulatory modules (Figure 1.3) [52, 53]. The conventional isozymes contain tandem C1 domains that bind diacylglycerol and a C2 domain that binds anionic lipids in a Ca\(^{2+}\)-dependent manner. Similarly, the novel isozymes also contain tandem C1 domains that bind diacylglycerol but contain a variant of the C2 domain (novel C2) that lacks key residues that bind to Ca\(^{2+}\) and, therefore, the novel isozymes are not sensitive to Ca\(^{2+}\). In contrast, atypical isozymes contain a variant of the C1 domain (atypical C1) that binds neither diacylglycerol nor phorbol esters [54, 55]. Nor are they regulated by Ca\(^{2+}\). Instead, protein-protein interactions provide the major driving force for controlling the function of these isozymes in cells. At the N-
terminal, they contain a PB1 domain (Figure 1.3) involved in protein interactions [56] as well as a carboxyl-terminal PDZ ligand. All isozymes have a conserved carboxyl-terminal tail (CT) that serves as a phosphorylation-dependent docking site for key regulatory molecules (Figure 1.3B). In addition, all isozymes have an autoinhibitory pseudosubstrate sequence (Figure 1.3, green rectangle) that maintains PKC in an inactive state by occupying the substrate-binding cavity.

The PKC isozymes can translocate to subcellular compartments including plasma membrane, mitochondria and nucleus in response to stimuli [57, 58] and some isoforms of PKC can target to caveolae to enhance the regulation of caveolar-localized proteins (they are also PKC downstream effectors) [59]. This facilitates signaling pathways related to PKC isoforms because it is occurred in a coordinated and efficient environment where caveolae/caveolin proteins serve as a central organizer of different signaling molecules. However, more research need to be done in order to know the specific downstream effects of PKC isoforms once they are activated and translocated to caveolae/caveolin proteins.

1.1.3 Caveolae and caveolins in cardioprotection against ischemia
Myocardial ischemia has been a major cause of morbidity and mortality worldwide. One of the interventions for protection from such injury is called ischemic preconditioning which is an experimental technique for producing protection to the loss of blood supply and oxygen in many types of tissues. In the heart, ischemic preconditioning can be achieved by repeated short episodes of ischemia in order to protect heart against a subsequent long-term ischemic damage to the heart. Although preconditioning was discovered more
than two decades ago by Murry and his colleagues [61], it has not yet been translated into a useful therapeutic approach partly because of its complicated underlying mechanisms which we have not obtained the complete map of it till now. Ischemic preconditioning involves the activation of multiple signaling pathways [62] in which spatial and temporal relationships still need to be well defined. Recent studies suggest the possibility of the involvement of caveolins and caveolae in protecting the heart from ischemia/reperfusion injury [63]. Caveolins, the principally structural components of caveolae, can function as chaperones and provide direct temporal and spatial regulations with numerous cardio-protective signaling molecules via their scaffolding domain [23]. As shown in Figure 1.4, opioids [64] and adenosine receptors [65], which can promote cardiac protection through their multi-signaling pathways, localize to caveolae and coimmunoprecipitate with caveolin-3 in the heart [66, 67]. In addition to this, downstream signaling molecules including endothelial nitric oxide synthase (eNOS), Src, PKC, G-protein α, PI3K, and ERK1/2 Akt, eNOS and GC etc., by which ischemic preconditioning produces are all localized to caveolae/caveolin-3 in the heart [23, 62]. Disruption of caveolae using MβCD eliminates the ability of ischemia and pharmacological preconditioning to protect cardiac cells from injury [67], implying that caveolae is an essential component for cardiac protection against ischemic injury. Evidence that caveolins are involved in protecting the heart includes the finding that the
disruption of caveolae attenuates protection of adult cardiac myocytes from ischemic damage and caveolin-3 knockout mice (Cav-3 KO) which leads to the lack of cardiac caveolar microstructure, are resistant to pharmacological preconditioning [63]. Thus identifying cell-specific expression of caveolins and formation of caveolae is a novel approach to achieve cardiac protection. In other research, it has been found out that lack of caveolae/caveolin-3 inhibited isoflurane-induced cardioprotection [57]. Interestingly ischemic preconditioning itself increases the number of plasma membrane caveolae in cardiac cells which might provide stronger protective effects against ischemic damage [63]. Also, ischemic preconditioning may modulate the microenvironment of caveolae and caveolin-associated protein interactions in order to facilitate the signaling that promote cardiac protection [68]. Such findings imply that cardiac cells’ caveolae/caveolin-3 are indispensable for the cardiac protection against ischemic injury. Future studies will still focus on identifying the proteins and signaling networks residing in caveolae that may mediate and modulate cardio-protection.

1.2 Cardiac ion channels
Cardiac K⁺ channels are a group of important ion channels in the heart not only because they determine the resting membrane potential, the heart rate, but also they determine and have a significant impact on the shape and duration of the action potential. Dysregulation of K⁺ channels will cause severe
outcomes including several types of cardiac arrhythmias such as LQTS [42, 43]. They are also important targets for the actions of neuro-transmitters, hormones, drugs and toxins known to modulate cardiac function since closure of them will make it easier to depolarize the membrane potential to the action potential threshold and thus induce action potential in order to release neuro-transmitters or hormones [1–3].

1.2.1 Cardiac action potential and major ionic currents

Cardiac K⁺ currents can be specified on the basis of differences in their functional and pharmacological properties. In mammalian cardiac cells, K⁺ channels can be generally identified and categorized as voltage-gated (Kᵥ) and ligand-gated channels [69-72]. The Kᵥ channel family is a big potassium channel superfamily which includes several potassium channel subfamilies. Simply to say, this big superfamily includes the rapidly activating and inactivating transient outward current (I_{to1}), the ultrarapid (I_{Kur}), rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed rectifier and the inward rectifier (I_{K1}). Besides these potassium channels, the ligand-gated channels are also from this big superfamily [73]. It includes those activated by a decrease in the intracellular concentration of adenosine triphosphate (K_{ATP}) which means it is inhibited at most of the time when the intracellular ATP concentration is maintained at normal value. It also includes those activated by acetylcholine (K_{Ach}) [73]. In addition to these, a newly discovered potassium channel family
-- two pore domain potassium channel family in which its members can open even at resting membrane potential are unique to above potassium channel families with their own specific properties [74]. This special channel family and one of its members TASK1 will be discussed in details in later in this chapter.

Generally speaking, the cardiac action potential is a short-lasting event and fast-speed process in which the membrane potential of a cardiac cell rises and falls following a consistent resistance to the change of membrane potential (if it has this change). The cardiac action potential is a complicated process because lots of ion channels with different ion flow directions are involved in this process [73]. Figure 1.5 is a simplified diagram which shows the flow of different ion currents at different time periods in a complete cardiac action potential. Basically, the initial upstroke of the cells (which we also call phase 0) is due to the activation and opening of the fast inward Na⁺ current (I_{Na}) which can increase the membrane potential to around +50 mV. After that, initial rapid repolarization (phase 1) occurs which is a consequence of the rapid voltage-dependent inactivation of I_{Na} current and the activation of I_{to1} and the I_{Kur}. Since the driving force for K⁺ is from inside of cells to outside, it causes K⁺ flowing from the inside of the cell to the outside which leads to the decrease in the membrane potential. During phase 2 inward depolarizing currents through Na⁺ (slowly inactivated) and L-type Ca²⁺ channels (I_{CaL})
generate inward flow of ion currents which are balanced by the different components of the delayed rectifier K\(^+\) current (\(I_{Kur}\), \(I_{Kr}\) and \(I_{Ks}\)) generating outflow of K\(^+\). As a result, a plateau phase is obtained from the graph. The terminal phase 3 of repolarization is due to the increasing activation of the \(I_{Kr}\), \(I_{Ks}\) and \(I_{K1}\). As much K\(^+\) ions flowing out of the cell, it largely decrease the membrane potential as shown as large drop of membrane potential value in the figure. \(I_{K1}\) is also responsible for the

**Figure 1.5** Representative scheme of typical cardiac action potential (A) and the schematic representation of the major ionic currents (B) contributing to different phases [73].
maintenance of the resting potential because K⁺ can flow into the cell and increase the membrane potential when the membrane potential is more negative to the E_K. Thus, membrane potential can be maintained around E_K.

1.2.2 Leak K⁺ currents and two pore domain potassium channels

1.2.2.1 Structural basis of two pore domain potassium channels

First, the two pore domain potassium channels possess two concatenated pore-forming subunits. Each pore-forming subunit contains two pore-forming domains with four membrane-spanning helix flanking the potassium ion-selectivity filter segment (Figure 1.6) [74]. In addition, each subunit contains one intracellular N-terminal domain and one intracellular C-terminal which are thought to be critical modulation sites for other effector proteins [74].

![Figure 1.6](image-url) Schematic transmembrane topology of human two-pore domain potassium channels [74].
The members of this family were divided into six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) based on their sequence similarity and functional resemblance.

1.2.2.2 Biophysical properties of two pore domain potassium channels

Theoretically, two pore potassium channel current (also called background K\(^+\) since they open even at resting membrane potential) is not voltage dependent, meaning that the probability of opening (Po) of the channels is the same at all membrane potential values. (Po is also independent from the K\(^+\) concentrations on the two sides of the plasma membrane) [75]. In addition, another important feature of the two pore domain potassium current is that the amplitude of the current instantaneously follows the changes of the membrane potential (also called time-independent or the channel does not have activation, deactivation, and inactivation kinetics) (Figure 1.7) [75]. On the contrary, the alteration of the voltage-gated K\(^+\) current is delayed after the change of the membrane potential (Fig. 1.7A shows it took around 30 ms to achieve the plateau current amplitude). Since the membrane potential does not influence the Po of the ideal two pore domain potassium channel, a rapid change of the membrane potential results in an instant alteration of the current amplitude (around 10 ms shown in Figure 1.7B) [74]. Furthermore, an ideal two pore domain potassium channel is not rectifying (they do not have preference for the direction of K\(^+\) flow across the cell membrane). This property is also named as open rectification [76]. Opposite
driving forces of equal amplitudes induce opposite currents of equal amplitudes. Needless to say, although biophysical properties of the real two pore domain potassium channels have some deviations from what we described above, we are still using this ideal model to investigate these channels.

![Graph A: Kv2.1 current traces](image)

![Graph B: TASK-3 current traces](image)

**Figure 1.7** Representative current traces of voltage-gated and background potassium channels [74].

**1.2.2.3** *TWIK-related acid-sensitive K⁺ channel (TASK)*

TASK (TWIK-related acid-sensitive K⁺ channel), with its name resulting from its extreme sensitivity to variations of extracellular pH in the physiological range
And later it was extended to TASK1, TASK3 and TASK5. On the basis of molecular structure, TASK3 has the closest structural relative to TASK1. TASK3 was found to share 55-60% sequence identity with TASK1 in different species [78]. It is also inhibited by extracellular acidification (it is reported TASK3 is less sensitive to extracellular acidification than TASK1) and activated by extracellular alkanization [79]. TASK3 are exclusively inhibited by ruthenium red and Zn$^{2+}$ but not TASK1 [80, 81]. Besides, several other regulators have similar influence on the activity of these two channels in the same direction (Figure 1.8) [74]. In addition to TASK1 or TASK3 homodimers identified in various types of tissues or cells, TASK1/TASK3 can also form functional heterodimers in vivo [79]. Surprisingly, in contrast to TASK1 and TASK3 channels, TASK5 cannot be functionally expressed, although its mRNA is abundantly expressed in several tissues. Although we observe that two other channels, TASK2 and TASK4, also have the name “TASK” but they do not belong to the TASK subfamily due to low amino acid sequence similarity to TASK1 and sensitivity to pH in the alkaline range not including acid range. Therefore, TASK2 and TASK4 (also called TALK2) are categorized in the TALK subfamily of alkaline pH-sensitive two pore domain potassium channels [74].
1.2.2.4. Regulations of TASK channels

Native TASK-like currents have been demonstrated that they are inhibited by the stimulation of Gq-coupled receptors in different tissues and mainly through Gα proteins [82]. A constitutively active form of Gα associated with TASK1 or TASK3 and inhibited the channels. In addition, PKC inhibits TASK1 through phosphorylation of specific sites at C-terminal of TASK1 (Ser358 and Thr383 in human TASK1) [83, 84]. PKC induces TASK1 internalization in a 14-3-3β-dependent manner [85]. Moreover, anandamide and some synthetic cannabinoid receptor agonists like methanandamide were found to be potent inhibitors of human TASK1 [86]. The effect was not mediated by cannabinoid...
receptors. Interaction with accessory or chaperone proteins modifies the targeting of TASK1 to different locations within the cell. Reports showed that deletion of the last three amino acids (SSV) of the COOH-terminal tail of TASK channels caused the inhibition of TASK forward trafficking the plasma membrane. The SSV motif is the binding site for 14-3-3 proteins, and their phosphorylation-dependent interaction cover the ER retention motif. This is required to prevent trapping of the channel in the ER [87]. On the contrary, the (coatamer) COPI decrease the surface expression of TASK channels and causes accumulation in ER [88]. Thus, COPI and 14-3-3 act in an opposite ways to regulate TASK channel trafficking. In addition, covalent attachment of p11 proteins, an annexin II subunit, to TASK1 also enhance the forward trafficking of TASK1 to the plasma membrane possibly through cover an another ER retention motif KRR preceding the SSV sequence [89].

1.2.2.5. *Physiological functions mediated by TASK channels*

Carotid and aortic bodies are the primary sensors of hypoxia, metabolic acidosis, all of which stimulate respiration. In type I (glomer) cells of carotid bodies, several ion channels are influenced by both acidosis and hypoxia, leading to depolarization, voltage-dependent calcium influx. TASK1 and TASK3 were detected in neurons of locus coeruleus [90] and raphe nuclei [91]. Acidosis increased the action potential firing of locus coeruleus cells mainly via
the inhibition of TASK-like channels [92]. Halothane activated the acid-sensitive TASK-like channels, hyperpolarized the resting membrane potentials of these neurons, and prevented their spontaneous excito-activity [93]. In contrast to the systemic circulation, the smooth muscle cells of pulmonary arteries depolarize in response to reduced \( P_{O_2} \) and elevated \( P_{CO_2} \) in the surroundings of hypoventilated alveoli. The resulting voltage-dependent calcium influx induces vasoconstriction when TASK channels are closed due to its sensitivity to extracellular acidosis and cells become easier to depolarize. Under physiological conditions, this mechanism directs the blood to better ventilated areas of the lung [94]. However, in chronic generalized lung disease, this mechanism is responsible for the development of the pulmonary hypertension. In addition to the above mentioned, TASK channels are also found playing critical roles in central chemoreceptors [95], motoneurons [79], cerebellar granule neurons [96], aldosterone production [97] and even in epilepsy [98].

The expression of TASK1 channels in the heart was described widely [46]. TASK3 expression was negligible [46], while TASK5 subunit expression was intermediate [99]. TASK1 plays a functional role in the repolazation of the cardiac APD and contribute to the maintenance of HRV while TASK1-deficient mice showed prolonged cardiac repolarization and reduced HRV which may cause cardiac arrhythmias [100]. And TASK1 knock-out mice revealed a prolonged QT interval and disturbed QRS complex [101]. These findings
suggest that TASK1 may also be a candidate gene for inherited forms of conduction disorders. In another research, TASK1 was found to make a substantial contribution to the outward current flow in the plateau range of potentials [46]. Despite its small current amplitude in cardiac cells, cardiac TASK channels are crucial determinants of cardiac physiological functions.

Furthermore, it has been revealed that the expression of TASK3 may significantly influence cell viability in either direction. Depending on the cell type and also on actual conditions of the particular tissue, TASK3 may induce apoptosis, but under other circumstances it can help cell survival or promote proliferation [96].

1.3 Role of ion channels in cardiac ischemia and cardiac protection

1.3.1. Introduction of cardiac ischemia

In general, cardiac ischemia is characterized by a shortage of energetic supply (like hypoxia) as well as a deficient waste removal. The result is failure of contraction, deterioration of electrical behavior, and eventual leading to the cell death. The mammalian heart is an aerobic organ with one of the highest O\textsubscript{2} consumption rates (the other one is the brain) requiring a constant supply of O\textsubscript{2} to maintain essential cardiac functions and sustain the basic physiological function of the mammalian species. During ischemia, since there is a sustained O\textsubscript{2} deprivation it can alter the cardiac action potential and lead to arrhythmia or
other cardiac dysfunctions. There are numerous biochemical and physiological signalings involved in ischemia and cardiac protection against ischemic damage [40, 102]. In addition, the heart has developed several defenses that allow these cells to rapidly adjust to changes in O$_2$ during ischemia. What we focus on are two parts. The first one is IPC which has been demonstrated its significant importance in cardiac protection. We have not yet had a complete map of signaling pathways in related to IPC and it has not been applied to the clinical studies since its discovery [62]. The second part is the change of ion channels' functions during ischemia and how ion channels alleviate the damage to the heart from ischemia especially specific types of potassium ion channels. Some potassium ion channels are also critical components of IPC in cardioprotection. Since ion flow is critical to normal cardiac functions, and there is significant evidence that hypoxia induced by ischemia alters the function of ion channels and membrane transporters in cardiomyocytes.

1.3.2. **Ischemic preconditioning against cardiac ischemia**

Ischemic preconditioning (IPC) which was found by Murry and colleagues in 1986 was a remarkable discovery in cardioprotection against ischemia [61]. It protects the heart from ischemia/reperfusion. Ever since its discovery a great deal of research has been conducted in order to know the underlying mechanisms involved in IPC. Until now, several critical components and their related signaling pathways have been identified and confirmed. Adenosine,
bradykinin, and opioids receptors in cardiac cells act in parallel to trigger the preconditioned state and their signaling pathways converge at one crucial step: activating PKC (Figure 1.4). While activation of adenosine receptors couples directly to the activation of PKC through the phospholipases, bradykinin and opioids receptors activate PKC through relative more complex pathways that includes activation of a variety of downstream effectors: phosphatidylinositol 3-kinase (PI3-kinase), Akt, nitric oxide synthase, guanylyl cyclase, PKG, opening of mitochondrial K$_{ATP}$ channels, and activation of PKC by redox signaling [60, 103]. There are several differences between the opioid and bradykinin receptors’ signaling as the former activates PI3-kinase through transactivation of the epidermal growth factor receptor while the latter has an unknown signaling mechanisms [60]. The end-point of IPC is that it inhibits the formation of mitochondrial permeability transition pores (MPTP) early in reperfusion through activation of the survival kinases, Akt and ERK [62]. These kinases are activated as a result of PKC somehow promoting signaling from adenosine A$_2$ receptors early in reperfusion [104]. The survival kinases are thought to inhibit pore formation by phosphorylating GSK-3β [105].

Although we have an overview of what is happening during IPC, detailed signalings has yet been fully identified. More and more identified signalings or effectors enrich our knowledge of IPC. One of the important signalings which is still not very clear is that activation of PKCε is required and sufficient to protect
the heart from ischemia and reperfusion (IR) injury [103, 106]. Upon the activation of PKC, it translocates from the cytosol to different subcellular particulate fractions and a variety of cytoprotective stimuli that activate PKC including IPC elevates mitochondrial PKC level and this enhancement of mitochondrial PKC is mainly on PKCε which provide cardioprotection in previous findings [106]. Thus, identification of signalings and/or other effectors contributing to PKCε translocation to mitochondria is not only making our signaling map clearer but also could be a newly promising target in inducing IPC’s protective effects in the future clinical studies [107]. Here, we examined the roles of caveolin-3, HSP90 and TOM70 in mediating mitochondrial translocation of PKC and its age-dependency (Chapter 2).

### 1.3.3. **Role of potassium channels in cardioprotection against cardiac ischemia**

1.3.3.1. **Inward rectifying K⁺ channels in cardiac protection**

I\textsubscript{K1} is considered as a time-independent background K⁺ current. It exhibits a large inward current at the membrane potential more negative than the Nernst potential of K⁺ (E\textsubscript{K}) based on the intracellular and extracellular concentration of K⁺ and exibits a relatively large outward current at the resting potential slightly higher than E\textsubscript{K}. This is essential for stabilizing the resting membrane potential even there is fluctuation of the potential especially these fluctuation are generated due to abnormal conditions [108]. Moreover,
I\textsubscript{K1} is critically involved in determining cardiac APD, not only they set the resting membrane potential but also they induces rapid final stages of repolarization [108]. However, ischemia induced hypoxia causes early action potential shortening and speed up of the late phase of repolarization which I\textsubscript{K1} is highly involved [109, 110]. Disturbances in I\textsubscript{K1} during ischemia result in alteration of action potential shape and APD which lead to cardiac arrhythmias and cardiac cell death.

In addition to I\textsubscript{K1}, K\textsubscript{ATP} is also a critical component of cardioprotein against ischemia [111-113]. K\textsubscript{ATP} is a highly abundant potassium channel at plasma membrane. They are responsible for linking the cellular energy levels to membrane potentials [114, 115]. In the heart, K\textsubscript{ATP} channels on the plasma membrane are critical regulators of cellular excitability and action potentials during ischemia [111-113]. They are closed under the basal conditions due to the inhibition by intracellular millimolar range of ATP, but they can open when ATP concentration is decreasing less than micromolar range when energy deprivation is occurred such as ischemia [114]. K\textsubscript{ATP} channels would shorten the cardiac action potential duration during ischemia and reduce harmful Ca\textsuperscript{2+} influx through VGCC [116, 117]. Besides, there is ample evidence showing that K\textsubscript{ATP} channels play a key role in triggering IPC against ischemic damage possibly mainly through mitochondrial K\textsubscript{ATP} which eventually inhibit MPTP [60, 62, 103, 106] in order to protect mitochondria.
1.3.3.2. TASK channels in cardiac protection

Opening of TASK1 channels decrease spontaneous neuronal firing rates [93, 118, 119]. Studies with TASK1 (−/−) mice showing significantly larger infarct area during ischemia demonstrated that TASK1 is critical for neuroprotection against cerebral ischemia [120]. Taken together, TASK1 plays a major role in protecting cells from damage throughout the major role of maintaining cell resting membrane potential and decrease cell excitability. The alteration of TASK1 functions is likely to have a significant systemic impact especially during pathophysiological conditions such as ischemia/hypoxia. Ischemia with low O$_2$ level and acid pH inhibit TASK1 channels [46] in the heart which cause more fluctuation of cardiac cell resting potential and eventually more depolarization would be occurred. This would eventually leads to excitotoxicity which causes damage to the cells. Thus, TASK1 makes cardiac cells more vulnerable to the reduction of O$_2$ and acid pH. Cardiac cells then would be easily dead because of excitotoxicity. In summary, TASK1 could be a newly promising target for cardioprotection against ischemia and its related regulation become more and more important topics for further identification (Chapter 3 and 4).

4. OBJECTIVES

It is well-accepted that PKC is the key mediator in ischemic preconditioning, but little is known about the how PKC isoforms translocate to mitochondria
and whether this process is age-dependent. The objectives of the present investigations were two-fold. First, to examine which effectors facilitate the translocation of the PKC isoform(s) to mitochondria by activation of adenosine receptor. Secondly, we were trying to investigate whether these effectors are decreased through aging and have significant effect on the efficiency of PKC isoforms translocation to mitochondria. The results indicated that the translocation of PKC isoform PKCε is caveolin-3-dependent. It is also age-dependent due to the age-dependency of HSP90 and TOM70. Both proteins are involved in PKCε translocation to mitochondria.

In addition to PKC isoforms translocation and its importance in ischemic preconditioning, TASK1 is critical of protecting cells especially under pathophysiological conditions. Our next studies was designed to determine whether TASK1 is functionally modulated by caveolin-3 via its physical association with caveolin-3. Consequently, we identified that TASK1 is negatively modulated by caveolin-3 via association with caveolin-3.

We also investigated the molecular mechanisms by which hypoxia regulates TASK1 channels. We examined the role of protein kinase C (PKC) in hypoxia or metabolic inhibition-induced modulation of TASK1 channels in HEK293T cells and adult rat cardiac myocytes. TASK1 is largely inhibited during hypoxia and this inhibition is mainly through the activation of PKC isoform(s).
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 cardioprotection during ischemia/hypoxia, but also for designing a new strategy of clinical application.
CHAPTER 2

Age-dependent alteration in targeting protein kinase C to mitochondria in cardiomyocytes

Abstract

Activation of adenosine receptors has been shown to promote translocation of protein kinase C (PKC) isoforms to both caveolar plasma membrane and mitochondria. Given that caveolin-3 plays an important roles in PKC targeting to the plasma membrane, we hypothesized that caveolin-3 and adenosine receptor signaling may be linked to adenosine-induced mitochondrial translocation of PKCε. The first part of the study was designed to determine whether adenosine-induced translocation of PKCε to mitochondria is mediated by caveolin-3, adenosine A2B receptor and/or PI3K and whether it is age-related. Freshly isolated adult rat cardiomyocytes and rat heart-derived H9c2 cells were used in the study. Mitochondrial localization of PKCε was quantified by the percent of PKCε-positive mitochondria from isolated mitochondria. Immunofluorescence imaging of isolated mitochondria showed
that adenosine-induced mitochondrial translocation of PKCε was not inhibited by pretreatment with adenosine A2B receptor specific inhibitor MRS 1754 or PI3K inhibitor Wortmannin. Interestingly, adenosine-induced increase in mitochondrial translocation of PKCε was significantly reduced by suppressing caveolin-3 expression with specific siRNA, whereas negative siRNA had no effect. Our data also show that adenosine treatment did not increase the protein expression of caveolin-3. Furthermore, mitochondrial localization of PKCε was significantly reduced along with largely decreased HSP90 and TOM70 expression in middle-aged rats. In conclusion, we demonstrate that adenosine-induced translocation of PKCε to mitochondria is mediated by caveolin-3-dependent PKC signaling and this process is age-related, possibly through regulation of HSP90 and TOM70 expression. These results point out a novel mechanism in regulating PKC function in mitochondria.
Introduction

Myocardial preconditioning has been shown to drastically reduce the ischemia and reperfusion injury [61, 121]. Over the past several decades, numerous research has revealed signaling pathways and it has been now well known that activation of protein kinase C (PKC) is a key step in conferring cardioprotection while inhibition of this process prevents the protection [122-127]. Specifically, activation of PKC isoform PKCε is critical for cardioprotection of myocardial preconditioning [124, 125, 128]. Upon activation, PKC translocates to multiple cellular localizations including mitochondria, nucleus and plasma membrane [57, 58, 128]. Numerous studies suggest that mitochondrial PKCε is required for cardioprotection [58, 128-131].

There is growing evidence that adenosine contributes to cardioprotection via adenosine receptor subtype and their distinct signal transduction pathways, such as PKC and their downstream signaling [132-136]. Adenosine receptors are G-protein-coupled receptors which consist of four subtypes- A1A, A2A, A2B and A3. While A1 and A3 receptors are generally believed to couple with Gi/Go proteins, A2A and A2B receptors are linked to Gs proteins. It was reported that in the adenosine-triggered ischemic preconditioning, PKC was on the upstream of adenosine A2B receptor and A2B receptor then activated survival kinases including PI3 Kinase which ultimately inhibited mitochondrial permeability
transition pore [60, 137, 138]. Because PI3 kinase can also activate PKC either via direct activation by lipid products or via phosphorylation by PDK1 [139], it is likely that activation of adenosine receptors may cause PKC activation and targeting to distinct subcellular localizations such as mitochondria via adenosine A2B receptor-dependent signaling pathways. Further, A2B receptors have shown to be present in or near mitochondria rather than on the sarcolemma, suggesting the possible involvement of mitochondria in adenosine A2B receptors-mediated signaling [140].

It has been shown that PKCε translocated to mitochondria following ischemia and reperfusion injury or by adenosine receptor activation is dependent on heat shock protein (HSP) and translocase of the outer mitochondrial membrane (TOM) [106, 107, 141]. It has also been shown that upon activation, PKC isoforms selectively target to caveolae and regulate caveolar-localized proteins in response to stimuli [59, 127]. Caveolin, the structural protein in caveolae microdomain [142], plays a pivotal role in regulating numerous downstream effectors and acts as an organizer of signal transduction pathways in many cell types [23]. In cardiac myocytes, caveolin-3 is the major subtype of caveolin [8].

In the present study, we tested the hypothesis that adenosine-induced mitochondrial translocation of PKCε is a caveolin-3-dependent process, possibly via activation of A2B receptor and/or PI3K. We examined the effect of
inhibition of A2B receptor or PI3K on PKCε translocation to mitochondria in the freshly isolated adult rat cardiomyocytes and rat heart-derived H9c2 cells. We also investigated the effect of suppressing caveolin-3 on mitochondrial targeting of PKCε. Furthermore, we evaluated whether translocation of PKCε to mitochondria is reduced in middle-aged rats and its underlying mechanism since the cardioprotection of ischemic preconditioning is also age-related [143, 144]. We demonstrated that adenosine-induced PKCε translocation to mitochondria was mediated by a caveolin-3-dependent and A2B receptor/PI3K-independent pathway. In addition, we showed that adenosine-induced PKCε translocation to mitochondria was reduced in middle-aged rat hearts, possibly via reduced expression of HSP90 and TOM70. Our data reveal a novel mechanism underlying adenosine-induced PKCε targeting to mitochondria and its association with age.

**Materials and Methods**

**Materials**

Rabbit IgG directed against PKCε, rat IgG against HSP90 and goat IgG against TOM70 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Type II collagenase was purchased from Worthington (Lakewood, NJ). MitoTracker Green was obtained from Invitrogen (Carlsbad, CA). Adenosine, adenosine
receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), adenosine A2B receptor antagonist 8-[4-([(4 cyanophenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS 1754) and phosphoinositide 3-kinase (PI3K) inhibitor Wortmannin 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 male Sprague–Dawley rats aged 2 to 3 months (young) and 10 to 12 months (middle-aged) by enzymatic dissociation [145]. 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 had the same composition. The hearts were perfused with the same solution containing collagenase for 20–30 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 (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 various agents for 10 min at 37 °C prior to subsequent biochemical and immunofluorescence experiments.

Preparation of mitochondrial fraction or intact mitochondria

Intact mitochondria or mitochondrial fraction was prepared from H9c2 cells and adult rat cardiomyocytes by differential centrifugation [127, 146, 147]. For Western blot experiments, mitochondrial fraction was further purified by 30% percoll ultracentrifugation [145, 146]. For mitochondrial colocalization experiments, cells were stained with a mitochondrial marker MitoTracker (200 nM) for 30 min 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, and protease inhibitor cocktail. Two 15 s homogenization cycles were performed on ice. The homogenate was centrifuged at 1000 g for 10 min at 4 °C to remove nuclei and debris. The pellet containing the mitochondrial fraction was prepared in the homogenizing buffer and centrifuged at 8500 g for 30 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
95,000 g for 30 min at 4 °C. Mitochondrial band was collected by a Pasteur pipette and washed in the homogenizing buffer.

Cell culture and transfection

The small interfering RNA (siRNA) oligonucleotide targeting rat caveolin-3 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 [146]. H9c2 cells were transfected with siRNA using Hi-perfect siRNA transfection reagent [146]. Experiments were carried out 48 to 72 h after transfection.

Western blotting

Immunoblot analysis was carried out from the mitochondrial fraction [145, 147]. The purity of mitochondrial fraction was evaluated using antibody against the mitochondrial marker protein prohibitin and the plasma membrane marker Na+/K+ ATPase to ensure there is no significant contamination in the mitochondrial fraction. Briefly, the percoll-purified mitochondrial fraction was 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, 0.05% Tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system. Quantitative analysis of Western blot bands was performed by with ImageJ software.

*Immunofluorescence microscopy*

As described previously, intact mitochondria were isolated from cells pretreated with MitoTracker and/or other agents. Mitochondria 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. Mitochondria were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 h. 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 ± SEM. Multiple group means were compared by one-way ANOVA followed by LSD post hoc test. A P-value of < 0.05 was considered as statistically significant.

Results

Role of adenosine A2B receptors and PI3K signaling pathway in the translocation of PKCε to mitochondria

Adenosine A2B receptors and PI3K have been shown to be required for adenosine A1 receptor-mediated cardioprotection [132-136]. To investigate signaling mechanism underlying translocation of PKCε to mitochondria induced by adenosine, we examined whether this translocation is dependent on adenosine A2B receptor and/or PI3K. We isolated mitochondria from young adult rat cardiomyocytes and analyzed PKCε-positive mitochondria under different treatments. This method has been demonstrated to be an effective way to localize proteins in mitochondria [147]. The freshly isolated adult rat cardiomyocytes were pretreated without or with adenosine (10 μM), adenosine plus DPCPX (100 nM), adenosine plus MRS 1754 (100 nM) or adenosine plus Wortmannin (200 nM) for 10 min. Cells were also stained with a mitochondria-selective probe MitoTracker green before mitochondrial fractionation. Intact mitochondria were obtained from cardiomyocytes by
differential centrifugation as described in Materials and Methods. The quantification analysis was done by dividing PKCε-positive mitochondria by total mitochondria. Then the percentage of PKCε-positive mitochondria from each treatment group were normalized to control group without any treatment. As we showed previously, a small portion of PKCε was localized in mitochondria identified by MitoTracker green without any treatments. The number of PKCε-positive mitochondria normalized to the total mitochondria was 28.39 ± 1.10%. Adenosine treatment significantly increased mitochondrial localization of PKCε, as indicated by increased yellow punctate staining. The adenosine-induced increase in mitochondrial PKCε was largely blocked by adenosine A1 receptor inhibitor DPCPX (71.58 ± 1.61% vs. 41.22 ± 1.95%, adenosine vs. adenosine + DPCPX, p<0.001) (Figure 2.1B). However, Treatment with adenosine plus MRS 1754 or adenosine plus Wortmannin did not inhibit the effect of adenosine on mitochondrial translocation of PKCε (73.95 ± 1.09%, adenosine + MRS 1754, 73.04 ± 2.66%, adenosine + Wortmannin) (Figure 2.1B). The data were collected from 3 independent experiments. These results indicate that adenosine-mediated mitochondrial translocation of PKCε is independent of adenosine A2B receptors and PI3K signaling.
Figure 2.1  Effect of adenosine on mitochondrial localization of PKCε from young adult rat cardiomyocytes. (A) Representative immunofluorescence images of isolated mitochondria double-labeled with anti-PKCε antibody and MitoTracker green from cells treated without or with adenosine (Ade), Ade+DPCPX, Ade+MRS 1754 or Ade+Wortmannin. (B) Percentage of PKCε-positive mitochondria. (C) Representative Western blots of mitochondrial PKCε under various treatment. In contrast to PKCε, all of the drug treatment did not alter mitochondrial PKCδ compared with non-treatment group. Prohibitin was used as loading control. Percoll purification of mitochondria was successfully conducted due to few Na+/K+ ATPase was identified in mitochondria. The data were obtained from 3 independent experiments. ***p<0.001 vs. Control, ###p<0.001 vs. adenosine (Ade). Scale bar: 10 μm.
Figure 2.1

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mitochondria</th>
<th>PKCe</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade + DPCPX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade + MRS 1754</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ade + Wortmannin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

PKCe-positive mitochondria (%)

C

Protein bands:
- PKCe: 150 kDa
- PKCδ: 75 kDa
- Prohibitin: 25 kDa
- Na+/K+-ATPase: 100 kDa
Effect of suppressing caveolin-3 on adenosine-mediated mitochondrial translocation of PKCε

Caveolin has already been known as a scaffolding protein in caveolae microdomain structure and acting as an organizer which modulates various downstream effectors including protein kinases [23]. Among three subtypes of caveolins (caveolin-1, -2, -3), caveolin-3 is the most abundant in the heart [23]. It’s been shown that adenosine receptors are localized to caveolae [148], and PKC isoforms target to caveolar plasma membrane to enhance the regulation of caveolar-localized proteins once they are activated [59]. Therefore, we sought to determine the role of caveolin-3 in adenosine-induced mitochondrial translocation of PKCε. We investigated whether the suppression of caveolin-3 with siRNA alters the mitochondrial translocation of PKCε induced by adenosine receptor signaling. We employed rat heart derived H9c2 cells because they display many properties similar to those in adult rat cardiomyocytes and are easily transfected [145]. These cells have been used extensively for studies including cardioprotection, heat shock proteins and protein kinases. As shown in Figure 2.2C, transfection of H9c2 cells with siRNA targeting caveolin-3 significantly reduced the expression of endogenous caveolin-3. Western Blot detected a faint band from cells treated with caveolin-3 siRNA whereas anti-caveolin-3 antibody revealed a prominent band under scrambled siRNA treatment. Interestingly, suppression of caveolin-3 with siRNA significantly
prevented adenosine-induced translocation of PKCε to mitochondria (52.00 ± 2.00% vs. 17.74 ± 2.00%, adenosine vs. adenosine + caveolin-3 siRNA, p<0.001) whereas the negative scrambled siRNA did not have any effects on adenosine-induced increase in PKCε-positive mitochondria (47.84 ± 3.00%) (Figure 2.2A, 2.2B). The data were obtained from 3 independent experiments. These data suggest that caveolin-3 is crucial for adenosine-induced translocation of PKCε to mitochondria.

Similar to young adult rat cardiomyocytes, adenosine pretreatment in H9c2 cells increased the mitochondrial targeting of PKCε while this effect was largely prevented by treatment with DPCPX (Figure 2.3, 55.55 ± 2.00% vs. 20.04 ± 3.00%, adenosine vs. adenosine + DPCPX, p<0.001). Both MRA1754 and wortmannin did not prevent adenosine-induced mitochondrial translocation of PKCε (55.07 ± 2.00%, adenosine + MRS 1754, 56.89 ± 3.00%, adenosine + Wortmannin).
Figure 2.2 Effect of suppressing caveolin-3 expression with caveolin-3 siRNA on adenosine-induced increase in mitochondrial PKCε from H9c2 cells. (A) Representative immunofluorescence images of isolated mitochondrial double-labeled with anti-PKCε antibody and MitoTracker green from cells transfected with siRNA targeting caveolin-3 (Cav-3 siRNA) or control siRNA (scrambled siRNA) in the absence or presence of adenosine (Ade). (B) Percentage of PKCε-positive mitochondria. (C) Representative Western blots showing reduction of endogenous caveolin-3 (Cav-3) by specific siRNA against Cav-3. The data were obtained from 3 independent experiments. ***p<0.001 vs. Control, ###p<0.001 vs. adenosine (Ade). Scale bar: 10 μm.
Effect of adenosine on caveolin-3 expression in cardiomyocytes

To determine whether adenosine-induced signaling affects caveolin-3 expression, isolated young adult rat cardiomyocytes were treated with or without adenosine before cell homogenates were prepared for Western Blot analysis. Immunoblots with antibody against caveolin-3 revealed that there was no significant difference in the expression of caveolin-3 between control and adenosine treatment group (Figure 2.4, 121.41 ± 22.33%, adenosine, normalized to control). Data analysis was performed by comparing density of bands with ImageJ software. Our data suggest that although caveolin-3 expression is essential for adenosine-induced mitochondrial translocation of PKCε, increased expression of caveolin-3 is not involved in this translocation.
Figure 2.3 Effect of adenosine on mitochondrial localization of PKCε from H9c2 cells. (A) Representative immunofluorescence images of isolated mitochondrial double-labeled with anti-PKCε antibody and MitoTracker green from cells treated without or with adenosine (Ade), Ade+DPCPX, Ade+MRS 1754 or Ade+Wortmannin. (B) Percentage of PKCε-positive mitochondria. The data were collected from 3 independent experiments. ***p<0.001 vs. Control, ###p<0.001 vs. adenosine (Ade). Scale bar: 10 μm.
Figure 2.3

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mitochondria</th>
<th>PKCε</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ade</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ade+ DPCPX</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ade+ MRS 1754</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ade+ Wortmannin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

B

![Bar chart showing PKCε-positive mitochondria (%)](chart)

- Control
- Ade
- Ade+ DPCPX
- Ade+ MRS 1754
- Ade+ Wortmannin

PKCε-positive mitochondria (%)
Figure 2.4 Effect of adenosine on the expression of caveolin-3 in young adult rat cardiomyocytes. Representative Western blots of caveolin-3 (Cav-3) with or without adenosine (Ade) treatment. The whole cell lysate was obtained from adult rat cardiomyocytes treated with or without adenosine (Ade). Western blot was performed with anti-Cav-3 antibody from the whole cell lysate. GAPDH was used as loading control. Results were data from 3 independent experiments. n.s. indicates no statistically significant difference.
Adenosine-induced PKCε translocation to mitochondria in the heart from aged rats

All rats used in the experiments described above were young adult rats (2 to 3-month old). To determine whether adenosine-induced mitochondrial translocation of PKCε is age-related, the middle-aged rats (10 to 12-month old) were employed. We were interested in determining whether adenosine-induced PKCε translocation to mitochondria was also applicable in aged rats and whether this effect could be altered due to aging. Freshly isolated cardiomyocytes from 2 to 3-month and 10 to 12-month were pretreated with or without adenosine, adenosine plus DPCPX. Cells were also stained with a mitochondria-selective probe MitoTracker green before mitochondrial fractionation. Intact mitochondria were obtained from cardiomyocytes by differential centrifugation as described above. The quantification analysis was obtained from calculating the percent of PKCε-positive mitochondria over total mitochondria and then normalizing them to control group. As shown in Figure 2.5, a small portion of PKCε was also detected in mitochondria without any treatments from the middle-aged rats. The basal level of mitochondrial PKCε was significantly lower in the middle-aged rats when compared with the young rats (Figure 2.5B, 6.00% ± 1.27% vs. 28.39 ± 1.10%, middle-aged vs. young, p<0.001). Similar to young adult rats, Adenosine treatment increased the translocation of PKCε to mitochondria in the middle-aged rats and this effect
was inhibited by DPCPX (Figure 2.5C, normalized to control without adenosine, 161.62 ± 5.43% vs. 89.38 ± 30.20%, adenosine vs. adenosine + DPCPX, p<0.01). However, this adenosine-induced translocation was significantly lower when compared with that from 2 to 3-month group (161.62 ± 5.43% vs. 252.09 ± 5.68%, adenosine in middle-aged vs. adenosine in young, p<0.001). Data were analyzed from 3 independent experiments. These results indicate that the level of mitochondrial PKCε under control or adenosine treatment was significantly lower in the middle-aged rats when compared with the young adult rats.

Expression of HSP90 and TOM70 in aged rats

Previous studies indicate that heat shock protein (HSP) and translocase of the outer mitochondrial membrane (TOM) complex are important for protein import into mitochondria [106, 107, 141, 149, 150]. Activation of the adenosine receptor induces a rapid association of PKCε with TOM70, which is dependent on HSP90. To verify whether lessened PKCε translocation to mitochondria in aged rats resulted from reduced expression of both HSP90 and TOM70, we assessed the expression levels of HSP90 and TOM70 from mitochondria in both 2 to 3-month and 10 to 12-month old rats. We isolated cardiomyocytes from the young and middle-aged rats, and then analyzed mitochondria for the presence of HSP90 and TOM70. Western blot analysis revealed the significant
reduction in the expression of both HSP90 and TOM70 in the middle-aged rats compared to the young adult rats, indicating that aging resulted in markedly decreased expression of HSP90 and TOM70 in mitochondria (Figure 2.6). We observed strong protein bands of HSP90 and TOM70 from two 2 to 3-month rat mitochondrial samples while only faint bands can be seen from two 10 to 12-month rat mitochondrial samples. Prohibitin, a mitochondrial marker, served as loading control. These results indicate that decreased expression of HSP90 and TOM70 may contribute, at least in part, to the reduction in PKCε translocation to mitochondria.
Figure 2.5  Effects of adenosine on mitochondrial localization of PKCε from young adult and middle-aged rat cardiomyocytes. (A) Representative immunofluorescence images of isolated mitochondrial double-labeled with anti-PKCε antibody and MitoTracker green. (B) The basal level of PKCε in mitochondria from young adult and middle-aged rat hearts. Isolated mitochondria were prepared from 2 to 3-month (young adult) and 10 to 12-month (middle aged) rat cardiomyocytes. (C) Quantification of PKCε-positive mitochondria from cells treated with nothing (control), adenosine (Ade) or Ade+DPCPX from either 2 to 3-month (young adult) or 10 to 12-month (middle aged) rat cardiomyocytes. The data were collected from 3 independent experiments. ***p<0.001 vs. Middle-aged in (B). In (C), ***p<0.001 vs. Control, or Ade in young adult vs. Ade in middle-aged; ###p<0.001 vs. Ade in young adult; *p<0.05 vs. Control in Middle-aged; #p<0.05 vs. Ade in Middle-aged.
Figure 2.5

A

 Mitochondria  PKCε  Merge

Young  

Middle-aged

B

Bar graph showing Basal PKCε-positive mitochondria (%) for Middle-aged and Young groups.

C

Bar graph showing PKCε-positive mitochondria (%) for Control, Ado, and Ado+10μM IP3R1 groups.
Figure 2.6 Expression of HSP90 and TOM70 from young adult or middle-aged rat cardiomyocytes. Representative Western blots of HSP90 and TOM70 in 2 to 3-month and 10 to 12-month old adult rats. Mitochondria were prepared from either 2 to 3-month or 10 to 12-month adult rat cardiomyocytes. Prohibitin was used as mitochondrial marker and loading control. Results were data from 3 independent experiments.
Figure 2.6

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Young</th>
<th>Middle-aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prohibitin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOM70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- HSP90: 100 kDa
- Prohibitin: 25 kDa
- TOM70: 75 kDa

B

Protein expression level (% of Young)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Middle-aged</th>
<th>Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>TOM70</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

* P < 0.05
** P < 0.01
Discussion

The present study has demonstrated that adenosine-induced PKCε translocation to mitochondria is through a caveolin-3-dependent and adenosine A2B receptor/PI3K-independent pathway. Specifically, we showed that application of adenosine A2B receptor or PI3K inhibitor did not prevent mitochondrial translocation of PKCε induced by adenosine. We found that suppression of cavelin-3 expression with siRNA significantly decreased adenosine-induced PKCε targeting to mitochondria. We also found that adenosine-induced mitochondrial translocation of PKCε was significantly reduced in middle-aged rats when compared with the young adult rats, possibly via decreased expression of HSP90 and TOM70.

We and others have demonstrated that PKCε translocates to mitochondria in response to the activation of adenosine A1 receptors or ischemia followed by reperfusion [106, 107]. It is believed that PKCε in mitochondria is critical for the cardioprotection of preconditioning [58, 128, 151]. However, it is not completely understood how PKCε is translocated to mitochondria. It has been shown that in addition to PKC isoforms, activation of adenosine A1 receptors is linked to the activation of PI3K in either parallel or the downstream effector of PKC isoforms [152]. Activated PKC by adenosine receptors also triggers adenosine A2B receptors which was found in or near mitochondria and regulate the survival
kinases, such as PI3K/Akt and ERK [137, 138, 153-156]. Thus, PI3K or adenosine A2B receptors might be involved in PKCε translocation to mitochondria upon activation of adenosine A1 receptors. Since PKCε translocation to mitochondria is an indispensable step for cardioprotection, we studied the mechanisms underlying PKCε translocation to mitochondria by adenosine A1 receptor, one of mediators in cardioprotection. We examined whether adenosine A2B receptor and/or PI3K are involved in adenosine-induced regulation of mitochondrial PKCε. Our data demonstrated that the treatment with adenosine A2B receptor inhibitor MRS1754 or PI3K inhibitor wortmannin did not prevent adenosine-induced mitochondrial translocation of PKCε in both adult rat cardiomyocytes and H9c2 cells. Both inhibitors at the same concentrations as we used have been shown to effectively block adenosine A2B receptors and PI3K PI3K [157, 158]. It is thus likely that other signaling protein (s) may underline PKCε translocation to mitochondria induced by adenosine receptors.

We have previously shown that adenosine receptor activation stimulates the translocation of PKC isoforms to the caveolar plasma membrane [127]. It is well known that PKC isoforms can directly bind to caveolin scaffolding domain in order to trigger subsequent signaling pathways [59]. We therefore studied whether caveolin-3, the main structural protein of caveolae in cardiac myocytes, is essential PKCε translocation to mitochondria induced by adenosine-mediated
PKC signaling. Our data demonstrated that the suppression of caveolin-3 expression with specific siRNA targeting caveolin-3 significantly reduced adenosine-induced PKCε targeting to mitochondria, although the activation of adenosine receptors did not alter the protein expression of caveolin-3. These observations suggest that the basal level of caveolin-3 is essential for adenosine receptor-mediated mitochondrial translocation of PKCε. It is possible that caveolin-3-dependent PKC signaling promotes the translocation of cytosolic PKCε to mitochondria.

Studies have shown that cardioprotection is age-related [144, 159]. Protein expression is highly associated with aging, especially proteins related to stress [160]. We are wondering whether weakened PKCε translocation to mitochondria at least, partly contributes to the weakened protection against cardiac ischemia in aged population. Our data showed that adenosine-induced translocation of PKCε in the heart was largely decreased in middle-aged rats. We also found out that the basal level of PKCε in cardiac mitochondria was lower in middle-aged than that in young adult rat groups. In addition, we found that the protein expression of cardiac HSP90 and TOM70 in middle-aged rats was prominently lower than that in young adult rats. Given the importance of HSP90 and TOM70 in PKCε translocation to mitochondria [107], the decreased translocation of PKCε to mitochondria at basal level or induced by adenosine could be, at least in part, due to altered expression of HSP90 and TOM70. There is evidence,
obtained in both cytosolic and membrane fractions of ventricular cardiac cells, indicating that the expression of PKCε was not altered [161]. It is likely that aging may diminish the cardioprotective role of PKCε because of reduced mitochondrial targeting of PKCε. Indeed, it has been reported that PKCε did not participate in ischemic preconditioning in old rabbits [162]. Since aging is an extremely complex physiological and biochemical process and many proteins or and signaling transduction pathways might have been changed, our observations do not exclude the possibility that altered expression of other proteins on the pathway leading to mitochondrial targeting of PKCε may underline the altered level of cardiac mitochondrial PKCε in middle-aged rats.

Our finding indicates a novel mechanism involved in the adenosine-induced PKCε translocation to mitochondria, an important step in the cardioprotection against ischemia and reperfusion injury [61, 128-131, 163]. PKCε in mitochondria has been associated with a number of substrates in cardioprotection, such as aldehyde dehydrogenase 2, cytochrome c oxidase subunit IV, mitochondrial permeability transition pore, and mitochondrial ATP-sensitive K+ channels [128]. The present study provides a further understanding of the molecular mechanism underlying adenosine-induced mitochondrial targeting of PKC isoforms, which should increase our knowledge about adenosine-mediated cardioprotection against ischemic injuries, and may lead to identification of potential therapeutic targets.
We demonstrate that adenosine-induced translocation of PKCε to mitochondria is dependent on caveolin-3, not adenosine A2B receptors and PI3K. And the mitochondrial targeting of PKCε is tightly associated with aging, at least in part, via reduced expression of HSP90 and TOM70.
CHAPTER 3

Distribution and functional modulation of TASK1

Abstract

The two-pore domain potassium channel TASK1 is strongly expressed in the heart and has been shown to regulate the resting membrane potential and action potential. Modulation of the TASK1 background current provides a mechanism for control of cellular excitability. However, little is known about the localization and regulation of TASK1 in the heart. The present study was designed to determine whether TASK1 is functionally modulated by caveolin-3 (Cav-3). HEK293T cells transfected with Cav-3 and TASK1 cDNA were used in the study. Functional studies with patch-clamp technique showed that the expression of Cav-3 in HEK293T cells decreased the recombinant TASK1 current density significantly when compared with HEK293T cells transfected with TASK1 plus empty vector (11.01±3.08 pA/pF vs. 23.92±1.96 pA/pF, p<0.05, n=6-8). Immuno-stained HEK293T cells co-transfected with Cav-3 and
TASK1 demonstrated co-localization of these two proteins. Co-immunoprecipitation analysis indicated that Cav-3 associated with TASK1. In addition, less wild-type Cav-3 is expressed at cell periphery when co-transfected with Cav-3 mutant P104L in HEK293T cells. This leads to no significant reduction to TASK1 current density indicating that wild-type Cav-3 affects TASK1 current amplitude mainly through functional modulation. In summary, our study indicates that TASK1 is functionally regulated by Cav-3. These results point out a novel mechanism in the regulation of TASK1.

**Introduction**

A new family of potassium channels has exploded into view, these are two-pore domain potassium channels which possess unique structure—two-pore forming domains in each subunit [75]. Two pore domain potassium channels are ubiquitous in human physiological system [164]. They are identified as background or K⁺-selective channels due to a time and voltage independency, playing a crucial role in maintaining the resting membrane potential and setting input resistance in excitable cells. TASK1 which is encoded by *KCNK3* gene, is one of the members of two-pore domain potassium channel family [165]. TASK1 is inhibited by acidic pH [166] and low O₂ levels [167] which are conditions observed during cellular metabolic challenge such as ischemia. Studies with TASK1 (−/−) mice showing significantly larger infarct area during ischemia
demonstrated that TASK1 is critical for neuroprotection against cerebral ischemia [120]. TASK1 plays a major role in protecting cells from damage throughout the major role of maintaining cell resting membrane potential and decrease cell excitability. This also maintains normal ion homeostasis on both sides of the cells in which its disruption leads to cell death. In addition, the expression of TASK1 channel and its contribution to action potential duration in mammalian cardiomyocytes has been demonstrated [46, 168]. Taken together, this suggests that regulation of TASK1 activity in cardiac cells is an important area of study.

Caveolae, a subcellular microdomain of membrane rafts, are flask-like invaginations of plasma membrane that contain caveolin proteins. Caveolae act as a central organizer essential to some signal transduction pathways [23]. In addition, caveolin possesses a pivotal domain (the caveolae scaffolding domain, CSD) with which signaling molecules interact in an inactive state. Activation of these signaling molecules leads to their release from caveolin, making them available to activate the downstream effectors [20]. Recent studies reported that Cav-3 interacts and modulates various ion channels including potassium channels [24, 28]. We wonder whether Cav-3 modulate TASK1 activities. The 14-3-3 protein acts as an adaptor protein during the transportation of the TASK1 channel to the cell membrane surface [169]. The 14-3-3 protein can also interact with caveolae microdomain [15] which enhances the possibility that
caveolin modulates TASK1 activities via association. Moreover, recent studies have also shown that p11 protein, which is a subunit of annexin II associates with TASK1 protein and enhance its membrane expression [89]. Annexin A2 binds to Cav-3, which provides a linker between TASK1 and Cav-3 [170]. Based on these investigations, we hypothesized that TASK1 activity can be modulated by Cav-3 mainly through their direct or indirect association with each other.

In the current study, we have shown that recombinant TASK1 activities is negatively regulated by Cav-3 and TASK1 associates with Cav-3 in HEK293T cells. These results indicated a novel regulatory mechanism of TASK1.

**MATERIALS AND METHODS**

*Materials.*

Goat IgG directed against caveolin-3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat IgG directed against HA tag was purchased from Roche Diagnostics (Indianapolis, IN, USA). Secondary antibody directed against goat conjugated with Cy2 or Cy3 and rat conjugated with Cy2 or Cy3 were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Dynabeads Protein A was obtained from Invitrogen
Methanandamide was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of cardiomyocytes.

Adult rat ventricular myocytes were isolated from male Sprague–Dawley rats aged 2 to 3 months (young) by enzymatic dissociation [145]. 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 had the same composition. The hearts were perfused with the same solution containing collagenase for 20–30 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 (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's solution. Cells were incubated in Tyrode's solution with various agents for 10 min at 37 °C prior to subsequent biochemical and immunofluorescence experiments.
Cell culture and transfection.

Expression plasmids. HA-tagged rat TASK1 (Genbank accession number AF031384) construct was a gift from Dr. Douglas A. Bayliss’s lab. A triple hemagglutinin (HA) epitope tag was added to the N terminus of wild-type channels in this construct. HA-tagged caveolin-3 mutant Cav-3 P104L was generously provided by Dr. Berthier’s lab [171]. TASK1, caveolin-3, and EGFP were all subcloned into mammalian expression vector pcDNA3, respectively. Cav-3 P104L mutant was subcloned into pCB6-KXHA constructs.

Cell culture and transfection. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin–streptomycin [146]. All constructs were transfected in HEK293T cells by using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Experiments were carried out 48 h after transfection.

Western blotting.

Proteins in whole-cell lysate was denatured in a sample buffer. Equal amounts of proteins was loaded and electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were incubated for 2 h at room temperature with primary antibodies. After washing,
the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system. For Co-IP, proteins were incubated with primary antibodies for 2 h at 4°C. Antigen-antibody complexes were captured by Dynabeads Protein A for overnight at 4°C. Beads were washed 3 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and analyzed by probing with various antibodies.

**Co-immunoprecipitation.**

Proteins in whole-cell lysate was 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 incubated for 2 h at room temperature with primary antibodies. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system. For Co-IP, proteins was incubated with primary antibodies for 2 h at 4°C. Antigen-antibody complexes were captured by Dynabeads Protein A for overnight at 4°C. Beads were washed 3 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE,
transferred onto nitrocellulose membranes and analyzed by probing with various antibodies.

**Immunofluorescence microscopy.**

As described previously, 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 of HEK293T cells was visualized with conventional Nikon fluorescence microscope and immunofluorescence of rat cardiomyocytes were visualized with confocal laser scanning microscopy. All images were analyzed using a background subtraction method offline.

**Electrophysiology studies.**
The membrane currents were recorded from HEK293T cells using the conventional whole cell configuration. In the TASK1 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, 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). Steady-state current voltage relations were obtained by applying voltage ramps between -100 mV to +50 mV. TASK1 currents were calculated as methanandamide-sensitive currents.

Data analysis.

Group data were presented as means ± SEM. Multiple group means were compared by one-way ANOVA followed by LSD post hoc test. A P-value of < 0.05 was considered as statistically significant.

RESULTS

TASK1 currents are pH-sensitive and methanandamide-sensitive

The current flowing through TASK1 channels in transfected HEK293T cells was quantified by analyzing the effects of extracellular pH on the steady-state current–voltage relation. Transfected HEK293T cells were seeded on coverslips
of a flow-through chamber, mounted on the platform of an inverted fluorescence microscope (Nikon Eclipse, TS100), and perfused with a bath solution of the composition shown in the Materials and methods. For HEK293T cells expressing TASK1, we also transfected pEGFP cDNA constructs. Only cells with green fluorescence were selected for electrophysiological studies. Voltage ramps from -100 mV to +50 mV over 2000 msec were applied to individual cells (Figure 3.1B). Current amplitudes were obtained at +50 mV while the pH of the bath solution was adjusted between pH 6.4 and pH 8.4. As depicted for representative cells (Figure 3.1A), extreme bath acidification (from pH 7.4 to pH 6.4) caused a sharp decrease in whole-cell currents in cells expressing TASK1 channel cDNA constructs while extreme alkalinization of bath solution (from pH 6.4 to pH 8.4) dramatically increased the whole-cell current amplitude (Fig. 1A-C). To further characterize TASK1 currents, we studied the effect of a specific TASK1 blocker, methanandamide, on the current–voltage relation of HEK293T cells transfected with TASK1 cDNA construct. A typical recording presented in Fig. 1D illustrates that TASK1 currents are reduced significantly by methanandamide as described previously. Application of 10 μM methanandamide inhibited steady-state outward currents by approximately 40% (Figure 3.1D, 3.1F). Methanandamide-sensitive current was obtained by subtracting current with methanandamide treatment (Figure 3.1E). The I-V relationship revealed a reversal potential of about -80 mV, close to the expected potassium equilibrium potential (-80~-90 mV), and outward rectification, which
is characteristic of TASK1 currents. These results are consistent with the known inhibition of TASK1 channels by extracellular acidification or specific inhibitor methanandamide and augmentation of TASK1 channels by extracellular alkalinization.

Figure 3.1 Current components sensitive to various pH and methanandamide. Current components sensitive to pH and methanandamide. HEK293T cells were transfected with TASK1 cDNA. (A), currents were evoked by ramping the membrane potential from -100 mV to +50 mV over 2000 msec as the pH value of the culture medium was varied. Current amplitudes measured at +50 mV are shown. (B) Representative current traces under different pH values. (C) Averaged current density as indicated, which was calculated by dividing current amplitude at +50 mV by cell capacitance. (D) Currents were evoked by ramping the membrane potential from -100 mV to +50 mV over 2000 msec without or with methanandamide. (E) Methanandamide-sensitive current was obtained by subtracting control current. (F) Averaged current density as indicated, which was calculated by dividing current amplitude at +50 mV by cell capacitance. In (C), **p<0.01 vs. pH=7.4, ***p<0.001 vs. pH=7.4, n=6-10. In (F), **p<0.01 vs. TASK1, n=6-10.
Figure 3.1

A

B

C

D

E

F

I (pA)

Time (min)

Current density (pA/pF)

0 5 10 15 20 25

pH=7.4

pH=6.4

pH=8.4

I (pA)

Time (ms)

Control

Meth

I (pA)

Time (ms)

Control

Meth

0 500 1000 1500 2000 2500

0 250

-250

0 10 15 25

0 500 1000 1500 2000 2500

-500 0

-500

75
**TASK1 currents are negatively regulated by caveolin-3 in HEK293T cells**

Functionally, Cav-3 is both an anchoring protein for molecules within caveolae and a regulatory element for protein signaling activities. Cav-3 acts as an organizer and modulates various downstream effectors including ion channels. It has been reported that several types of potassium channels locate within caveolar microdomain and can be regulated by caveolin proteins [23].

We hypothesized that TASK1 might be regulated by Cav-3. To test such a possibility, we performed electrophysiological studies in HEK29T cells transfected with either TASK1 alone or co-transfected with TASK1 and Cav-3 (Figure 3.2C). Similarly, voltage ramps from -100 mV to +50 mV over 2000 msec were applied to individual cells. Current amplitudes were obtained at +50 mV and averaged current density was calculated by dividing by averaged cell capacitance. The TASK1 outward current was largely decreased in cells co-expressing Cav-3 compared with non-Cav-3 transfected cells (Figure 3.2A, Figure 3.2B). The average peak outward current density at + 50 mV for TASK1 was 23.92±1.96 pA/pF (n=6-8), whereas TASK1/Cav-3 was 11.01±3.08 pA/pF (p<0.05, n=6-8). We have shown a significant, 54% decrease in TASK1 current density with Cav-3 co-transfection. Western blot bands indicated successful transfection of either TASK1 alone or TASK/Cav-3 in heterologous expression system (Figure 3.2C).
Expression of TASK1 channels in HEK293T cells and its association with caveolin-3

Due to the large decrease of TASK1 current amplitude when co-expressed with Cav-3, we proposed that Cav-3 modulates TASK1 functions possibly through direct or indirect association. To test this possibility, we first determined whether TASK1 associates with Cav-3 in HEK293T cells transfected with both TASK1 and Cav-3 cDNA constructs and only cells expressing both proteins were included. HEK293T cells were assayed for immunofluorescence 48 h post-transfection. Regions of interest were selected at the edge of the cell and also at punctuate staining patterns on the cell. As seen in Figure 3.3, Cav-3 and TASK1 were both localized as punctate staining along the cell periphery. The merged images showed significant areas of co-localization for Cav-3 and TASK1 along the cell periphery as yellow color.

In order to further confirm that the association of TASK1 and Cav-3, we also performed co-immunoprecipitation using cell lysates from HEK293T cells transfected with TASK1 and Cav-3 cDNA constructs. We also transfected cells with either TASK1 or Cav-3 construct respectively in separate experiments. Anti-TASK1 antibody was used to immunoprecipitate Cav-3 and anti-Cav-3 antibody was used to immunoprecipitate TASK1. The immunoblot analysis of a co-immunoprecipitation experiment showed that, in HEK293T cells, when
TASK1/Cav-3 co-transfected, TASK1 and Cav-3 associates, either directly or indirectly with each other (Figure 3.4). For HEK293T cells only expressed with TASK1 or Cav-3, no association were identified as we expected. Rabbit IgG was used as a negative control.
Figure 3.2  Current components decreased by caveolin-3 (Cav-3) overexpression. Caveolin-3 (Cav-3) overexpression decreases TASK1 current. HEK293T cells were transfected with either TASK1 or TASK1 plus Cav-3 cDNA constructs. (A) Currents were evoked by ramping the membrane potential from -100 mV to +50 mV over 2000 msec at pH 7.4. (B) Averaged current density as indicated, which was calculated by dividing current amplitude at +50 mV by cell capacitance. *p<0.05 vs. TASK1, n=6-8. (C) Representative Western blot bands showed that successful transfections of either TASK1 or TASK1/Cav-3 in HEK293T cells.
Figure 3.3  TASK1 is associated with caveolin-3 (Cav-3) in transfected HEK293T cells. TASK1 is associated with caveolin-3 (Cav-3) in transfected HEK293T cells. HEK293T cells were transfected with both TASK1 and Cav-3 cDNA constructs and assayed 48 h post-transfection. Representative images from HEK293T cells co-transfected with TASK1 and Cav-3 cDNA constructs are shown. Arrow indicates colocalization at cell peripheral areas as indicated by yellow color. The lower panel is an enlargement of the area outlined in the upper panel. Data were obtained from 3 independent experiments. Scale bar: 20 μm.
Figure 3.4  Co-immunoprecipitation of TASK1 and caveolin-3 (Cav-3) from transfected HEK293T cells. Co-immunoprecipitation of TASK1 and caveolin-3 (Cav-3). HEK293T cells were transfected with TASK1 and/or Cav-3 cDNA constructs and assayed 48 h post-transfection. Co-immunoprecipitation was performed with anti-TASK1 or anti-Cav-3 antibodies. Rabbit IgG was used as negative control. Data were obtained from 3 independent experiments.
**TASK1 currents are not affected by caveolin-3 mutant in HEK293T cells**

In order to further demonstrate that cell surface Cav-3 negatively regulates TASK1 current amplitude in HEK293T cells. One of the Cav-3 mutant types Cav-3 P104L which is unable to transport to the plasma membrane correctly was applied in the experiments. Expression of Cav-3 P104L caused significant reduction of Cav-3 cell surface expression level even when Cav-3 wild-type was also present [172]. In addition, Cav-3 P104L has been shown previously to accumulate in the Golgi [173]. Based on these findings, we assume that transfection of Cav-3 P104L would not have significant effect on TASK1 current amplitude. In various types of transfected HEK293T cells, steady-state current voltage relations were obtained by applying voltage ramps between -100 mV to +50 mV. Current amplitudes were obtained at +50 mV. IV relationships from TASK1/Cav-3 compared with TASK1 alone or Cav-3 P104L or wild-type Cav-3 plus Cav-3 P104L showed a significant decrease in TASK1 current density. The average peak outward current of TASK1/Cav-3 at + 50 mV was 11.01±3.08 pA/pF (n=6-8) whereas compared with TASK1 alone, the peak outward current was 23.92±1.96 pA/pF (p<0.05, n=6-8). Current densities of TASK1/Cav-3 P104L or TASK1/Cav-3/Cav-3 P104L do not have significant difference compared with TASK1 alone, 20.28±1.96 pA/pF, 21.02±1.62 pA/pF respectively (n=6-10) (Figure 3.5A, 3.5B). Interestingly, Cav-3 P104L or Cav-3 P104L/Cav-3 did not affect TASK1 whole cell expression. We verified this by performing
Western blot experiments using GAPDH as an internal control of protein expression (Figure 3.5C).
Figure 3.5  Current components unaffected by caveolin-3 mutant (Cav-3 P104L) overexpression. Caveolin-3 mutant (Cav-3 P104L) overexpression does not affect TASK1 current. HEK293T cells were transfected with either TASK1 plus wild-type Cav-3, TASK1 plus wild-type Cav-3 and Cav-3 P104L mutant, or TASK1 plus Cav-3 P104L mutant cDNA constructs. (A) Currents were evoked by ramping the membrane potential from -100 mV to +50 mV over 2000 msec at pH 7.4. (B) Averaged current density as indicated, which was calculated by dividing current amplitude at +50 mV by cell capacitance. *p<0.05 vs. TASK1, n=6-10. (C) Representative Western blot bands showed expression of TASK1, Cav-3 or Cav-3 P104L mutant in HEK293T cells. Data were obtained from 3 independent experiments.
**TASK1 is affected by caveolin-3 possibly mainly through functional modulation in HEK293T cells**

Given that Cav-3 P104L or Cav-3/Cav-3 P104L did not have functional consequences on TASK1 current density, we identified whether there are different expression patterns between wild-type Cav-3 and double transfection of wild-type Cav-3 and Cav-3 P104L in HEK293T cells. Intracellular Cav-3 P104L has been shown previously to accumulate in the Golgi [174]. Hence we investigated if Cav-3 P104L also cause the accumulation of wild-type Cav-3 when co-transfected together. In this section, heterologous expression system in HEK293T cells were also applied. From Figure 3.6A we clearly observed that wild-type Cav-3 was localized as punctate staining along the cell periphery of HEK293T cells while cell peripheral staining in HEK293T cells with Cav-3/Cav-3 P104L double transfection was largely decreased. In the middle panel of Figure 3.6A, decreased cell peripheral staining were observed in wild-type Cav-3 when co-expressed with Cav-3 P104L. This also caused decreased Cav-3 fluorescence intensity compared with wild-type Cav-3 alone. Above all, these results indicate that the negative regulation of Cav-3 on TASK1 is mainly through cell surface Cav-3 possibly via association with TASK1.

**Figure 3.6** The effect of mutant caveolin-3 (Cav-3 P104L) on wild-type caveolin-3 (Cav-3) expression level at cell peripheral area. The effect of
caveolin-3 mutant (Cav-3 P104L) on wild-type caveolin-3 (Cav-3) expression level at cell peripheral areas. HEK293T cells were transfected with Cav-3 or Cav-3 plus Cav-3 P104L cDNA. (A) Representative immunofluorescence images of Cav-3 without Cav-3 P104L transfection (left panel) or with Cav-3 P104L transfection (right panel). Scale bar: 10 μm. (B) Representative Western blot bands of either Cav-3 or Cav-3 P104L.

Figure 3.6
Expression of TASK1 channels in adult rat cardiomyocytes and its association with caveolin-3

The results shown above suggest that TASK1 and caveolin-3 interact with each other in HEK293T cells overexpressing both proteins. Based on these findings, we hypothesized that TASK1 may localize to caveolar microdomain in cardiac myocytes. To test this possibility, freshly isolated adult rat cardiac myocytes were used in this study. As shown in Figure 3.7, caveolin-3 was localized as punctate staining along the cell peripherals, T-tubular-like structures and cell-to-cell contact which is similar to TASK1. The merged images showed significant areas of colocalization for caveolin-3 and TASK1 along the T-tubular-like structures and cell-to-cell contact as yellow color.

We performed co-immunoprecipitation assay in the cell lysates from cardiac myocytes in order to further confirm that the association of TASK1 and caveolin-3 in cardiomyocytes. Similar to assays for HEK293T cells shown above, cell lysates containing equal amounts of total proteins were immuno-precipitated with either anti-caveolin-3 antibody or anti-HA antibody. The immune complex was collected with Dynabeads Protein A. Samples were resolved, transferred and analyzed by immunoblotting with antibody against caveolin-3 and HA tag. As shown in Figure 3.8, TASK1 co-precipitated with caveolin-3 and vice versa. Our data suggests that TASK1 associates with caveolin-3 in adult rat cardiac myocytes.
Figure 3.7 TASK1 is associated with caveolin-3 (Cav-3) in adult rat cardiomyocytes. (A) Representative confocal images from freshly isolated adult rat cardiomyocytes. Arrows indicated colocalization which was shown as yellow color. The lower panel is an enlargement of the area outlined in the upper panel. Data were obtained from 3 independent experiments. Scale bar: 20 um.
Figure 3.8 Co-immunoprecipitation of TASK1 and caveolin-3 (Cav-3) from freshly isolated adult rat cardiomyocytes. Co-immunoprecipitation was performed with anti-TASK1 or anti-Cav-3 antibodies. Rabbit IgG was used as negative control. Data were obtained from 3 independent experiments.
Discussion

In this study, we present the first report of the modulation of Cav-3 on TASK1 and their association. Cav-3 reduces TASK1 current amplitude possibly via the association of Cav-3 and TASK1. We also demonstrated this association in adult rat cardiomyocytes. We investigated the effect of Cav-3 on TASK1 by studying TASK1 current amplitude via electrophysiological technique and, for comparison, a Cav-3 mutation, P104L, associated with limb girdle muscular dystrophy [171, 172]. This mutation did not decrease TASK1 current density, compared with TASK1, when co-expressed with wild-type Cav-3. As demonstrated by multiple complementary techniques, our data showed a physical correlation between TASK1 and Cav-3 in heterologous expression system by using co-immunoprecipitation, immunofluorescence. Taken together, our data support negative modulation of Cav-3 on TASK1 and a relevant association between TASK1 and Cav-3. Given the importance of TASK1 in the maintenance of cell resting membrane potential, inhibition or reduction of TASK1 causes unstabilization of cell membrane potential which might lead to excessive excitability, cell death from excitotoxicity especially under pathophysiological conditions.

We hypothesized that Cav-3 may be a channel-associating protein of TASK1. 14-3-3 and p11 proteins are essential for TASK1 trafficking to cellular
membrane [88, 89, 175-177] and both proteins can associate with Cav-3 [15, 170] which might leads to functional modulation of Cav-3 on TASK1 [15, 89, 178]. We then speculated that TASK1 might be modulated by Cav-3 via direct or indirect association. The first step to answer the above questions was to investigate whether cell surface Cav-3 modulates TASK1. To test the hypothesis that the cell surface Cav-3 have functional modulations on TASK1, we investigated the co-expression of TASK1 with wild-type Cav-3 or Cav-3 P104L and compared them with TASK1 co-expressed with empty vector. In Figure 3.2, we demonstrated that Cav-3 reduced TASK1 current amplitude. This finding broadens the scope of regulations of TASK1, to include TASK1 with channel-associating and regulatory proteins such as Cav-3. Whether there are other components contributing to the modulation of TASK1 by Cav-3 is not fully known but is important, and will be fully discovered in the future studies. In our study, Cav-3 P104L and wild-type Cav-3 had a different cellular localization pattern (Figure 3.6). Cav-3 P104L accumulate intracellularly while had much less expression along cell peripheral areas [41]. This was not surprising for Cav-3 P104L because it has been reported previously that Cav-3 P104L is trafficking-defective and accumulates in the Golgi compartment of cells [171, 172]. We chose to include this particular mutation in our study in order to identify the whether modulation of TASK1 by Cav-3 is basically through cell surface Cav-3. We investigated this by utilizing electrophysiological technique patch clamp on different transfected HEK293T cells (Figure 3.5). TASK1/Cav-3
co-expression showed a decrease in current amplitude as shown above but TASK1/Cav-3 P104L or TASK1/Cav-3/Cav-3 P104L did not decrease TASK1 current amplitude significantly. This unchanged current amplitude further demonstrated that wild-type Cav-3 which has the ability to traffick to the cell membrane reducing TASK1 currents. On the contrary, Cav-3 P104L or Cav-3/Cav-3 P104L, which are traffick-defective, do not affect TASK1.

The overexpression of the ion channels of interest in a heterologous expression system is useful for studying electro-physiological properties. The most advantage is that we can obtain much larger current amplitude than that through endogenous TASK1 channels. In addition, HEK293T cells, do not express any endogenous caveolins which Cav-3 can be expressed exogenously in order to analyze its functional modulation on other proteins. Only a few endogenous currents were identified in HEK293T which are much smaller than overexpressed current amplitude facilitating our analysis on TASK1 currents. Thus, they were chosen for our modeling system.

The next step is to determine whether the modulation of TASK1 by Cav-3 is based on the association of these two proteins. As shown in Figure 3.3 and Figure 3.4, Cav-3 and TASK1 co-localize in a heterologous expression system as shown by immunofluorescence. Areas of strong co-localization were found along cell peripherals. Co-localization also were found out in adult rat
cardiomyocytes which were along cell peripherals, T-tubules-like structure and cell-to-cell contact (Figure 3.7). We complemented this part of research by performing co-immunoprecipitation of Cav-3 and TASK1. Our co-immunoprecipitation result also indicated an association between these two proteins. These results pointed out that the effect of Cav-3 on TASK1 is probably mainly through association with each other. However, whether wild-type Cav-3 has any significant effects on TASK1 expression along cell peripheral areas needs to be further identified. The result may suggest whether major effect of Cav-3 on TASK1 is due to its surface protein modulation. This may also indicate whether modulatory mechanism occur before the channel reaches the membrane. In other words, whether Cav-3 play a role in modulating the trafficking of TASK1 will be identified in the future studies.

TASK1 interaction with 14-3-3 and/or p11 may be important for targeting newly synthesized TASK1 proteins to specific microdomains, such as caveolar lipid rafts [88, 89, 170, 176, 177]. The relationship between Cav-3 and TASK1 is a crucial step in understanding the components of the possible TASK1 macromolecular complex, regulation, and modification. Dimers of TASK1 make up the predominant component of TASK1 channel. However, TASK3 also contribute to the formation of TASK channels and can create homotetramers with itself [78] or heterotramers with TASK1 [79, 179]. It is possible that Cav-3
associates with TASK3 or TASK1/TASK3 heterodimers. This association, as well as the functional effects need to be further identified in the future studies.

The two pore domain potassium channel family is a recently identified family of potassium-selective channel family. They are neither voltage-gated nor time-dependent which means they open even at resting membrane potential [165]. This could let them help set the membrane potential and contribute to membrane repolarization in excitable cells [165]. TASK1 is one of their important members which has been found abundantly in neurons and cardiac cells [46, 84, 100, 180, 181]. Opening of TASK1 channels increases K⁺ leak currents which stabilizes cells at hyperpolarized voltages below the firing threshold of nerves or muscles whereas loss or close of TASK1 channels leads to depolarization and excitation which causes cell death mainly due to excitotoxicity. Thus, TASK1 is critically involved in the control of excitability and contribute to the cell protection especially during ischemia when cell membrane potential are easily to depolarize [120]. In this report, we have shown that Cav-3 decreases TASK1 currents. In order to protect cardiac cells during ischemia, the expression level of Cav-3 would be enhanced due to its significance in cardio-protection [63]. This might further decrease TASK1 current amplitude which eventually leads to more cell death. Whether this side-effect of increasing Cav-3 during ischemia is balanced by other protective effectors associated with Cav-3 needs to be elucidated in the future studies. It is important to know more about
modulation of TASK1 channels. Thus, implications of the association of these two important cardiac proteins may extend our knowledge of cardioprotection.

In summary, we have demonstrated for the first time that Cav-3 causes the reduction of TASK1 current amplitude via association between Cav-3 and TASK1. This has important implications for the maintenance of cell membrane potential and the whole cell stability.
CHAPTER 4

TASK1 is regulated by hypoxia-induced protein kinase C signaling

Abstract

TASK1 is a member of the two-pore domain potassium channel family that is pH and O$_2$ sensitive. It is strongly expressed in the heart and functions as a background potassium channel. In addition to its modulation by caveolin-3, we were further interest in investigating the molecular mechanisms by which hypoxia regulates TASK1 channels. We examined the role of protein kinase C (PKC) in metabolic inhibition-induced modulation of TASK1 channels in adult rat cardio- myocytes. TASK1-like currents of rat cardiac cells were significantly reduced by metabolic inhibition with the metabolic inhibitor sodium cyanide (NaCN). However, this effect was largely prevented by pretreatment with the PKC selective inhibitor bisindolylmaleimide (BIM), indicating PKC-mediated phosphorylation may play a role in the NaCN-induced reduction of TASK1 activities. Similar results were obtained from HEK293T cells with TASK1 expression. Moreover, we also identified the protective role of TASK1 during
hypoxia. Hypoxia was induced by incubating cells in an airtight chamber in which O$_2$ was replaced by N$_2$ with glucose-free Tyrode’s solution. Cell viability assay after prolonged hypoxia (4 hours) was conducted. While hypoxia caused a significant reduction in the viability of cardiomyocytes, the inhibition of TASK1 channels by the selective inhibitor methanandamide further increased hypoxia-induced death of cardiomyocytes. Similar results were obtained from HEK293T cells. In addition, overexpression of TASK1 in HEK293T cells significantly decreased the number of cell death when compared with cells without TASK1 expression, indicating a potential cardioprotective role of TASK1. Western blot analysis showed that 4 hours of hypoxia led to a decrease in the protein expression of TASK1 in cardiomyocytes, suggesting suppressed expression of TASK1 during hypoxia may contribute, at least in part, to the hypoxia-induced cell death. In summary, we provided the evidence that TASK1 is negatively regulated by hypoxia or metabolic inhibition, possibly via activation of PKC isoforms. Our data are supportive for a possible role of TASK1 channels in the cardioprotection against hypoxia.
INTRODUCTION

Two pore domain potassium channels which are also know as leak channels are major determinants of excitable cell membrane potential and its excitability [118, 119]. Channels are composed of two monomers, each with two pore-forming domains, with each monomer contributing two pores [75] which is unique to its name two pore domain potassium channels and different from other types of potassium channels. There are 15 members in this family. One of its members, TASK1 channels are widely expressed, with enriched expression reported in motor neurons [79, 91], cerebellar granule neurons [91, 182], the carotid body [179] and heart [46, 84, 100]. TASK1 channels are acid-sensitive (activated by alkalinization and inhibited by acidification) [46, 168] and are activated by volatile anesthetics [183]. Opening of TASK1 channels decreases spontaneous neuronal firing rates [183-185]. Studies with TASK1 (−/−) mice demonstrated that TASK1 is critical for neuroprotection during stroke [120]. Taken together, TASK1 plays a major role in protecting cells from damage through the major role of preserving cell resting membrane potential so that alterations of TASK1 functions and availability are likely to have a significant systemic impact on cell viability especially during pathophysiological conditions such as ischemia/hypoxia.
Multiple regulatory or scaffolding proteins control TASK1 maturation and channel surface expression. There are some proteins contributing to the upregulation of TASK1 expression at cell membrane areas. *In vitro* studies demonstrate that the C-terminal of TASK1 phosphorylation by PKA is related to the enhancement of TASK1 cell surface expression, probably by increased forward trafficking from the ER to the cell membrane [186]. 14-3-3 and p11, for example, bind to the TASK1 C-terminus directly and increase TASK1 cell surface expression [87, 89, 176, 177]. On the contrary, βCOP can bind to the the N terminus of TASK1 and prevents egress from the ER and Golgi to the plasma membrane [87, 88].

Abundant evidence demonstrates that PKC subtypes, especially PKCε decrease TASK1 channel activities by phosphorylating specific C terminal sites [83-85]. In addition, TASK1 channels are acutely inhibited during ischemia/hypoxia but the underlying mechanisms mediating TASK1 inhibition are not clear. Given the importance of TASK1 in the maintenance of cell resting membrane potential and cell protection especially in ischemia/hypoxia, we investigated the potential role of TASK1 in cardiac hypoxia. In the current study, we showed that TASK1 was inhibited by hypoxia mimicked by metabolic inhibition mainly through activation of PKC. Moreover, we demonstrate that inhibition of TASK1 channels in adult rat cardiomyocytes during long term hypoxia largely decrease cell viability while overexpression of TASK1 in
HEK293T cells prevented more damage compared with non-transfected ones. Taken together, these results demonstrate that TASK1 is crucial for preventing cell death during hypoxia and the suppression of TASK1 channels during hypoxia is mainly through activation of PKC which may lead to a new target in protecting cardiac cells from damage during hypoxia.

MATERIALS AND METHODS

Materials.

Methanandamide, 4-AP, CdCl₂, TEA and NaCN were purchased from Sigma-Aldrich (St. Louis, MO, USA). 0.4% trypan blue was obtained from GIBCO (Grand Island, NY, USA). Methanandamide, CdCl₂, TEA and NaCN were dissolved in deionized water. 4-AP was dissolved in dimethyl sulfoxide (DMSO), which did not exceed a final concentration of 0.1%.

Isolation of cardiomyocytes.

Adult rat ventricular myocytes were isolated from male Sprague–Dawley rats aged 2 to 3 months (young) by enzymatic dissociation [145]. 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 Ca$^{2+}$ free but otherwise had the same composition. The hearts were perfused with the same solution containing collagenase for 20–30 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 (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’s solution. Cells were incubated in Tyrode’s solution with various agents for 10 min at 37 °C prior to subsequent biochemical and immunofluorescence experiments.

*Cell culture and transfection.*

*Expression plasmids.* HA-tagged rat TASK1 (Genbank accession number AF031384) construct was a gift from Dr. Douglas A. Bayliss’s lab. A triple hemagglutinin (HA) epitope tag was added to the N terminus of wild-type channels in this construct. HA-tagged caveolin-3 mutant Cav-3 P104L was generously provided by Dr. Berthier’s lab [171]. TASK1, caveolin-3, and EGFP were all subcloned into mammalian expression vector pcDNA3, respectively. Cav-3 P104L mutant was subcloned into pCB6-KXHA constructs.
**Cell culture and transfection.** HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin–streptomycin [146]. All constructs were transfected in HEK293T cells by using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Experiments were carried out 48 h after transfection.

**Induction of hypoxia.**

The general experimental protocols employed was described as hypoxia was induced by incubating the cells in an airtight chamber in which O₂ was replaced by N₂ with glucose-free Tyrode’s solution that contains (in mmol/L) 139 NaCl, 4.7 KCl, 0.5 MgCl₂, 1.0 CaCl₂, and 5 HEPES, pH 7.4, at 37°C for 4 hrs.

**Cell viability assay.**

Centrifuge an aliquot of cell suspension being tested for viability 5 min at 5000 × g and discard supernatant. The aliquot should contain a convenient number of cells to count in a hemacytometer when suspended in 1 ml PBS and then diluted again by mixing with 0.4% trypan blue (e.g., 5 × 10⁵ cells/ml). Resuspend the cell pellet in 1 ml PBS or serum-free complete medium.
Mix 1 part of 0.4% trypan blue and 1 part cell suspension (dilution of cells).
Allow mixture to incubate ~3 min at room temperature. Mixing can be performed in a well of a microtiter plate or a small plastic tube using 10 to 20 µl each of cell suspension and trypan blue. Apply a drop of the trypan blue/cell mixture to a hemacytometer. Place the hemacytometer on the stage of a binocular microscope and focus on the cells. Count the unstained (viable) and stained (nonviable) cells separately in the hemacytometer. To obtain the total number of viable cells per ml of aliquot, multiply the total number of viable cells by 2 (the dilution factor for trypan blue). To obtain the total number of cells per ml of aliquot, add up the total number of viable and nonviable cells and multiply by calculate the percentage of viable cells as follows:

Viable cells (%) = total number of viable cells per ml of aliquot /

    total number of cells per ml of aliquot × 100

Electrophysiology studies

The membrane currents were recorded from adult rat cardiomyocytes or HEK293T cells using the conventional whole cell configuration. In the TASK1 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, 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). Steady-state current voltage relations were obtained by applying voltage ramps between -100 mV to +50 mV. In adult rat cardiomyocytes, cells were held at 0 mV and prepulsed to +40 mV in order to inactivate voltage-activated Ca²⁺, Na⁺ and K⁺ channels. 4-AP, CdCl₂ and TEA were included in the bath solution to inhibit the residual voltage-activated channels. Steady-state current voltage relations were obtained by applying voltage ramps between +40 mV to -100 mV. 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). TASK1 currents were calculated as methanandamide-sensitive currents.

Data analysis.

Group data were presented as means ± SEM. Multiple group means were compared by one-way ANOVA followed by LSD post hoc test. A P-value of < 0.05 was considered as statistically significant.
RESULTS

*pH-sensitive currents are down-regulated by extracellular acidification and up-regulated by alkalinization in cardiomyocytes*

We first tried to quantify currents which are pH-sensitive in rat ventricular cardiomyocytes by analyzing the effects of extracellular pH on the steady-state current-voltage relation. Since mounting evidence has shown that TASK1 channels can be activated by alkalinization and blocked by acidification, the first step is to identify pH-sensitive currents. The voltage protocol was designed to inactivate voltage-dependent ion channels. Freshly isolated cardiomyocytes were used for the study. Cells were held at 0 mV and prepulsed to +40 mV in order to inactivate voltage-activated Ca$^{2+}$, Na$^+$ channels. In addition, we used a variety of blockers which included 4-AP (2.5 mM), CdCl$_2$ (1 mM) and TEA (10 mM). The blockers were included in the bath solution to inhibit the residual voltage-activated channels. In the presence of the blockers, outward currents were observed and recorded during the initial voltage clamp step to +40 mV. Previous research has shown that these blockers had no effects on TASK1 channels expressed in Xenopus oocytes [46]. In the presence of the blockers, extracellular acidification to pH 6.3 reduced the outward current measured at potential + 40 mV (Figure 4.1) while pH 8.4 increased the outward current measured at the same potential (Figure 4.1). The pH-sensitive currents were
subtracted from pH 7.4 with pH 6.4 treatment group (data not shown). The pH-sensitive currents also showed reversal potentials around -80 mV which is the typical reversal potential of potassium channels. These findings are consistent with blockage of TASK1-like channels by extracellular acidification and promotion of TASK1-like channels by extracellular alkalinization which should give a good approximation of the amplitude of TASK1 currents.

**Figure 4.1  Current components sensitive to various pH in cardiomyocytes.** Freshly isolated adult rat cardiomyocytes were treated with various pH. (A) Time course of current amplitudes at +40 mV under various pH treatments. (B) Representative ramp traces from (A). (C) Relative current density as indicated, averaged current density was calculated by dividing current amplitude at +40 mV by averaged cell capacitance. *p<0.05 vs. pH 8.4, #p<0.05 vs. pH=7.4, n=6. Data were given as mean ± S.E.M.
Figure 4.1

A

I (pA) vs Time (min)

pH=7.4  pH=6.4  pH=8.4

B

I (pA) vs Time (ms)

pH=8.4  pH=7.4

C

Current density (pA/pF)

pH 7.4  pH 6.4  pH 8.4
Effect of TASK1 channel blocker methanandamide on steady-state outward currents in cardiomyocytes

To further characterize TASK1-like currents and to test whether TASK1-like channels might generate a measurable whole-cell current, we studied the effect of a TASK1 channel specific blocker, methanandamide, on the current-voltage relation of rat cardiomyocytes (Figure 4.2). As a first step, we analyzed the pharmacological properties of methanandamide by measuring its effects on steady-state outward currents in rat cardiomyocytes. The typical recording presented illustrated that methanandamide-sensitive currents were generated by perfusing methanandamide as described previously [15, 22]. Application of 10 μM of methanandamide inhibited steady-state outward currents by approximately 15%.

The effects of methanandamide on the current-voltage relation of cardiac cells in the presence of the blockers were shown in Figure 4.2A. At potentials positive to +40 mV, application of 10 μM methanandamide inhibited outward currents that were similar to the pH-sensitive outward currents. The methanandamide-sensitive currents also showed reversal potentials around -80 mV which is the typical reversal potential of potassium channels (data not shown). These findings are consistent with blockage of TASK1 channels by its specific inhibitor methannandamide which were reported before [79, 187, 188].
Taken together, the data described above support the idea that the pH-sensitive currents and methanandamide-sensitive currents measured in the presence of the blockers consisted mainly of TASK1 component.

*TASK1-like currents are down-regulated by metabolic inhibitor sodium cyanide in cardiomyocytes*

Tissue acidosis and hypoxia are pathogenetic hallmarks of ischemia and occur within minutes after cessation of cerebral blood flow. We therefore want to analyze the effects of acute ischemia on the electrophysiological properties of TASK1 channels *in vitro*. In order to confirm the effects of acute ischemia on the electrophysiological properties of TASK1 channels *in vitro*, freshly isolated adult rat cardiomyocytes were used in the study. We also used NaCN plus Tyrode’s solution with 2-deoxyglucose to induce metabolic inhibition which mimicked acute ischemia. Throughout the experiments, methanandamide was present in order to demonstrate the blockage of ion channels was mainly from TASK1. Current-voltage relationships (I/V) were investigated by ramping the membrane potential from +40 mV to -100 mV. When the perfusate was added into NaCN, it significantly reduced the steady-state outward currents compared with control levels at + 40 mV potential (Figure 4.2B). NaCN-sensitive currents showed reversal potentials around -80 mV which is the typical reversal potential of potassium channels. The current amplitude plotted over time also
showed a typical U-shape indicating the change of outward current amplitude under NaCN perfusion. The effect of NaCN can be reversed by perfusing cells with normal physiological solution. Methanandamide was applied to further demonstrate TASK1 identity in NaCN-sensitive currents. After perfused NaCN with the cells, we changed the solution into NaCN plus methanandamide. As we expected, NaCN did not cause more significant reduction to the outward current since most of TASK1 channels were blocked by methanandamide.
Figure 4.2  Current components sensitive to methanandamide and sodium cyanide were mainly TASK1-like currents in cardiomyocytes. Cells were held at 0 mV and currents were evoked by ramping the membrane potential from +40 mV to -100 mV to inactivate voltage-gated K+, Ca2+ and Na+ channels. Also, residual voltage-gated K+ channels were inhibited by 4-AP (2.5 mM) and TEA (10 mM) while residual voltage-gated Ca2+ channels were blocked by CdCl2 (1 mM). (A) Representative time course of current amplitude under methanandamide treatment. 10 μM methanandamide were perfused in the solution. Currents were evoked by ramping the membrane potential from +40 mV to -100 mV and measured at +40 mV. (B) Representative time course of current amplitude under NaCN treatment. 5 mM NaCN were perfused in the solution. (C) Representative time course of current amplitude under methanandamide or methanandamide plus NaCN treatment. (D) Relative current density as indicated, which was calculated by dividing current amplitude at +40 mV by averaged cell capacitance. *p<0.05 vs. Control, n=7~8. Data were given as mean ± S.E.M.
TASK1-like currents were down-regulated by metabolic inhibition mainly through PKC in cardiomyocytes

We next investigated the underlying mechanisms of metabolic inhibition-induced TASK1 current reduction. We proposed that PKC might be the important player causing the reduction of TASK1 currents during the metabolic inhibition treatment [83-85, 189]. To test this possibility, we asked whether PKC mediated down-regulation of TASK1-like currents in cardiomyocytes, by measuring the whole cell outward currents in the presence of PKC specific inhibitor—BIM. The individual traces shown in Figure 3 illustrated the absence of decrease in current amplitude with NaCN perfusion which was pretreated with 1 μM BIM for 5 min. Therefore, we found out that the decrease in current amplitude with NaCN perfusion was prevented by BIM which inactivates PKC. The average decrease in current amplitude over time can be observed and contrasted with currents recorded in the absence of BIM. These results suggested that the NaCN-induced reduction in TASK1-like currents was mainly due to the activation of PKC.

Taken these together, the above results indicated that ischemia which was mimicked by NaCN-induced reduction, caused the decrease of TASK1 currents mainly through the activation of PKC (Figure 4.3).
Figure 4.3  Current components sensitive to sodium cyanide and its loss-of-sensitivity after treated with BIM in cardiomyocytes. (A) Time course of current amplitudes at 0 mV under BIM (100 nM) plus NaCN (5 mM) treatment. (B) Representative ramp traces from A. Currents were evoked by ramping the membrane potential from +40 mV to -100 mV. (C) Relative current density as indicated, which was calculated by dividing current amplitude at +40 mV by averaged cell capacitance, n=9. Data were given as mean ± S.E.M.
Protective role of TASK1 in prolonged hypoxia in cardiomyocytes or HEK293T cells

We next investigated functional roles of TASK1 channels in vitro during experimental ischemia mimicked by long-term hypoxia. In the first set of experiments we analyzed the effect of TASK1 channels on cell protection by placing cardiomyocytes in an air-tight chamber full of nitrogen for 4 hours before further assessment. Hypoxia caused a much more damage to the cells compared with non-treatment group. To demonstrate the protective role of TASK1 channels on cardiomyocytes during hypoxia, we also used methanandamide to block TASK1 and after that we observed more damage occurred than non-methanandamide treatment group (Figure 4.4A).

In the second set of experiments we induced hypoxia by placing HEK293T cells in an air-tight chamber full of nitrogen for 4 hours. This leads to enormous cell death (Figure 4.4B). In contrast, group with cells over-expressed TASK1 channels had higher cell viability after long-term hypoxia. To further prove specificity of methanandamide actions on TASK1 channels, we applied methanandamide to HEK293T cells with TASK1 overexpression and observed the prevention of protection from TASK1. In addition, methanandamide did not influence cell viability when cells were not suffered from hypoxia.
In addition, long-term hypoxia caused TASK1 protein expression in whole cell largely decreased when we detected proteins by using Western blot analysis (Figure 4.5) which probably accounted for the significantly lower cell viability after 4 hour hypoxia treatment.

Taken together, these findings indicated that TASK1 channels critically contribute to the limitation of tissue damage during ischemia.
**Figure 4.4** Cell viability assays for cardiomyocytes or HEK293T cells under different treatment. Cell viability assays were conducted by trypan blue. (A) Cell viability assay for cardiomyocytes under different treatment. (B) HEK293T cells either transfected with TASK1 cDNA or not (non-transfection, NT) were used in the study. For hypoxia group, cells were treated with 4h-hypoxic treatment. In (A), ***p<0.001 vs. Control, ###p<0.001 vs. Hypoxia, n=3. In (B), **p<0.01 vs. TASK1+Hypoxia, ***p<0.001 vs. TASK1+Hypoxia, n=3.
Figure 4.5  TASK1 expression with or without hypoxia in adult rat cardiomyocytes. Representative Western blot bands of TASK1 with or without hypoxic treatment. GAPDH was used as loading control. Results were data from 3 independent experiments. *p<0.05 vs. Control, n=3.
DISCUSSION

Our findings first demonstrated that TASK1-like channels plays a protective role in the cardiac ischemia mimicked by cardiac hypoxia. We found that TASK1-like channels were blocked by hypoxia mimicked by metabolic inhibition and this inhibition was mainly through the activation of PKC. During long-term hypoxia, the inhibition of TASK1-like channels by administration of methanandamide -- a specific inhibitor acting on TASK1 channels -- resulted in accelerated larger amount of cell death. Moreover, overexpression of TASK1 channels alleviated the damage to the cells during hypoxia. In the presence of the inhibitor methanandamide treatment again, it resulted in significantly decrease in cell viability which further indicate that methanandamide predominantly altered TASK1-like channel function and TASK1-like channel plays an important role in protecting cells from hypoxic damage.

It is widely accepted that cardiovascular system have developed a broad array of immediate and long term homeostatic protective mechanisms “on demand” to suppress hyperexcitability induced by brief hypoxic and/or ischemic episodes [40]. Other potassium channels such as $K_{ATP}$ channels [112, 113, 190] or the voltage-gated potassium channel $I_{K1}$ [110, 191] could already be linked to the maintenance of cell resting membrane potential which contribute to the depression of cell excitability during hypoxia/ischemia. Previous research have
shown that TASK1 channel which is one of the members of two pore domain potassium channel family, plays a protective role in lessening the injury from cerebral ischemia [192] and stroke development in mice [120]. Since the identification of TASK1 and its abundancy in cardiomyocytes [46], we are now focusing on its potential role in cardioprotection during ischemia. Based on our above studies, we now add evidence that TASK1 plays an important role against ischemic heart injury. Thereby the hyperpolarizing effect of the functional leak potassium channel probably represents one major mechanism [164, 193]. This is corroborated by the findings that TASK1-like channels were inhibited in experimental settings by hypoxia mimicked by metabolic inhibition induced by NaCN. Although we observed that inhibition of TASK1-like channels caused much more damage to the cell viability while over-expression of this channel lessened the damage effects of hypoxia on the cells, the detailed mechanisms by which TASK1 channels mediate cardioprotection need to be further elucidated and, besides their hyperpolarizing effect, might include other unknown mechanisms as well.

Although TASK1 channels are widely expressed in the heart as demonstrated by PCR and protein assays [46, 194], there is quite a few research focusing on its role in cardioprotection in hypoxia. Our electrophysiological data has shown that TASK1-like currents were decreased by hypoxia mimicked by metabolic inhibition and this reduction was might largely responsible for the severe cell
death in the cell viability experiments mentioned above. Opening TASK1 channels during hypoxia might prevent severe cell death. Thus, TASK1 might be a new promising target for the future study regarding to cardioprotection during hypoxia [120]. Characteristic features of the TASK1-like channel mediated currents are outward rectification [78], reversal near the K⁺ equilibrium potential and a characteristic pharmacological profile, e.g. current inhibition by pH-lowering [46], or methanandamide [86], increasing by high pH [180] which were all demonstrated by our experiments.

We investigated one of the key components playing a pivotal role in hypoxia mimicked by metabolic-inhibition which induced TASK1 current reduction. Most evidence suggests that the effects of acute hypoxia on cardiomyocyte ion channels are due to a change in the phosphorylation state of the channel or in the redox status of the cell. And previous research indicated that the reduction in VGCC activity occurs both in native cardiomyocytes and in HEK cells expressing a recombinant protein where phosphorylation of the channel by protein kinase A (PKA) augments the current and prevents inhibition by hypoxia [195, 196]. TASK1 channels can also be activated by PKA [186] while numerous studies suggest its inhibiton by phosphorylation is from PKC especially PKC ε [83-85, 189]. Thus, we were interested in whether the activation of PKC is the fundamental element for the reduction or inhibiton of TASK1 during hypoxia. We first demonstrated that the reduction of outward
currents caused by metabolic inhibition was mainly through the inhibition of TASK1-like channels. While we observed the decrease of outward currents during NaCN treatment, we did not notice further significant reduction of the current amplitude by perfusing TASK1 specific inhibitor -- methanandamide which implies most of the TASK1-like channels were inhibited during NaCN treatment. Then, we applied PKC specific inhibitor BIM before any other treatment. Interestingly, after the treatment of BIM, there was no obvious reduction of outward currents when perfused with NaCN. Collectively, these findings suggest that the PKC is the key factor for the reduction/inhibition of TASK1-like channels during hypoxia in cardiomyocytes. However, whether there are other effectors have influence on TASK1-like channel activities during metabolic inhibition need to be illustrated in the future studies. Interestingly, we know that Cav-3 decrease TASK1 channels from Chapter 3 and activated PKCε translocates to Cav-3 in order to elicit downstream effectors from Chapter 2. We can make an assumption that activated PKCε translocates to Cav-3 which facilitates its suppression on TASK1 channels in cardiac cells during hypoxia. Therefore, Cav-3, PKCε and TASK1 are all key factors in preventing cell death during hypoxia. Future studies will focus on testifying these promising targets.

In summary, this work provides evidence for a major involvement of TASK1 against hypoxia in cardiomyocytes. Our in vitro experiments indicate that inhibition of TASK1-like channels accelerates and worsens hypoxic damage. In
addition, metabolic inhibition-mimicked hypoxia reduced TASK1-like currents via activation of PKC. Thus, stabilization of TASK1 channel function appears as a novel therapeutic target to protect heart from hypoxic damage.
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

We first demonstrate that adenosine-induced translocation of PKCε to mitochondria is mediated by caveolin-3-dependent PKC signaling and this process is age-related, possibly through regulation of HSP90 and TOM70 expression. Moreover, we identified that TASK1 is functionally regulated by caveolin-3 probably mainly through association with Cav-3 and TASK1-like channels plays a protective role in cardiac hypoxia. Furthermore, it has been illustrated that TASK1-like channels were blocked by hypoxia mimicked by metabolic inhibition and this inhibition was mainly through the activation of PKC.

The work presented in this thesis is not only important for a better understanding of downstream signaling pathways involved in ischemic preconditioning but also modulation of potassium channels critical of protecting cardiomyocytes against myocardial hypoxia. Our first observations may have important implications in cardioprotection associated with mitochondrial targeting of PKCε and its age-dependency. The study has demonstrated that
adenosine-induced PKCε translocation to mitochondria is through a caveolin-3-dependent and adenosine A2B receptor/PI3K-independent pathway. We and others have demonstrated that PKCε translocates to mitochondria in response to the activation of adenosine A1 receptors or ischemia followed by reperfusion [106, 107]. It is believed that PKCε in mitochondria is critical for the cardioprotection of preconditioning [58, 128, 151]. Future studies will focus on investigating other possible effectors involved in the translocation process. By identifying underlying mechanisms of PKCε translocation to mitochondria upon activation of A1 receptors, it might provide potential effectors which elicit IPC effectively and efficiently during future therapeutic studies.

In the following study, our data supports a relevant association and interaction between TASK1 and Cav-3. In addition to this, our findings also demonstrated that TASK1 channels are critically involved in the cardiac hypoxia and contributing to cardio-protection during hypoxia. We found that TASK1 channels were blocked by metabolic inhibition induced hypoxia and this inhibition was mainly through the activation of PKC. The two pore domain potassium channel family is a recently identified family of potassium-selective channel family. They are neither voltage-gated nor time-dependent which means they open even at resting membrane potential [165]. This could let them help set the membrane potential and contribute to membrane repolarization in excitable cells [165]. TASK1 is one of their important members which has been found abundantly in
neurons and cardiac cells [46, 84, 100, 180, 181]. Opening of TASK1 channels increases K⁺ leak currents which stabilizes cells at hyperpolarized voltages below the firing threshold of nerves or muscles whereas loss or close of TASK1 channels leads to depolarization and excitation which could contribute to cell death mainly due to excitotoxicity. Thus, TASK1 is critically involved in the control of excitability and contributing to protection during ischemia [120]. We proposed that the loss of TASK1 function is involved in cell death during heart ischemia or hypoxia. Therefore, it is important to know more about modulation of TASK1 channels. In this report, we have shown that TASK1 is negatively regulated by Cav-3 probably based on their association at cell membrane. It could be protential target for decreasing cell death during ischemia if we find out a way to suppress the inhibiton of Cav-3 on TASK1. Hence, implications of the association of these two important cardiac proteins may extend our knowledge of cardioprotection. Furthermore, we know that TASK1 is inhibited by localizing to Cav-3 and activated PKC translocates to Cav-3 in cardiomyocytes in order to facilitate inducing downstream effectors. It is very obvious that Cav-3, PKC and TASK1 are connected to each other for the modulation of TASK1. This connection might play more important role during hypoxia. We might try either opening TASK1 channels or suppressing the phosphorylation of TASK1 by PKC or reducing the association between TASK1 and Cav-3 to testify their impacts on cell viability during hypoxia. Also, the detailed mechanisms by which TASK1
channels mediate cardioprotection need to be further elucidated and, besides their hyperpolarizing effect, might include other unknown mechanisms as well.
BIBLIOGRAPHY


caveolin to the necks of caveolae in adipocytes, Molecular biology of the cell, 14 (2003) 3967-3976.


[38] J. Abi-Char, A. Maguy, A. Coulombe, E. Balse, P. Ratajczak, J.L. Samuel, S. Nattel, S.N. Hatem, Membrane cholesterol modulates Kv1.5 potassium


[56] T. Lamark, M. Perander, H. Outzen, K. Kristiansen, A. Overvatn, E. Michaelsen, G. Bjorkoy, T. Johansen, Interaction codes within the family of


[102] E. Carmeliet, Cardiac ionic currents and acute ischemia: from channels to arrhythmias, Physiological reviews, 79 (1999) 917-1017.


[106] G.R. Budas, E.N. Churchill, M.H. Disatnik, L. Sun, D. Mochly-Rosen, Mitochondrial import of PKCepsilon is mediated by HSP90: a role in


sensing receptors to inhibit endo(sarco)plasmic reticulum-mitochondria crosstalk, Molecular and cellular biochemistry, 341 (2010) 195-206.


[138] A. Kuno, S.D. Critz, L. Cui, V. Solodushko, X.M. Yang, T. Krahn, B. Albrecht, S. Philipp, M.V. Cohen, J.M. Downey, Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A2b-


phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction, Molecular and cellular biology, 16 (1996) 1722-1733.


[182] J.A. Millar, L. Barratt, A.P. Southan, K.M. Page, R.E. Fyffe, B. Robertson, A. Mathie, A functional role for the two-pore domain potassium channel TASK-1


contribution of TWIK-related acid-sensitive K+-containing channels to the function of dorsal lateral geniculate thalamocortical relay neurons, Molecular pharmacology, 69 (2006) 1468-1476.


