

Georgia State University

## ScholarWorks @ Georgia State University

---

Biology Dissertations

Department of Biology

---

12-3-2007

# KATP Channel Phosphorylation: Mechanisms and Contribution to Vascular Tone Regulation by Vasodilating and Vasoconstricting Hormones and Neurotransmitters

Yun Shi

Follow this and additional works at: [https://scholarworks.gsu.edu/biology\\_diss](https://scholarworks.gsu.edu/biology_diss)



Part of the [Biology Commons](#)

---

### Recommended Citation

Shi, Yun, "KATP Channel Phosphorylation: Mechanisms and Contribution to Vascular Tone Regulation by Vasodilating and Vasoconstricting Hormones and Neurotransmitters." Dissertation, Georgia State University, 2007.

doi: <https://doi.org/10.57709/1063865>

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact [scholarworks@gsu.edu](mailto:scholarworks@gsu.edu).

**K<sub>ATP</sub> Channel Phosphorylation: Mechanisms and Contribution to  
Vascular Tone Regulation by Vasodilating and Vasoconstricting  
Hormones and Neurotransmitters**

by

Yun Shi

Under the Direction of Chun Jiang

**ABSTRACT**

Contractility of vascular smooth muscles (VSMs) in resistance arteries determines systemic blood pressure and blood supplies to local tissues, in which ATP sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels play a role. The K<sub>ATP</sub> channels that couple metabolic state to cellular activity are activated by multiple hormonal vasodilators and inhibited by vasoconstrictors.

To understand the molecular mechanisms for the channel regulation by vasodilators, we studied the effects of β-adrenergic receptors on Kir6.1/SUR2B in HEK cells. Stimulation of β-adrenergic receptors activated the channels, which relied on the G<sub>s</sub>-protein, adenylyl cyclase, cAMP and PKA system. Using mutational analysis, we scanned all the putative PKA sites on Kir6.1 and SUR2B subunits and identified two residues (Ser1351 and Ser1387) in SUR2B critical for channel activation. *In vitro* phosphorylation experiments confirmed that Ser1387 but not

Ser1351 was phosphorylated in isolated SUR2B peptides. Molecular modeling and molecular dynamics simulations reveal that phosphorylation at Ser1387 causes interdomain movements in SUR2B subunit. Blockage of the movements by engineering a disulfide bond across NBD<sub>2</sub> and TMD<sub>1</sub> eliminated the PKA-dependent channel activation.

We also studied the molecular basis for the inhibition of vascular K<sub>ATP</sub> channels by PKC. In the HEK expression system, we found that the Kir6.1/SUR2B channel but not the Kir6.2/SUR2B was drastically inhibited by PKC stimulation. We constructed Kir6.1/Kir6.2 chimeras and identified two critical protein domains for the Kir6.1 channel inhibition by PKC. The distal C-terminus was the direct target of PKC where multiple phosphorylation sites were identified. These phosphorylation sites were located in a short sequence with stereotypical sequence repeats. Mutation of any decreased the effects of PKC. Joint mutation of all of them prevented the channel inhibition by PKC. The proximal N-terminus is also involved in PKC effects without phosphorylation sites, suggesting it may play a role in channel gating.

Thus, this thesis provides experimental evidence for the vascular K<sub>ATP</sub> channel modulation by PKA and PKC. Phosphorylation of the Kir6.1 and SUR2B subunits by PKC and PKA produce inhibition and activation of the vascular K<sub>ATP</sub> channel, respectively, which appears to be one of the molecular bases contributing to vascular tone regulation by both vasoconstricting and vasodilating hormones and neurotransmitters.

INDEX WORDS: Kir6.1, SUR2B, ATP-sensitive K<sup>+</sup> channels, Vascular tone, Protein kinase A, Protein kinase C, Vasodilators, Vasoconstrictors, Adrenergic receptors

**K<sub>ATP</sub> Channel Phosphorylation: Mechanisms and Contribution to  
Vascular Tone Regulation by Vasodilating and Vasoconstricting  
Hormones and Neurotransmitters**

by

YUN SHI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

In the College of Arts and Sciences  
Georgia State University

2007

**Copyright by  
Yun Shi  
2007**

**K<sub>ATP</sub> Channel Phosphorylation: Mechanisms and Contribution to  
Vascular Tone Regulation by Vasodilating and Vasoconstricting  
Hormones and Neurotransmitters**

by

Yun Shi

Major Professor: Chun Jiang

Committee: Delon Barfuss  
Deborah Baro  
Teryl Frey

Electronic Version Approved:

Office of Graduate Studies  
College of Arts and Sciences  
Georgia State University  
December 2007

## **1. Acknowledgements**

Pursuing a doctoral degree is one of the most challenging and important events in my life which requires not only my personal efforts but also supports from my surroundings. My sincere appreciation is therefore extended to all who have provided assistance, advice and encouragement to me during my Ph.D. study.

First of all, I would like to thank Dr. Chun Jiang, for his continuous instruction and encouragement. He has a tremendous influence not only to my career, but also to my personality. I am also indebted to the faculty and staff of the Department of Biology at Georgia State University for their advice and generous assistance during my Ph.D. program. I am grateful to Drs. Deborah Baro, Delon Barfuss, and Teryl Frey for serving on my dissertation committee, and providing me many valuable suggestions in my experimental designs. I would like to thank Dr. Phang C. Tai for his special guidance in my graduate studies.

I would like to express my appreciation to the members in Dr. Jiang's lab for their involvements in my research projects. Special thanks to Drs. Jianping Wu, Ningren Cui, Jinzhe Mao, Li Li, Runping Wang and Hainan Piao for their efforts in teaching me experimental techniques. I appreciate Mr. Weiwei Shi, Dr. Asheebo Rojas, Ms Xiaoli Zhang, Ms. Zhongyin Wu, Ms. Nah Ta and Mr. Binh Ha for their excellent technique supports. Without their efforts, my studies would be impractical or delayed. My thanks are extended to Dr. Harrison in Computer Science Department and my friend Mr. Jeff Chen. Collaboration with them makes one of the major studies in the thesis reality.

I also give special thanks to Ms. Ping Liang-Jiang for her help with the DNA sequencing and making primers. My appreciations are also paid to many members of the Departments of Biology and Chemistry for their advice and assistance during my Ph. D. program.

Finally, I wholeheartedly thank my family, my wife Suyun Yang and my lovely daughter Carol for their love, encouragement and supports to me and my career pursuit. I am extremely grateful to my parents and all of my extended family for all of their supports for my oversea studies.



## 2. Table of Contents

1. Acknowledgements.....	IV
2. Table of Contents.....	VI
3. List of Tables .....	IX
4. List of Figures .....	X
5. Abbreviations .....	XI
6. Specific Aims and Hypotheses .....	1
7. General Introduction: Vascular K <sub>ATP</sub> Channels in Vascular Tone Regulation.....	3
7.1. Vascular tone and blood supply .....	3
7.1.1. Contractility of VSMs and vascular tone.....	3
7.1.2. Ca <sup>2+</sup> and contractile state of VSMs .....	4
7.1.3. The membrane potential in VSMs and K <sup>+</sup> channels .....	7
7.1.4. K <sub>ATP</sub> channels are involved in regulation of VSM membrane potential.....	7
7.2. Molecular aspects of K <sub>ATP</sub> Channels .....	8
7.2.1. Discovery of K <sub>ATP</sub> channels .....	8
7.2.2. K <sub>ATP</sub> channels are composed of Kir6.x and SURx .....	9
7.2.3. Functions of Kir6 subunits .....	11
7.2.4. Functions of SUR subunits .....	13
7.2.5. Coupling between SUR and Kir subunits in channel gating.....	14
7.3. Molecular components of Vascular K <sub>ATP</sub> Channels .....	15
7.3.1. Kir6.1/SUR2B isoform resembles a native K <sub>ATP</sub> channel in VSMs .....	15
7.3.2. Vascular expression .....	16
7.3.3. Evidence from gene knockout animals .....	19
7.4. Regulation of the Channel Activity .....	20
7.4.1. Metabolites.....	20
7.4.2. Hormonal vasodilators .....	20
7.4.3. Vasoconstrictors.....	22
7.5. Questions and potential solutions .....	23
7.5.1. The questions about vascular regulators .....	23
7.5.2. Potential solutions .....	24
8. Significance.....	28
9. Material and Methods .....	31
9.1. Cell culture .....	31
9.2. Molecular biology .....	31
9.3. Artery rings .....	32
9.4. Dissociated vascular smooth muscle cells .....	33
9.5. Patch clamp experiments .....	34
9.6. Phosphorylation sites prediction .....	35
9.7. Western blot .....	35
9.8. <i>In vitro</i> phosphorylation.....	35
9.9. Molecular modeling .....	36
9.10. Molecular dynamics simulation .....	37
9.11. Chemicals.....	38
9.12. Data analysis .....	39

10. Result 1: PKA Phosphorylation of SUR2B Subunit Underscores Vascular K <sub>ATP</sub> Channel Activation by B-Adrenergic Receptors .....	40
10.1. Abstract .....	41
10.2. Introduction.....	42
10.3. Results.....	43
10.3.1. Baseline Kir6.1/SUR2B channel activity .....	43
10.3.2. Activation of the Kir6.1/SUR2B channel by $\beta_2$ receptor stimulation.....	45
10.3.3. PKA dependence.....	47
10.3.4. PKA phosphorylation sites .....	52
10.3.5. Activation of vascular K <sub>ATP</sub> channels by isoproterenol.....	60
10.4. Discussion.....	62
10.4.1. PKA mediates Kir6.1/SUR2B channel activation by isoproterenol. ....	62
10.4.2. Why can't we repeat the others' sites? .....	64
10.4.3. What is the explanation for Ser1351 mutations? .....	64
10.4.4. The Ser1387 is an important PKA site: its functional implication in SUR1 and SUR2A channels. ....	66
11. Result 2: PKA Phosphorylation Produces Interdomain Movement in SUR2B Suggesting a Gating mechanism for Activation of the Vascular K <sub>ATP</sub> Channel.....	70
11.1. Abstract.....	71
11.2. Introduction.....	72
11.3. Results.....	76
11.3.1. Alignment of SUR2B with SAV1866 .....	76
11.3.2. Modeling of the SUR2B_core .....	76
11.3.3. Activation of the Kir6.1/SUR2B channel by forskolin.....	81
11.3.4. Elimination of PKA activation by mutation at Arg1462 or Tyr506 .....	81
11.3.5. Triad formation by Arg1462, Tyr506 and Ser1387 following PKA phosphorylation.....	82
11.3.6. The movement of ICL <sub>2</sub> .....	85
11.3.7. The NBD <sub>2</sub> groove is reshaped by phosphorylation.....	87
11.3.8. Restriction of the interdomain movement by disulfide bond.....	90
11.4. Discussion.....	91
11.4.1. The rationale to choose SAV1866 to model SUR2B_core. ....	91
11.4.2. What is new in our SUR2B_core model? .....	93
11.4.3. The importance of defining TMD-NBD interaction in SURx. ....	93
11.4.4. The mechanism of PKA activation. ....	94
11.4.5. Possible cooperation of PKA and nucleotide activators. ....	97
12. Result 3: A Unique Motif in Kir6.1 Consisting of 4 Phosphorylation Repeats Underlies the Vascular K <sub>ATP</sub> Channel Inhibition by Protein Kinase C.....	98
12.1. Abstract.....	99
12.2. Introduction.....	100
12.3. Results.....	101
12.3.1. PKC-dependent inhibition of the Kir6.1/SUR2B channel.....	101
12.3.2. Critical protein domains for the PKC-dependent channel inhibition.....	105
12.3.3. Phosphorylation of a stretch of serine repeats in the C terminus by PKC .....	109
12.4. Discussion.....	113
12.4.1. PKC inhibition is unique in Kir6.1/SUR2B channels.....	113

12.4.2. The N-terminus might be involved in channel gating .....	114
12.4.3. The C-terminus contains a PKC motif with phosphorylation repeats ...	115
12.4.4. A hypothetic model for gating mechanisms of PKC phosphorylation ..	116
12.4.5. The formation of multiple PKC sites may undergo evolutionary selection. .....	116
13. General Discussion .....	118
13.1. Comparison of different working patterns in PKA and PKC phosphorylations .....	118
13.2. Unique bi-directional regulation of the vascular K <sub>ATP</sub> channels.....	120
13.3. Functional impacts of the PKA and PKC phosphorylation. ....	123
13.3.1. Circulating hormones.....	124
13.3.2. Other factors released from nerve terminals.....	124
13.3.3. Endothelium-derived vasoregulators .....	125
13.3.4. Local mediators released from damaged tissues.....	125
13.3.5. Channel regulation by anesthetics. ....	126
13.4. The vascular K <sub>ATP</sub> channel as a key integration molecule in vascular tone regulation .....	126
14. Reference List .....	131
15. Appendix: Publications.....	152

### 3. List of Tables

Table 7-1. Expression of $K_{ATP}$ channels in vascular smooth muscles. ....	18
Table 10-1. The effect of forskolin on Kir6.1/SUR2B and SUR2B mutants. ....	56
Table 13-1. Summary of PKA effects on recombinant $K_{ATP}$ channels. ....	121
Table 13-2. Summary of PKC effects on recombinant $K_{ATP}$ channels. ....	121

## 4. List of Figures

Figure 7-1. Vascular smooth muscle contraction. ....	5
Figure 7-2. List of vasoactive agents that affect $K_{ATP}$ channel activity.....	21
Figure 10-1. Kir6.1/SUR2B channels expressed in HEK293 cells. ....	43
Figure 10-2. Concentration-dependent activation of Kir6.1/SUR2B currents by isoproterenol. ....	46
Figure 10-3. Dissection of signal pathway for Kir6.1/SUR2B channel activation by isoproterenol. ....	47
Figure 10-4. Augmentation of Kir6.1/SUR2B channel activity by the catalytic subunit of PKA. ....	51
Figure 10-5. Mutation on potential PKA sites. ....	53
Figure 10-6. Identification of PKA phosphorylation sites in SUR2B. ....	57
Figure 10-7. Characterization of Ser1387 in PKA phosphorylation.....	58
Figure 10-8. Effects of isoproterenol on vascular smooth muscles. ....	61
Figure 10-9. The sites important for PKA regulation on $K_{ATP}$ channels. ....	67
Figure 11-1. Alignment of SUR2B and SAV1866. ....	74
Figure 11-2. SUR2B core Model. ....	78
Figure 11-3. Forskolin effects on wt and mutant Kir6.1/SUR2B. ....	80
Figure 11-4. Formation of a compact triad among Tyr506, Ser1387 and Arg1462 following phosphorylation. ....	83
Figure 11-5. The movement of ICL <sub>2</sub> following phosphorylation. ....	86
Figure 11-6. The conformational changes in NBD <sub>2</sub> interface. ....	88
Figure 11-7. Introduction of a disulfide bond between ICL <sub>2</sub> and NBD <sub>2</sub> blocked PKA effects. ....	92
Figure 11-8. Schematics of the PKA-dependent channel gating. ....	95
Figure 12-1. Kir6.1/SUR2B and Kir6.2/SUR2B channels expressed in HEK293 cells. .....	102
Figure 12-2. Effects of the recombinant catalytic fragment of PKC on Kir6.1/SUR2B and Kir6.2/SUR2B currents in inside-out patches. ....	104
Figure 12-3. Responses of Kir6.1-Kir6.2 chimeras to PMA. ....	105
Figure 12-4. Dissection of critical protein domains for the PKC-dependent channel inhibition. ....	107
Figure 12-5. Mutational analysis of potential PKC phosphorylation sites. ....	109
Figure 12-6. <i>In vitro</i> phosphorylation on MBP-fusion peptides.....	111
Figure 12-7. A hypothetical model of PKC inhibition on Kir6.1/SUR2B channel. ...	117
Figure 13-1. Regulation of vascular $K_{ATP}$ channels by metabolites and vasoactive hormones. ....	128

## 5. Abbreviations

3AA	Kir6.1_S385A/SUR2B_T633A-S1465A
ABC	ATP binding cassette
AC	adenylyl cyclase
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ADRB2	$\beta_2$ -adrenergic receptor
AMMP	Advanced molecular modeling program
ATP	adenosine triphosphate
C	closed state
cAMP	cyclic adenosine monophosphate
C <sub><math>\alpha</math></sub>	alpha carbon in an amino acid residue
cGMP	cyclic guanosine monophosphate
CO <sub>2</sub>	carbon dioxide
DAG	diacylglycerol
2deox-ATP	2',5'-dideoxyadenosine 3'-triphosphate
DMSO	Dimethyl sulfoxide
DOI	25-dimethoxy-4-iodophenyl-2-aminopropane
EDHF	endothelium-derived hyperpolarizing factor
EET	epoxyeicosatrienoic acid
EGTA	ethylene glycol-bis- $\beta$ -aminoethylether-N,N,N',N'-tetraacetic acid
GFP	green fluorescent protein
GirK	protein-coupled inward rectifier K <sup>+</sup> channels
GK	K <sup>+</sup> conductance
GPCR	G protein coupled receptors
H	hill coefficient
HEK	human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EC <sub>50</sub>	concentration for 50% inhibition
ICL	intracellular linker
IP <sub>3</sub>	inositol-1, 4, 5-triphosphate

K <sub>ATP</sub>	ATP-sensitive K <sup>+</sup> channel
K <sub>Ca</sub>	Ca <sup>2+</sup> -activated K <sup>+</sup> channel
KCO	K <sup>+</sup> channel opener
Kir	inward rectifier K <sup>+</sup> channel
Kir6.x	members in the sixth subfamily of inward rectifier K <sup>+</sup> channels
Kir6.2ΔC36	inward rectifier K <sup>+</sup> channel 6.2 with 36 amino acids truncated
K <sub>NDP</sub>	Nucleotide diphosphate activated K <sup>+</sup> channel
KO	gene Knockout
Kv	voltage-gated K <sup>+</sup> channel
LC-CoA	long chain acyl CoA
MLC	myosin light chain
MLCK	myosin light chain kinase
NBD	nucleotide bonding domain
NBT	nitro blue tetrazolium
NDP	nucleotide diphosphate
NO	nitric oxide
O	open state
O <sub>g</sub>	γ-oxygen
PCR	polymerase chain reaction
PDB	protein database
PDD	phorbol 12,13-didecanoate
PE	phenylephrine
PIP <sub>2</sub>	phosphatidylinositol bisphosphate
PIPES	piperazine-N,N'-bis-2-ethanesulfonic acid
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMA	phorbol 12-myristate 13-acetate
P <sub>o</sub>	open-state probability
pS(er)	phosphorylated serine residue
RMSD	root mean square deviation
RT-PCR	reverse transcriptive polymerase chain reaction

S.E.	standard error
SR	sarcoplasmic reticulum
SS	signature sequence (a conserved motif for nucleotide binding in all ABC proteins)
SUR	sulphonylurea receptor
TAP1	transporter 1, ATP-binding cassette, sub-family B
TM1	the first transmembrane segment in Kir
TM2	the second transmembrane segment in Kir
TMD	transmembrane domain
TMH	transmembrane helix
TRP	transient potential channels
VDCC	voltage-dependent $\text{Ca}^{2+}$ channel
VIP	vasoactive intestinal polypeptide
VSM	vascular smooth muscle
$W_A$	Walker A motif (a conserved motif for nucleotide binding in all ABC proteins)
$W_B$	Walker B motif (a conserved motif for nucleotide binding in all ABC proteins)
WT	wild type



## 6. Specific Aims and Hypotheses

ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ) play an important role in vascular tone regulation. The channels couple cellular metabolic activity to the contractility of vascular smooth muscles (VSMs). Both vasodilating and vasoconstricting hormones and neurotransmitters act on the vascular  $K_{ATP}$  channels, through which they produce changes in membrane potentials, intracellular  $Ca^{2+}$  concentrations and vascular tone.

Previous studies have shown that the activation of the vascular  $K_{ATP}$  channels by vasodilating hormones relies on the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway, while the activation of the PKC pathway is necessary for the channel inhibition by vasoconstricting hormones. However, the molecular basis for the PKA and PKC-dependent modulation of the channels remains illusive. Whether these kinases directly phosphorylate the channel proteins is uncertain; there is no information about where the phosphorylation sites are located, whether there are other signal pathways between these kinases and the channels, and how the channel activity is affected by these kinases.

The studies herein address these issues. The specific aims of this dissertation are:

**A. To elucidate the role of PKA in the vascular  $K_{ATP}$  channel activation by vasodilators and identify the molecular substrates of PKA.**

**B. To understand the structural basis and gating mechanisms underlying PKA-mediated channel activation.**

**C. To identify critical protein domains and phosphorylation sites for the vascular  $K_{ATP}$  channels inhibition by PKC.**

The hypothesis tested for specific aim 1: Activation of the PKA signaling pathway underlie the opening of vascular  $K_{ATP}$  channels by stimulation of  $\beta$ -adrenergic receptors via direct phosphorylation of the channel proteins.

The hypothesis for specific aim 2: PKA phosphorylation leads to conformational changes in the specific protein domains of SUR2B that eventually alter the channel activity.

The hypothesis addressing specific aim 3: The inhibitory effect of PKC relies on pore-forming Kir6.1 subunit instead of the SUR2B subunit that host critical protein domains and phosphorylation sites.

## **7. General Introduction: Vascular K<sub>ATP</sub> Channels in Vascular Tone Regulation**

### **7.1. Vascular tone and blood supply**

Systemic blood pressure and blood supplies to local tissues and organs are of crucial importance for the human body and are tightly regulated by physiological functions, and are controlled by small arteries and arterioles. Their dysregulation often leads to catastrophic consequences. The diastolic blood pressure is the foundation of blood supply and O<sub>2</sub> delivery to all tissues and organs, while regional blood flow underlies the functions of individual organs or tissues. Normally, the small arteries and arterioles maintain a certain level of contraction, referred to basal vascular tension or tone and that maintains systemic blood pressure and various blood flows to the local tissues.

Vascular tone is tightly regulated by a complex of signaling systems involving autonomic nerve activity, circulating hormones and local metabolites. Dysregulation of vascular tone leads to vascular diseases related to blood pressure such as hypertension and abnormality in the functioning of individual organs, such as ischemia, hyperemia, shock, etc. The understanding the cellular and molecular basis of vascular tone regulation thus will provide information for development of therapeutic strategies for vascular diseases.

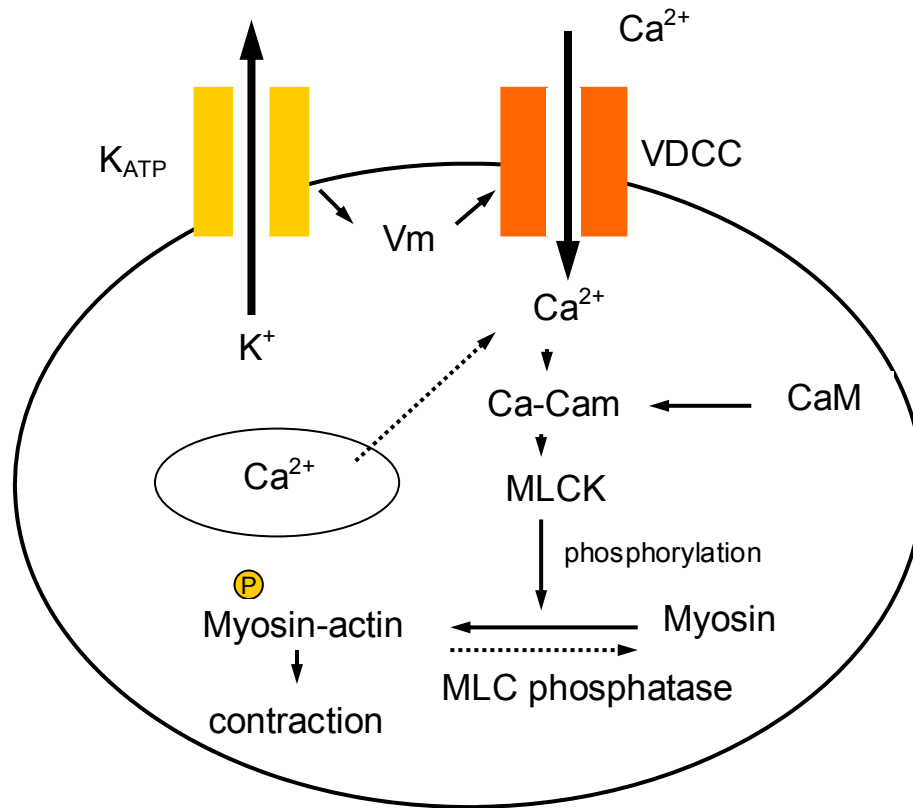
#### **7.1.1. Contractility of VSMs and vascular tone**

Smooth muscle cell contractility determines vascular diameters and tone. When vascular smooth muscles (VSMs) contract, the vessel constricts, increasing

perfusion resistance and reducing blood supplies to the local tissue. When VSMs are relaxed, the blood vessel becomes wider, reducing perfusion resistance and increasing blood supplies to the tissue. The contractility of VSMs is controlled by autonomic nervous system, circulating hormones, local tissue mediators and mechanical forces. Regardless of the stimuli, VSM cells contract and develop force utilizing cross-bridge cycling of actin and myosin filaments, where  $\text{Ca}^{2+}$  serves to initiate contraction.

#### **7.1.2. $\text{Ca}^{2+}$ and contractile state of VSMs**

Upon receiving contractive signals, cytosolic free  $\text{Ca}^{2+}$  in smooth myocytes rises from resting level of ~100nM to 500-700nM (Williams & Fay, 1986; Knot *et al.*, 1998). At the elevated concentrations,  $\text{Ca}^{2+}$  binds to calmodulin, a  $\text{Ca}^{2+}$ -binding protein in VSMs. Binding of  $\text{Ca}^{2+}$  causes conformational changes in calmodulin and exposure of its interaction sites to many target proteins, including myosin light chain kinase (MLCK). Activated by a  $\text{Ca}^{2+}$ -calmodulin complex, MLCK phosphorylates myosin light chain (MLC). The MLC phosphorylation augments cycling of myosin cross-bridges along actin filaments, and enhances contraction forces of VSMs (Seeholzer & Wand, 1989; Stull *et al.*, 1997; Fajmut *et al.*, 2005). Removal of  $\text{Ca}^{2+}$  from the cytosol leads to the dissociation of calmodulin from MLCK, dephosphorylation of MLC by MLC phosphatase and relaxation of the VSMs (Fig. 7-1).



**Figure 7-1. Vascular smooth muscle contraction.**

$Ca^{2+}$  influx from VDCCs is the major source contributing to intracellular  $Ca^{2+}$  elevation.  $Ca^{2+}$  causes muscle contraction through a series of biochemical reactions.

The cytosolic  $\text{Ca}^{2+}$  comes from two sources, extracellular spaces and intracellular  $\text{Ca}^{2+}$  stores (mostly from sarcoplasmic reticulum, or SR).  $\text{Ca}^{2+}$  concentrations in both the extracellular space and SR are in the millimolar to hundreds of millimolar range, much higher than that in the cytosol (~100nM at rest). Depolarization of the cell activates voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs) on the sarcolemma triggering  $\text{Ca}^{2+}$  influx (Nelson *et al.*, 1990b; Thorneloe & Nelson, 2005). Changes in blood pressure and shear force also affect  $\text{Ca}^{2+}$  entry via transient potential channels (TRP)(Beech *et al.*, 2004; Remillard & Yuan, 2006).  $\text{Ca}^{2+}$ -release from the SR via  $\text{IP}_3$  receptors (Missiaen *et al.*, 1999) and/or ryanodine receptors (Hisayama *et al.*, 1990; Neylon *et al.*, 1995) is another source of cytosolic  $\text{Ca}^{2+}$ . Unlike cardiac and skeletal muscles where  $\text{Ca}^{2+}$  mainly comes from the SR,  $\text{Ca}^{2+}$  influx through the VDCCs is the major source of cytosolic  $\text{Ca}^{2+}$  in VSMs (Thorneloe & Nelson, 2005). Therefore, agents and cellular activity affecting VDCC activity have a major impact on cytosolic  $\text{Ca}^{2+}$  levels and thus vascular tone.

As the name indicates, the VDCCs open or close depending on the voltage across the plasma membrane. VDCCs in VSMs consist of L-type and T-type  $\text{Ca}^{2+}$  channels (Ganitkevich & Isenberg, 1990; Smirnov & Aaronson, 1992; Snutch *et al.*, 2001), both of whose activities increase upon depolarization and decrease upon hyperpolarization. The membrane potentials reported in variety of blood vessels are typically between -40 and -60mV (Nelson & Quayle, 1995). In this range, the relationship between activity of  $\text{Ca}^{2+}$  channels and membrane potentials is rather linear and steep, allowing a small change in membrane potential to cause a large alteration in  $\text{Ca}^{2+}$  influx (Nelson *et al.*, 1990b).

### **7.1.3. The membrane potential in VSMs and K<sup>+</sup> channels**

The membrane potentials in VSMs rely on K<sup>+</sup>, Cl<sup>-</sup> and leak Na<sup>+</sup> currents (Nelson & Quayle, 1995). The K<sup>+</sup> conductance (GK) is regulated by environmental factors and contributes to moment-to-moment changes in vascular tone. Several types of K<sup>+</sup> channels exist in VSMs, including voltage-gated K<sup>+</sup> channels (Kv), Ca<sup>2+</sup>-activated K<sup>+</sup> channels (K<sub>Ca</sub>), inward rectifier K<sup>+</sup> channels (Kir) and ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub>) (Nelson & Quayle, 1995; Jackson, 2005).

Activity of these channels is differentially regulated by changes in extracellular signals, intracellular second messengers and other chemical and physical stimuli. The Kv channels are open upon depolarization; bring the membrane potentials back to the rest level or repolarization. Because of the existence of Kv channels, the membrane potentials of VSMs generally cannot go above 0mV under physiological conditions. The Ca<sup>2+</sup>-activated K<sup>+</sup> channels act as a feedback system that repolarizes the VSM cells, decreases cytosolic Ca<sup>2+</sup>, and prevents sustained contraction. The inward rectifier K<sup>+</sup> channels alter the vascular tone according to changes in K<sup>+</sup> levels in the blood. The K<sub>ATP</sub> channels are thought to be particularly important as their activity is subject to regulation by various pharmacological and endogenous vasoregulators, as well as metabolic activity that can directly affect blood flow (Quayle *et al.*, 1997; Cole & Clement-Chomienne, 2003; Seino & Miki, 2003).

### **7.1.4. K<sub>ATP</sub> channels are involved in regulation of VSM membrane potential**

The role of vascular K<sub>ATP</sub> channels in controlling VSM membrane potential has been established by early pharmacological studies. A number of synthetic compounds, known as K<sup>+</sup> channel openers (KCOs, including diazoxide, nicorandil, cromakalim and pinacidil), increase K<sup>+</sup> conductance in VSMs and decrease systemic

blood pressure (Rhodes & Sutter, 1971; Andersson, 1973; Standen *et al.*, 1989; Winquist *et al.*, 1989; Longmore *et al.*, 1991). Despite the structural diversity, KCOs work exclusively on  $K_{ATP}$  channels, resulting in hyperpolarization in VSM membrane potential and vasodilation (Standen *et al.*, 1989; Edwards & Weston, 1993). For example, 10 $\mu$ M cromakalim activates  $K_{ATP}$  channels in rabbit cerebral artery and human pulmonary artery, and hyperpolarizes the VSM cell by  $\sim 20$ mV (Nelson & Quayle, 1995; Cui *et al.*, 2002). Such large hyperpolarization closes the voltage-gated  $Ca^{2+}$  channels and leads to complete relaxation of vessels. In contrast, glibenclamide, which inhibits  $K_{ATP}$  channels, causes a significant depolarization in some vessel beds such as coronary and mesenteric arteries (Nelson *et al.*, 1990b; Garland & McPherson, 1992), suggesting the involvement of  $K_{ATP}$  activity in basal vascular tone. Although in renal, cerebral and pulmonary arteries, glibenclamide does not affect basal tone probably because the basal activity of  $K_{ATP}$  channels is low in these artery beds, the  $K_{ATP}$  channels regulate tone during metabolic press (Wiener *et al.*, 1991; Reid *et al.*, 1993; Loutzenhiser & Parker, 1994).

## **7.2. Molecular aspects of $K_{ATP}$ Channels**

### **7.2.1. Discovery of $K_{ATP}$ channels**

Using inside-out patches, Noma (Noma, 1983) reported that  $K^+$  channels in the cardiac muscle were strongly inhibited by intracellular ATP. Such ATP-sensitive  $K^+$  channels have also been observed in other tissues such as pancreas (Ashcroft *et al.*, 1984; Cook & Hales, 1984), skeletal muscles (Spruce *et al.*, 1985), smooth muscles (Standen *et al.*, 1989) and neurons (Ashford *et al.*, 1988; Amoroso *et al.*, 1990;



Bernardi *et al.*, 1993). Studies on the native K<sub>ATP</sub> channels have revealed variability in their biophysical and pharmacological properties.

In general, K<sub>ATP</sub> channels are inhibited by ATP and activated by Mg<sup>2+</sup>-nucleotide diphosphates (NDP) in the cytosol (Zhang & Bolton, 1995; Aguilar-Bryan *et al.*, 1998; Ashcroft & Gribble, 1998; Masia *et al.*, 2005; Nichols, 2006). These channels are also activated by some, if not all, KCOs such as diazoxide, pinacidil and cromakalim (Edwards & Weston, 1993; Ashcroft & Gribble, 2000; Matsuoka *et al.*, 2000; Yamada & Kurachi, 2004; Moreau *et al.*, 2005). In addition, they are inhibited by sulfonylureas including glibenclamide (or glybenclamide, glyburide), glimepiride, glipizide, gliquidone, gliclazide, tolazamide and tolbutamide (Standen *et al.*, 1989; Zhang & Bolton, 1995; Ashcroft & Gribble, 2000; Reimann *et al.*, 2001). K<sub>ATP</sub> channels are defined by a combination of biophysical and pharmacological properties. K<sub>ATP</sub> channels in different tissues, however, display different single channel conductance as well as different sensitivities to physiological and pharmacological agents (nucleotides, KCOs and sulfonylureas) (Ashcroft, 1988; Quayle *et al.*, 1997), suggesting diversity of K<sub>ATP</sub> channels. Indeed multiple genes encoding the channel proteins and splicing variants have been found.

### **7.2.2. K<sub>ATP</sub> channels are composed of Kir6.x and SURx**

K<sub>ATP</sub> channels are composed of two different subunits, Kir6.x and SURx, with the 4:4 stoichiometry (Clement *et al.*, 1997; Inagaki *et al.*, 1997; Shyng & Nichols, 1997). Each type of subunit is encoded by a different gene family: pore-forming subunits (Kir6.x,  $\alpha$ -subunits) and auxiliary sulfonylurea receptors (SURx,  $\beta$ -subunits). Using a partial sequence of Kir3.1 cDNA as probe, Inagaki *et al.* (Inagaki *et al.*, 1995b) cloned the *Kcnj8* (*Kir6.1*) gene from a rat pancreatic islet cDNA library. The

*Kir6.1* gene is expressed ubiquitously in all tissues, encoding a protein of 424 amino acids. Using *Kir6.1* cDNA as a probe, the *Kcnj11* (*Kir6.2*) gene was cloned from a human genomic library and a rat cDNA library (Inagaki *et al.*, 1995a) as well as a mouse cDNA library (Sakura *et al.*, 1995). Abundant expression of *Kir6.2* was found in the pancreas as well as heart, brain and skeletal muscles. The *Kir6.2* protein has 390 amino acids in length, sharing ~70% identity with *Kir6.1*. *Kir6.1* and *Kir6.2* belong to the inward rectifier K<sup>+</sup>-channel subfamily based on their homology; they are 43-46% identical to other members in Kir subfamily (Inagaki *et al.*, 1995a).

Two genes encoding sulfonylurea receptors have been cloned so far, namely *abcc8* and *abcc9* (*SUR1* and *SUR2*) (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1996). There are splicing variants of *SUR2* mRNAs, the major ones encoding *SUR2A* and *SUR2B* (Isomoto *et al.*, 1996). The *SUR2B* differs from *SUR2A* in the last 42 and 45 residues in rodents and humans respectively. The *SUR2B* C-terminus is more homologous to *SUR1* than is *SUR2A*. Splicing variances in *SUR1* have also been reported (Sakura *et al.*, 1999; Gloyn *et al.*, 2001; Hambrock *et al.*, 2002). *In vivo*, the *Kir6.x* and the *SURx* are expressed in plasma membranes to form functional channels guided by ER retention signaling sequences in both subunits (Zerangue *et al.*, 1999).

Since *Kir6* and *SUR* subunits were cloned, expression systems have been used to clarify biophysical and pharmacological properties of the channels and compare to their native counterparts. All combinations of *Kir6.x* (*Kir6.1* and *Kir6.2*) and *SURx* (*SUR1*, *SUR2A* and *SUR2B*) can form functional channels (Aguilar-Bryan *et al.*, 1998; Ashcroft & Gribble, 1998). It is now known that all *Kir6.2*-containing channels have a unitary conductance of 70-80pS while *Kir6.1*-containing channels are 30-35pS (Inagaki *et al.*, 1995a; Inagaki *et al.*, 1995b; Isomoto *et al.*, 1996; Yamada *et al.*,

1997; Wang *et al.*, 2003). Kir6.1/Kir6.2 can also form heteromeric channels in expression system with single channel conductance between typical Kir6.2 and Kir6.1 channels (Cole *et al.*, 2000; Cui *et al.*, 2001), although whether there are such channels in real tissues is still unknown. In inside-out patches, Kir6.2 channels are spontaneously open while Kir6.1 channels are completely closed in the absence of nucleotides (Kondo *et al.*, 1998). Original reports suggested that Kir6.1 and Kir6.2 channels were clearly different in their sensitivity to ATP inhibition (Yamada *et al.*, 1997); however, this has not been completely accepted in the field (Babenko *et al.*, 2000b; Babenko & Bryan, 2001). SURx are known to determine the channel sensitivity to activation by  $Mg^{2+}$ -nucleotides and KCOs, and to determine inhibition by sulfonylureas (Ashcroft & Gribble, 1998; Bryan & Aguilar-Bryan, 1999; Seino, 1999). Therefore channels consisting of different SURx have different pharmacological properties. In contrast to Kir6.x, SURx cannot form heterotetramers in expression systems (Giblin *et al.*, 2002). Combinations of Kir6.x and SURx form  $K_{ATP}$  channels accounting for the variety of channel properties found in various tissues. With pharmacological evidence as well as mRNA expression data in tissues, it is now accepted that the Kir6.2/SUR1 channel is the pancreatic isoform (Ashcroft & Gribble, 2000), the Kir6.2/SUR2A combination represents the  $K_{ATP}$  channels in cardiac and skeletal muscles (Isomoto *et al.*, 1997), and the  $K_{ATP}$  channels in vascular smooth muscles are likely to be made of Kir6.1/SUR2B (see Chapter 7.3).

### **7.2.3. Functions of Kir6 subunits**

The Kir6 pore-forming subunits belong to the inward rectifier  $K^+$  subfamily. Like other members in the subfamily, Kir6.1 and Kir6.2 possess two transmembrane segments (TM1 and TM2) with both N- and C-termini located intracellularly. A pore-

loop is located between TM1 and TM2. Four Kir6 proteins form a tetramer in which four 'GFG' filtering segments form a selective pore for  $K^+$  to pass (Clement *et al.*, 1997; Inagaki *et al.*, 1997; Shyng & Nichols, 1997; Doyle *et al.*, 1998). TM2s line the permeation pathway and converge to form a gate by phenylalanine residues at the cytoplasmic face of the pore (Rojas *et al.*, 2007). TM1s stand surrounding TM2s and transfer N-terminus gating signals to the pore by interacting with TM2 (Cui *et al.*, 2003; Wang *et al.*, 2005; Wang *et al.*, 2007).

Tucker et al (Tucker *et al.*) have found that by deleting the distal C-terminus (26-36 residues, where the ER retention signal is located), Kir6.2 can form functional  $K^+$  channels in the absence of SURx. The engineered Kir6.2s are inhibited by ATP but not activated by NDP, indicating that ATP inhibition is an intrinsic property of Kir6 subunits. Direct evidence for ATP-binding has been shown using photoaffinity labeling of Kir6.2 by [ $\gamma$ - $^{32}P$ ]-8-azido-ATP (Tanabe *et al.*, 2000). Further studies demonstrated the ATP binding sites are likely located on both N-terminus and C-terminus including Arg50, Lys185, Ile183, Arg201 and Gly334 (Tucker *et al.*, 1997; Drain *et al.*, 1998; Tucker *et al.*, 1998; Shyng *et al.*, 2000a). Via homology modeling based on the bacterial Kir channel (Kuo *et al.*, 2003) and the cytoplasmic portion of the Girk1 (Kir3.1) channel (Nishida & MacKinnon, 2002), ATP binding pockets in Kir6.2 have been described (Antcliff *et al.*, 2005). These binding pockets involve binding sites from adjacent Kir6.2 subunits.

Using the truncated forms of Kir6.2, studies in our lab have demonstrated that the  $K_{ATP}$  channels are sensitive to pH changes in the physiological range, with His175 being the titration site (Xu *et al.*, 2001a; Wu *et al.*, 2002a; Wu *et al.*, 2002b). The pH sensitivity of  $K_{ATP}$  channels is involved in modulation of contractility of cardiac

muscles under heavy exercise (Li *et al.*, 2005) and regulation of vascular tone by acidosis (Wang *et al.*, 2003). Other modulators such as phospholipids and long chain acyl CoA (LC-CoA) have been shown to antagonize the ATP inhibitory effects on  $K_{ATP}$  channels (Hilgemann & Ball, 1996; Fan & Makielski, 1997; Baukrowitz *et al.*, 1998; Shyng & Nichols, 1998). The molecular and structural basis for channel regulation by PIP2 has been studied using mutational analysis and homology modeling. Multiple positive charged residues in N- and C-termini on Kir6.2 bind PIP2 (Shyng *et al.*, 2000b; Schulze *et al.*, 2003a), are shown to form binding pockets in a homology modeling study (Haider *et al.*, 2007). LC-CoAs share some of those positive charged residues and might use same binding pockets (Schulze *et al.*, 2003b; Manning Fox *et al.*, 2004).

#### **7.2.4. Functions of SUR subunits**

The SUR subunits are large proteins with multiple domains. They belong to the ABC transporter superfamily rendering a typical ABC ‘core’, two clusters of transmembrane domains (TMD<sub>1</sub> and TMD<sub>2</sub>) each containing 6 transmembrane helices (TMHs) and two nucleotide-binding domains (NBD<sub>1</sub> and NBD<sub>2</sub>). NBD<sub>1</sub> is linked to the C-terminal of TMD<sub>1</sub>, and NBD<sub>2</sub> to TMD<sub>2</sub>. Besides the ‘core’, there is an additional TMD<sub>0</sub> containing 5 helices linked to TMD<sub>1</sub> with intracellular linker-0 (ICL<sub>0</sub>). Therefore, the whole protein spans the membrane 17 times with the N-terminus located extracellularly. Both NBDs are located inside, containing the canonical phosphate-binding Walker A and Walker B motifs and other conserved motifs such as signature sequence, Q-loop and H-loop, hallmarks in all ABC proteins. SURx tightly bind 8-azido ATP in NBD<sub>1</sub>, suggesting NBD<sub>1</sub> is a high-affinity ATP binding motif (Matsuo *et al.*, 2000).  $Mg^{2+}$  potentiates nucleotide binding in NBD<sub>2</sub>,

where hydrolysis seems to occur (Matsuo *et al.*, 1999; Matsuo *et al.*, 2000). The hydrolysis of ATP by NBD<sub>2</sub> is proposed to provide energy to rearrange TMD<sub>1</sub> and TMD<sub>2</sub> (Yamada & Kurachi, 2004). However, this process is still not understood. First, ADP can cause channel activation in the absence of ATP, suggesting the energy from hydrolysis might not be necessary. Second, ATP hydrolysis and TMD rearrangements are associated with substrate transport in other ABC transporters, whereas in SURx such a process is not required. Third, how NBDs interact with TMDs and how nucleotide-binding causes TMDs rearrangements is not clear.

The SURx subunits are targets for binding of multiple inhibitory and stimulatory compounds. Sulfonylureas such as glibenclamide and tolbutamide, inhibit all K<sub>ATP</sub> channels. K<sup>+</sup> channel openers, such as diazoxide, pinacidil and lecovromakalin, activate K<sub>ATP</sub> channels with variable potency depending on the SUR subunits. The molecular basis for sulfonylureas and KCOs' effects are far from being well understood although some critical sites have been revealed in the TMDs (Babenko *et al.*, 2000a; Moreau *et al.*, 2005).

#### **7.2.5. Coupling between SUR and Kir subunits in channel gating**

How do SURx and Kir6.x interact? This issue is obviously important for understanding SUR-mediated channel gating. Using chimeras between TAP1, a human multiple drug-resistance protein, and SUR1, Schwanpasch *et al.* (2000) demonstrate that TMDs in SURx are critical for interaction with Kir6.x. Rainbow *et al.* (2004) propose that segments of the NBD<sub>2</sub> directly interact with Kir6.2. The TM1 in Kir6.2 and N-terminal flanking sequences are believed to be involved in the Kir-SUR interaction (Schwappach *et al.*, 2000). The proximal C-terminus of Kir6.2 is also proposed to be another site of interaction (Giblin *et al.*, 1999). Two groups have

reported that SUR1-TMD<sub>0</sub> forms complexes with Kir6.2 (Babenko & Bryan, 2003; Chan *et al.*, 2003). The TMD<sub>0</sub> also enhances the Kir6.2 (C-terminus truncated form) channel activity in the absence of the core of SUR1. This ‘mini-K<sub>ATP</sub>’ channel clearly lacks many properties of full channels and is insensitive to sulfonylureas, Mg<sup>2+</sup>-NDP and KCOs, but retains ATP inhibition (Babenko & Bryan, 2003), implying the important roles of SUR-core in sulfonylurea-mediated inhibition and NDP-mediated activation. However, even with these data in hand, how SUR<sub>x</sub> transfers gating signal to Kir6.<sub>x</sub> is far from clear. One of the reasons is the lack of the understanding of SUR structures, such as how TMD assembly and how NBD interact. Structural information about SUR<sub>x</sub> will certainly help the understanding of SUR-mediated channel gating.

### **7.3. Molecular components of Vascular K<sub>ATP</sub> Channels**

#### **7.3.1. Kir6.1/SUR2B isoform resembles a native K<sub>ATP</sub> channel in VSMs**

The single channel conductance of native vascular K<sub>ATP</sub> channels is reported to be from 10 to 280pS, a range so wide that it can only be explained by multiforms of channels (Quayle *et al.*, 1997). The channels with small-middle conductance (20-50pS) recorded under symmetric ~140mM K<sup>+</sup> (Quayle *et al.*, 1997) are not spontaneously open in inside-out patches. To record currents from the channels requires the presence of MgNDP or KCOs at cytosolic side of the patches. The channel sensitivity to ATP inhibition is also variable. Some reports suggest the channels are inhibited by 10μM to several hundred μM ATP (See review by (Quayle *et al.*, 1997)), while some report ATP-insensitivity (Beech *et al.*, 1993; Zhang & Bolton, 1995; Zhang & Bolton, 1996). This group of channels has mostly been studied as recombinant Kir6.1/SUR2B channels in expression systems (Yamada *et al.*,

1997). Some channels with a slightly larger conductance of 70-80pS in high  $K^+$  (Zhang & Bolton, 1996) and ~50pS at 60/140mM  $K^+$  (Cole *et al.*, 2000) can be explained as the Kir6.2/SUR2B isoform (Isomoto *et al.*, 1996). The ' $K_{ATP}$ ' channels with large conductance over 100pS are also sensitive to ATP, Glibenclamide and KCOs (Standen *et al.*, 1989). Such large conductance 'ATP-sensitive'  $K^+$  channels are also reported in neurons (Jiang *et al.*, ; Jiang & Haddad, 1997). Their molecular basis, however, is still unknown.

When Kir6.1 and SUR2B were cloned, both cDNAs were reported to be ubiquitously expressed, consistent with the distribution of blood vessels. The Kir6.1/SUR2B channel has a unitary conductance of ~30pS with no obvious voltage-dependent rectification (Yamada *et al.*, 1997). The Kir6.1/SUR2B channel is completely closed in the absence of nucleotides and reactivated by NDPs. The channel is activated by KCOs and inhibited by glibenclamide. ATP appears to be required to maintain the channel activity and only causes inhibition at high concentration (>5mM). Therefore, the Kir6.1/SUR2B channel has most properties of the  $K_{NDP}$  channels found in vascular smooth muscles (Beech *et al.*, 1993; Zhang & Bolton, 1996; Cole *et al.*, 2000).

### **7.3.2. Vascular expression**

The molecular properties of native  $K_{ATP}$  channels in VSMs have been investigated in mRNA/protein expression analysis in addition to electrophysiological observations. Table 7-1 summarizes the published molecular properties of native  $K_{ATP}$  channels in VSMs. In cultured human pulmonary artery smooth muscles, levcromackalim activated glibenclamide sensitive  $K^+$ -currents with a unitary conductance of ~29pS, comparable to the Kir6.1/SUR2B expressed in HEK293 (Cui



*et al.*, 2002). The expression of all possible  $K_{ATP}$  subunits was checked using RT-PCR in the same study; Kir6.1 and SUR2B mRNAs but not Kir6.2, SUR1 and SUR2A were found in cultured smooth muscles from the tissue (Table 7-1). In rat aorta, the Kir6.1 and SUR2B mRNAs were found to be downregulated by growth hormones using microarrays (Tivesten *et al.*, 2004). In addition to Kir6.1 and SUR2, the mRNA of Kir6.2 is reported (Ren *et al.*, 2003; Gao *et al.*, 2005) in rat aorta. Kir6.2 was also found in human corpus cavernosum but appeared to be much less than Kir6.1 suggesting a ratio of 3:1 or 4:0 (Insuk *et al.*, 2003). In our lab, we found the Kir6.1 and SUR2B mRNA were more abundantly expressed in resistance arteries than large arteries, supporting the function of the channels in regulation of vascular tone (Li *et al.*, 2003). The channel expression at protein level has been reported. In the coronary resistance arteries, both SUR2 and Kir6.1 are expressed, while Kir6.2 and SUR1 are absent (Morrissey *et al.*, 2005). In rat basilar and middle cerebral arteries, the expression of Kir6.1 and SUR2B is predominant while moderate expression of Kir6.2 and SUR1 are detected using real-time PCR and western blot (Ploug *et al.*, 2006). In cultured smooth muscle cells from rat basilar artery, however, another research group did not observe Kir6.2 mRNA using RT-PCR (Santa *et al.*, 2003). It is not known whether the cultured cells have altered expression pattern or the real tissues contain contamination from endothelial  $K_{ATP}$  channels. Therefore, the reports of channel expression patterns in VSMs are not consistent even from the same tissues. Difference in tissue preparation or the sensitivity of detecting methods might contribute to the discrepancy. Nevertheless, these studies suggest that the Kir6.1/SUR2B is the major, if not the only, component in VSMs.

**Table 7-1. Expression of K<sub>ATP</sub> channels in vascular smooth muscles.**

Tissues	Kir6.1	Kir6.2	SUR1	SUR2A	SUR2B	SUR2	methods	References
coronary resistance vessels	+	-	-			+	IH	Morrissey, 2005
human placental vasculature	+	NA	NA	NA	NA		RT-PCR/Western blot	Wareing, 2006
rat tail artery and aorta	+	+	NA			+	RT-PCR	Gao, 2005
rat aorta	+	NA	NA	NA	+		microarray	Tivesten, 2004
rat aorta	+	+	NA	NA	+		RT-PCR	Ren, 2003
human corpus carvernosum	+	+	-	-	+		RT-PCR	Insuk, 2003
cultured SM from pulmonary arteries	+	-	-	-	+		RT-PCR	Cui, 2002
kidney vascular smooth muscles	+	NA	NA	NA	NA		Western, IH, <i>in situ</i> , EM	Zhou, 2007
rat basilar and middle cerebral arteries	+	+	+	-	+		Western, Real-Time PCR	Ploug, 2006
cultured SM from rat basilar artery	+	-	NA	NA	+		RT-PCR	Santa, 2003

List of published data for expression of K<sub>ATP</sub> channels in vascular smooth muscles. SM, smooth muscle cells. +, positively detected. -, negatively detected. NA, not tested. IH, immunohistochemistry. EM, electron microscopy. RT-PCR, reverse transcriptive PCR. *in situ*, *in situ* hybridization.

In some vessels such as aorta, Kir6.2 expression is significant, which could form Kir6.2/SUR2B channel or potentially heteromeric channels in the combination of Kir6.1/Kir6.2/SUR2B. The presence of SUR2A and SUR1 is not widely accepted in VSMs (Cole & Clement-Chomienne, 2003; Seino & Miki, 2003).

### **7.3.3. Evidence from gene knockout animals**

Recently all four genes encoding  $K_{ATP}$  channels have been successfully knocked out in mice (Miki *et al.*, 1998; Seghers *et al.*, 2000; Chutkow *et al.*, 2001; Miki *et al.*, 2002; Shiota *et al.*, 2002). While *Kir6.2* or *SUR1* knockout mostly damages the glucose homeostasis (Miki *et al.*, 1998; Seghers *et al.*, 2000; Shiota *et al.*, 2002), knockout of *Kir6.1* or *SUR2* impairs coronary artery function (Chutkow *et al.*, 2001; Miki *et al.*, 2002). These animals show high death rates resulting from myocardial ischemia. Electrophysiological recording demonstrates that the animals develop spontaneous elongation of ST segments. In *Kir6.1* knockout mice, pinacidil can induce  $K_{ATP}$  currents in cardiac muscles but not in coronary artery smooth muscles, suggesting *Kir6.1* is the critical component of  $K_{ATP}$  channels in coronary artery. Without the protection by  $K_{ATP}$  channels in coronary arteries, the animals develop Prinzmetal angina and die suddenly (Chutkow *et al.*, 2001; Miki *et al.*, 2002). Studies in  $K_{ATP}$  KO mice thus further demonstrate *Kir6.1* and *SUR2* encode vascular  $K_{ATP}$  channels.

## **7.4. Regulation of the Channel Activity**

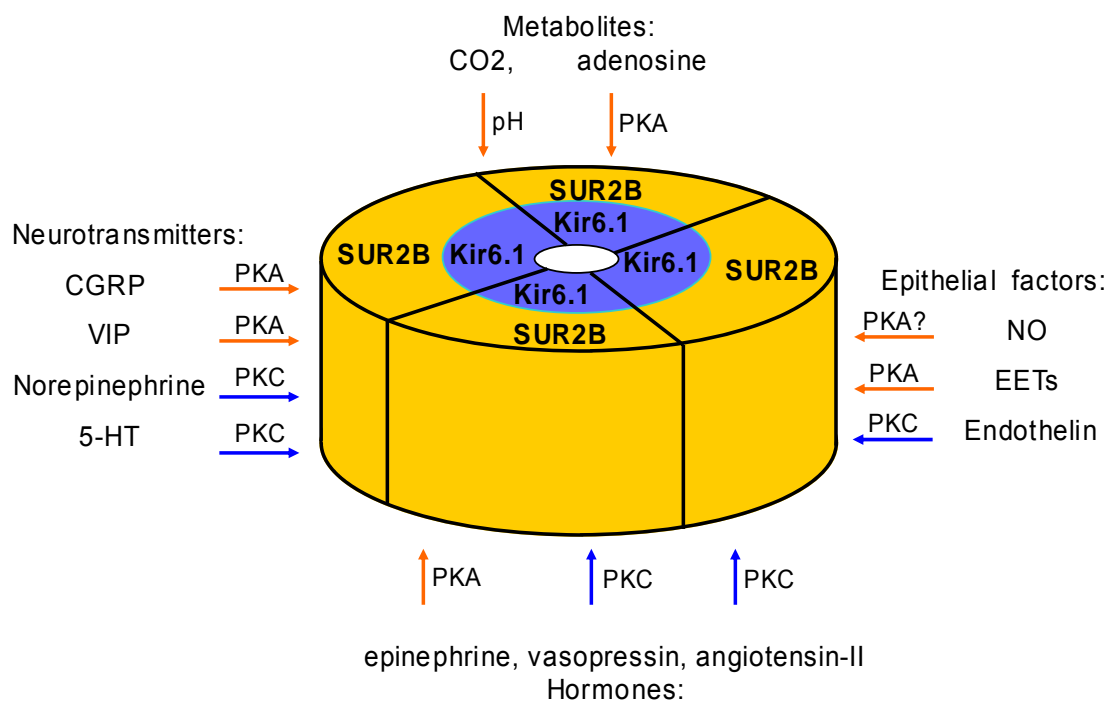
### **7.4.1. Metabolites**

Like  $K_{ATP}$  channels in other tissues, vascular  $K_{ATP}$  channels can couple intracellular metabolic state to membrane potentials. Inhibition of cellular metabolism with metabolic poisons, substrate depletion, or hypoxia has been reported to cause the channel activation in several tissues (for review, see (Quayle *et al.*, 1997)). ATP, ADP and pH are three major factors that change during metabolic stress and contribute to  $K_{ATP}$  channel modulation. ATP inhibits vascular  $K_{ATP}$  channels, although the sensitivity varies among reports (Quayle *et al.*, 1997). ATP concentrations, however, are well maintained in smooth muscles under physiological condition. ATP depletion only happens under extreme pathophysiological conditions (Quayle *et al.*, 1997). Therefore, the release of ATP inhibition might contribute to channel activity only under severe conditions. In contrast, the ADP concentrations change in a relatively wider range, thus it is one of the critical factors mediating metabolic regulation on vascular  $K_{ATP}$  channels. With stress challenges such as hypoxia and hypercapnia, extracellular and intracellular pH also drops significantly, which also drastically activate vascular  $K_{ATP}$  in basilar artery and mesenteric artery (Santa *et al.*, 2003; Wang *et al.*, 2003).

### **7.4.2. Hormonal vasodilators**

Multiple vasodilating neurotransmitters and hormones have been shown to activate  $K_{ATP}$  channels in several vessel beds (Fig. 7-2). Calcitonin gene-related peptide (CGRP) is a potent endogenous vasodilator found in perivascular nerves in many tissues. CGRP activates  $K_{ATP}$  channels in smooth muscle cells isolated from porcine coronary

arteries (Miyoshi & Nakaya, 1995) and rabbit mesenteric arteries (Nelson *et al.*, 1990a; Quayle *et al.*, 1994). CGRP hyperpolarizes the smooth muscle cells in intact mesenteric arteries and causes vasodilation that is partially abolished by glibenclamide. The cAMP-PKA pathway is involved in the CGRP effect.



**Figure 7-2. List of vasoactive agents that affect K<sub>ATP</sub> channel activity**

CO<sub>2</sub> activates K<sub>ATP</sub> channel via decrease in intracellular pH. Other vasodilators activate K<sub>ATP</sub> channels in a PKA dependent manner. Most vasoconstrictors inhibit K<sub>ATP</sub> channels in a PKC dependent manner.

Vasoactive intestinal polypeptide (VIP) is another potent vasodilator found in perivascular nerve terminals in coronary and cerebral artery beds. VIP activates  $K_{ATP}$  channels in smooth muscles isolated from rat mesenteric artery and Kir6.1/SUR2B channel expressed in HEK293 cells via protein kinase A (Yang *et al.*, 2007).

Adenosine is an important endogenous vasodilator in the coronary circulation, in which adenosine induces vasodilation that is inhibited by glibenclamide (Belloni & Hintze, 1991; Nakhostine & Lamontagne, 1993; Orito *et al.*, 1993; Toombs *et al.*, 1993; Akatsuka *et al.*, 1994; Randall, 1995). The effects of adenosine seem to be mediated by adenosine A2 receptors known to activate the PKA signaling pathway (Akatsuka *et al.*, 1994; Kleppisch & Nelson, 1995).

Endothelium-release nitric oxide (NO) relaxes VSMs by several mechanisms, one of which is mediated via activation of  $K_{ATP}$  channels (Murphy & Brayden, 1995; Ishibashi *et al.*, 1998). The intracellular signal pathway presumably involves cGMP-PKG, while cGMP might also activate PKA as a potential mechanism of the channel activation (Quayle *et al.*, 1997). Another  $K_{ATP}$  channel activator is endothelium-derived hyperpolarizing factor (EDHF) (Brayden, 1990). EDHF is a mixture of several epoxyeicosatrienoic acids (EETs) that activate  $K_{ATP}$  channels in mesenteric artery through the cAMP-PKA pathway (Ye *et al.*, 2005; Ye *et al.*, 2006).

#### **7.4.3. Vasoconstrictors**

Vasoconstrictors contract VSMs through multiple mechanisms. One of them is via inhibition of  $K_{ATP}$  channels. Endothelin (Miyoshi *et al.*, 1992; Kasemsri & Armstead, 1997; Thorin *et al.*, 1998), vasopressin (Wakatsuki *et al.*, 1992) and angiotensin II

(Miyoshi & Nakaya, 1991; Kubo *et al.*, 1997; Kawabata *et al.*, 2001; Sampson *et al.*, 2007) activate  $G_q$ , phospholipase C and protein kinase C. Blockade of PKC activity using pharmacological tools eliminates the inhibitory effects on  $K_{ATP}$  channels. Furthermore, angiotensin II may inhibit  $K_{ATP}$  channels by inhibiting PKA activity (Hayabuchi *et al.*, 2001b). Since the  $K_{ATP}$  channels are involved in rest membrane potential, the PKC inhibition is significant in basal tone regulation. Besides, and probably more importantly, the PKC inhibition facilitates vasoconstriction by blockade of  $K_{ATP}$  activation.

## **7.5. Questions and potential solutions**

### **7.5.1. The questions about vascular regulators**

Experimental evidence suggests that the effects of PKA and PKC on  $K_{ATP}$  channel activity are critical for vascular tone regulation. However, the underlying mechanisms remain elusive, though the phenomena have been discovered for more than ten years. The molecular substrates of PKA and PKC are unclear. Do PKA and/or PKC directly phosphorylate the channel proteins? If so, which subunit is the target? Where are the phosphorylation sites? If both kinases directly target the channel proteins, why can one produce channel activation and the other cause channel inhibition? What is the underlying gating mechanism upon phosphorylation? If the kinase(s) does not phosphorylate the channel proteins directly, what are the signaling molecules targeted directly by the kinase(s)? It is clear that addressing these questions has a major impact on the understanding of circulation regulation regarding the importance of the channels in vascular tone regulation.

### **7.5.2. Potential solutions**

To address the channel regulations by PKA and PKC, several experimental techniques have been considered in the original experimental design conducted in this thesis.

#### **7.5.2.1. Heterologous expression systems and real tissues**

The heterologous expression systems and mutational analysis have been used to identify the critical sites for ATP and PIP<sub>2</sub> binding on Kir6.2 discussed in above. The same strategy has been used to identify phosphorylation sites in Kir6.2/SUR1 (Beguin *et al.*, 1999; Lin *et al.*, 2000). Comparing to real tissues, the heterologous expression systems allow us to manipulate channel proteins. However, when the data from the expression systems are translated to the real tissues, we need pay attention: the real tissues may not possess the same signaling pathway as that in expression system. For instance, the Kir6.2/SUR1 channel is activated by PKA in expression systems (Beguin *et al.*, 1999; Lin *et al.*, 2000), while activation of G<sub>s</sub> system in pancreatic  $\beta$ -cells seems to inhibit the channel (Kang *et al.*, 2006). A minimal requirement for using heterologous expression systems is that the factor investigated should has comparable effects on channels in expression systems and in real tissues. The Kir6.1/SUR2B channels are activated by protein kinase A in HEK293 cells with comparable sensitivity to those in real tissue (Quinn *et al.*, 2004). The Kir6.1/SUR2B channels also present the same PKC inhibitory effects as native channels (Thorneloe *et al.*, 2002). Therefore, using a combination of heterologous expression systems and mutational analysis might provide



valuable information for understanding the underlying mechanisms of PKA activation and PKC inhibition.

#### **7.5.3.2. Chimeras vs. mutational analysis**

Mutational analysis is a traditional method in identification of phosphorylation sites. PKC and PKA phosphorylate serine and threonine residues within a consensus sequence, thus allowing us to predict the putative sites. With the putative phosphorylation sites in hand, mutagenesis can be applied to target them one by one to analyze their function in phosphorylation. Mutational analysis has been used in our lab in identification of PKC sites in Girk channels (Mao *et al.*, 2004) and identification of the PKA sites in Kir6.1/SUR2B channel in this thesis. However, this method has drawbacks. If the protein has multiple functional phosphorylation sites, the effects may not be strictly additive. In this case, other strategies can be considered such as chimeras. To identify critical protein domains using a chimeric approach, two proteins must be similar in their protein sequences, and have clearly different responses (better if completely opposite). The Kir6.1 and Kir6.2 are highly similar to each other with ~70% identity in the primary sequences. However, all Kir6.2-containing channels are activated or do not respond to PKC, while Kir6.1-containing channels are inhibited by PKC (detailed in chapter 12). The effects are clearly opposite. So in this thesis we adopted a chimeric strategy to complement the mutational analysis and found critical motives for PKC inhibition.

### **7.5.3.3. Molecular modeling in understanding phosphorylation-associated channel gating**

The molecular modeling strategy can further explain the molecular basis of the channel gating from structural views, providing a more detailed understanding of the basic mechanism. This has been applied to understanding the ATP and PIP2 binding pockets in Kir6.2 (Antcliff *et al.*, 2005; Haider *et al.*, 2007). To use this strategy it is necessary to find the appropriate structural template that generally comes from the same protein family. Kir6.1 belongs to the inward rectifier K<sup>+</sup> channel subfamily. The cytosolic portions of mammalian Kir3.1 (Nishida & MacKinnon, 2002) and Kir2.1 (Pegan *et al.*, 2005) have been crystallized with high resolution. The transmembrane and core regions have been resolved for the entire bacterial KirBac1.1 (Kuo *et al.*, 2003). Therefore Kir6.1 and Kir6.2 subunits can be modeled using these known crystal structures. SUR subunits belong to ATP-binding cassette transporter (ABC) superfamily. The ABC transporters are large proteins with multiple domains including an ABC-core that is composed of two nucleotide-binding domains (TMDs) and two clusters of transmembrane domains (TMDs). In SURx, the additional TMD<sub>0</sub> is linked to the N-terminus of the ABC-core. Recently several ABC proteins have been crystallized. Among them is SAV1866, a homologue of SURx. In this thesis, we have found a critical PKA site is located on the SUR2B subunit. Thereby the SAV1866-based SUR2B model may provide structural information for the PKA-mediated gating. On the other hand, we have found that the PKC sites are all located in distal C-terminus of Kir6.1. Unfortunately, the resolved

crystal structures of Kir2.1 and Kir3.1 lack this region, and cannot be used to understand PKC-mediated gating mechanisms.

## 8. Significance

Small arteries and arterioles regulate diastolic blood pressure and regional blood flow. Diastolic blood pressure controls systemic functions, while the regional blood flow determines activity of each individual organ and tissue. Therefore, the vascular tone or tension, to a large degree, determines the functional status of the system and local tissues. It is known that the vascular tone is tightly controlled by complex neural, humoral and metabolic mechanisms. It is the primary target of the autonomic nervous system. A number of circulating hormones released from adrenal glands, kidneys and the central nervous system work on local tissues via their action on the vascular tone. Metabolic activity of local tissues can affect vascular tone through a number of metabolites.

The contractility of vascular smooth muscles (VSMs) determines the vascular tone. It is known that smooth muscle contraction relies on a chain of biochemical reactions leading to the interaction of contractile filaments, where intracellular  $\text{Ca}^{2+}$  elevation serves as an initiating factor. Most vasoactive signals are transduced to a  $\text{Ca}^{2+}$  signal in the VSMs by a large number of receptors, ion channels and intracellular signaling molecules. Several key molecules have been found to integrate multiple sources of vasoactive signals and converge the information into the common  $\text{Ca}^{2+}$  signal in VSMs. The understanding of the molecular mechanisms for the actions of these key molecules would shed insight into the cellular basis of vascular tone regulation and provide the basis for designing effective therapeutical methods for hypertension, shock, and other cardiovascular disorders.

$\text{Ca}^{2+}$  entry through voltage-gated  $\text{Ca}^{2+}$  channels (VDCCs) is one of the major sources of intracellular  $\text{Ca}^{2+}$  elevation in VSMs. The primary factor affecting VDCCs activity is membrane potential. Because membrane potentials are generated and controlled by  $\text{K}^{+}$  channels, the knowledge of how  $\text{K}^{+}$  channels change their activity in VSMs becomes critical for understanding vascular tone regulation.

$\text{K}_{\text{ATP}}$  channels are probably the most important  $\text{K}^{+}$  channels in vascular tone regulation. Previous studies in our laboratory have shown the vascular  $\text{K}_{\text{ATP}}$  channels are activated by hypercapnic acidosis. Others have demonstrated that the channels are activated by a change in intracellular ATP and ADP concentrations under metabolic stress. In addition to the above metabolic products, hormones and neurotransmitters tightly regulate vascular  $\text{K}_{\text{ATP}}$  channels. A numbers of vasodilating hormones and neurotransmitters activate  $\text{K}_{\text{ATP}}$  channels in vascular smooth muscles and various vasoconstrictors inhibit the channels. Thus, vascular  $\text{K}_{\text{ATP}}$  channels must integrate multiple vasoactive inputs and produce corresponding changes in vascular tone, affecting systemic blood pressure and local blood distribution.

Accumulative experimental evidence indicates the PKA and PKC signaling pathways regulate channel activity by vasodilators and vasoconstrictors, respectively. It is assumed that the channel proteins are phosphorylated directly by these kinases. However, the molecular substrates for those protein kinases remain unknown, and other possibilities exist. For example, intermediate molecules downstream of the kinases may be involved, or changes in channel activity may be the result of alteration in membrane trafficking. The dissertation research was thus aimed at addressing the molecular basis of vascular

K<sub>ATP</sub> channel regulation by PKA and PKC systems. A combination of electrophysiological, molecular biological, pharmacological and protein structural techniques were employed. Our data showed that the vascular K<sub>ATP</sub> channels were directly phosphorylated by both kinases. By systemic analysis of the primary sequence as well as the tertiary structure of the channel proteins, potential mechanisms underlying the channel modulation by phosphorylations of the channel proteins were proposed. The mechanisms of the channel regulation discovered in this thesis provide fundamental information for the channel regulation by various vasoconstricting and vasodilating hormones and neurotransmitters that will eventually help in development of therapeutic strategies in the treatment and prevention of several cardiovascular diseases.

## **9. Material and Methods**

### **9.1. Cell culture**

Human embryonic kidney cells (HEK293, CRL-1573, Batch #2187595, ATCC, Rockville, MD) were chosen to express the  $K_{ATP}$  channels. The HEK293 cells were cultured as a monolayer in the MEM-E medium with 10% fetal bovine serum and penicillin/streptomycin added. Cultured at 37°C with 5% CO<sub>2</sub> in the atmosphere, the cells were split twice weekly. The HEK293 cells were transfected using Lipofectamine<sup>2000</sup> (Invitrogen, Carlsbad, CA) with 1µg Kir6.1 and 3µg SUR2B per 35mm petri dish. To facilitate the identification of positively transfected cells, 0.5µg green fluorescent protein (GFP) cDNA (pEGFP-N2, Clontech, Palo Alto, CA) was added to the cDNA mixture. Cells were disassociated from the monolayer using 0.25% trypsin ~24hrs post-transfection. A few drops of the cell suspension were added on to 5×5mm<sup>2</sup> cover slips in a 35mm petri dish. The cells were then cultured for 24-48hrs before experiments

### **9.2. Molecular biology**

Rat Kir6.1 (GenBank #D42145) and mouse SUR2B (GenBank #D86038) were used in studies in this thesis. Both cDNAs were cloned in the eukaryotic expression vector pcDNA3.1 and used for mammalian cell expression. The human  $\beta_2$ -adrenergic receptor (ADRB2) (GenBank #NM\_000024) was purchased from Origene (Rockville, MD).

Site-specific mutations were made using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Kir6.1-Kir6.2 chimeras were produced by overlap extension using PCR (*pfu* DNA polymerase, Stratagene) (Piao *et al.*, 2001). The orientation of the constructs and correct mutations were confirmed with DNA sequencing. The constructs were expressed in HEK293.

pMal vector (New England Biolabs) was used to express fusion proteins. The cDNAs of target peptides were amplified with PCR and inserted into pMal vector. The constructs were transformed into protease-deficient *E. coli* BL21, in which MBP-fusion peptides were induced with 0.3mmol/L isopropylthiogalactoside for 2 hours. The MBP-fusion peptides were purified using amylose resin according to the procedure by the provider (New England Biolabs).

### **9.3. Artery rings**

Mesenteric arterial rings were obtained from Sprague-Dawley rats (250-350g) in accordance with the guidelines for the care and use of laboratory animals by Georgia State University. The rats were anesthetized by inhaling saturated halothane vapor followed by cervical dislocation. The mesenteric arteries were dissected free and transferred to ice-cold Krebs solution containing (in mM): NaCl 118.0, NaHCO<sub>3</sub> 25.0, KCl 3.6, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11.0, and CaCl<sub>2</sub> 2.5 bubbled with 5% CO<sub>2</sub>. The arteries were cut into 6-8 endothelium-intact rings of 2mm in length and stored in Krebs solution. Endothelium-denuded rings were also used in which the endothelium was removed by a rough plastic tube and tested by loss of response to acetylcholine. During the experiment, a



ring was mounted on a force-electricity transducer (Model FT-302, iWorx/CBSciences, Inc. Dover, NH) in a tissue bath. With a 0.8g preload, the ring was allowed to equilibrate in the tissue bath for 30min when the tension was reduced to ~0.6g. The tissue bath was filled with Krebs solution and perfused with 5% CO<sub>2</sub> at 36°C. Arterial tone was measured as changes in isometric force. Only rings that showed a clear vasoconstriction response to 1.0μM phenylephrine were used in the study.

#### **9.4. Dissociated vascular smooth muscle cells**

Acutely dissociated vascular smooth muscles were prepared with a two-step enzyme digestion. Mesenteric arteries were obtained as previously described, cut into small segments (1-2mm), and placed in a 5ml saline solution containing (in mM): NaCl 140, KCl 5.4, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1, HEPES 10 and D-glucose 10 at room temperature for 10 min. The tissues were then placed in 1ml solution containing 20 units of papain (Worthington, New Jersey) and 1.25mg dithiothreitol (DTT). After incubation for 30min at 35°C, the tissue was washed once and then transferred to 1ml solution containing 440 units of collagenase (CLS II, Worthington) and 1.25mg trypsin inhibitor (Sigma) for 15min. After a thorough wash the tissue was moved to 1ml solution containing 20% fetal bovine serum and triturated with a fire polished Pasteur pipette to yield single smooth muscle cells. The cells were stored on ice and used within 8hrs. A drop of cells was placed in a 35mm tissue culture dish where the cells were allowed to attach to the surface. The cells that had typical smooth muscle morphology and did not show evident swelling or shrinkage were used for further studies.

## 9.5. Patch clamp experiments

Patch clamp experiments were performed at room temperature as described previously. In brief, fire polished patch pipettes were made with 1.2mm borosilicate glass capillaries. Whole-cell recording was performed using single-cell voltage clamp with recording pipettes of 4-6M $\Omega$ . Current records were low-pass filtered (2kHz, Bessel 4-pole filter, -3dB), digitized (10kHz, 16-bit resolution), and stored on computer disk for later analysis using the Clampfit 6 and 9 software (Axon Instruments Inc.). The bath solution contained (in mM): KCl 10, potassium gluconate 135, EGTA 5, glucose 5, and HEPES 10 (pH = 7.4). The pipette solution contained (in mM): KCl 10, potassium gluconate 133, EGTA 5, glucose 5, K<sub>2</sub>ATP 1, NaADP 0.5 and HEPES 10 (pH = 7.4), in which the free Mg<sup>2+</sup> concentration was adjusted to 1mM using MgCl<sub>2</sub>. Since the variation of Cl<sup>-</sup> concentrations in solutions was rather small, the resulted liquid junction potential was less than 1mV according to the Henderson equation, and was thereby not corrected. Inside-out patches were performed with symmetric high K<sup>+</sup> in the bath and pipette: KCl 10, potassium gluconate 135, EGTA 5, glucose 5, and HEPES 10 (pH = 7.4), with [Mg<sup>2+</sup>] adjusted to 1mM using MgCl<sub>2</sub>. After formation of a giga-seal, the patch was excised and the intracellular side was exposed to bath solution. The holding potential was 0mV and a constant single voltage of -60mV was applied to the patch. All currents recorded from the inside-out patches were digitized in a higher sampling rate (20 kHz).

## 9.6. Phosphorylation sites prediction

PKA and PKC phosphorylation sites were predicted using two online programs *Kinasephos*, (<http://kinasephos.mbc.nctu.edu.tw/>) (Huang *et al.*, 2005) and *NetPhosK* (<http://www.cbs.dtu.dk/services/NetPhosK>) (Blom *et al.*, 2004). A serine or threonine was considered for further studies as a putative PKA site if there was an alkaline amino acid at the -2 or -3 position. A typical PKC site is serine or threonine followed by basic residues at +2 or +3 position.

## 9.7. Western blot

MBP-Kir6.1Cs with or without mutants were generated with the distal C-terminus segment (residues 346-420) of Kir6.1 fused to C-terminus of Maltose binding protein (MBP) using same methods detailed in above. The peptides were run in 10% SDS-page gel and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Western blot was carried out using rabbit primary antibody against Kir6.1 C-terminus (KRNSMRRNNSMRRSN, corresponding to amino acid residues 382-396 of rat Kir6.1, Sigma, 1:2000) and secondary goat anti-rabbit IgG conjugated with alkaline phosphatase. The protein bands were depicted with bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

## 9.8. *In vitro* phosphorylation

*In vitro* phosphorylation was performed on SUR2B and Kir6.1Cs peptides fused to maltose-binding protein (MBP). Mutants were produced either using the site-directed

mutagenesis kit (Stratagene) or just using the templates with mutants in pcDNA3.1. The SUR2B fusion peptides were subsequently incubated with the catalytic subunit of PKA (cPKA, P2645 from Sigma) in the following reaction: 5µg fusion peptides in 5µl elution buffer (in mM) (NaCl 200, tris-HCl 30, EDTA 1, maltose 10, pH 7.4), 5µl of 5X reaction buffer (tris-HCl 125, EGTA 0.1, pH 7.5), 5µl Mg-ATP solution (MOPS 20, β-glycerophosphate 25, EGTA 5, Na<sub>3</sub>VO<sub>4</sub> 1, DTT 1, MgCl<sub>2</sub> 75, ATP 0.5 and pH 7.2), 10 units of cPKA in 10µl H<sub>2</sub>O and 1µl of 5µCi/µl of [<sup>32</sup>P-γ]-ATP (Perkin-Elmer, San Francisco, CA). After 1 hour 5µl of 5X protein loading buffer were added to each sample to terminate the reaction. The samples were then subjected to electrophoresis in 10% SDS-page gel, stained with Coomassie blue, and photographed. The gel was then fixed, dried. Autoradiography was carried out using a Fuji BAS 2500 Imaging Plate. This experiment was repeated twice. Same procedure was conducted for PKC *in vitro* phosphorylation on Kir6.1Cs peptides by replacing cPKA with cPKC.

## 9.9. Molecular modeling

Modeling of the SUR2B\_core was based on the crystal structure of SAV1866 (PDB: 2HYD). The sequences of SAV1866 were aligned with both halves of the SUR2B\_core (TM-NBD<sub>1</sub> and TM-NBD<sub>2</sub>) using ClustalW. The atomic coordinates of amino acids, nucleotides and water molecules in the template (SAV1866) were transferred to the SUR2B\_core model. The ADP molecule in first nucleotide binding pocket was replaced with ATP. The coordinates of ATP-binding Mg<sup>2+</sup> and ADP-binding Mg<sup>2+</sup> were obtained using the crystal structures of ABC transporters HlyB (PDB: 1XEF)

and TAP1 (PDB: 1JJ7), respectively. The linker regions between TMD<sub>1</sub> and NBD<sub>1</sub> (residues 618-665), and between TMD<sub>2</sub> and NBD<sub>2</sub> (residues 1290-1309) were modeled as surface loops in the structure. The linker between NBD<sub>1</sub> and TMD<sub>2</sub> (residues 914-975) was omitted from the model due to lack of a template. Energy minimization was performed using 1000 steps of conjugate gradients optimization with the latest AMMP potential set (version tuna) (Chen *et al.*, 2004). The model was viewed with PyMOL (<http://pymol.sourceforge.net/>).

#### **9.10. Molecular dynamics simulation**

Molecular dynamics (MD) simulations were carried out to study the conformational change in the NBD<sub>2</sub>-ICL<sub>2</sub> interface. The SUR2B\_core model contained many hydrophobic transmembrane segments that would require a very large molecular dynamics simulation including the lipid bilayer. Therefore, we constructed two models containing NBDs and an extended segment of ICL<sub>2</sub> (residues 500-512) with and without a phosphate group linked to atom O<sub>g</sub> of Ser1387. Seven thousand and five hundred H<sub>2</sub>O were added to the model molecules to ensure an aqueous environment. Additional ions were added to neutralize the total charge of the protein. No screening dielectric term or bulk solvent correction was included. A constant dielectric of one was used. The amortized fast multipole algorithm in AMMP was used for the long-range terms in the non-bonded and electrostatic potentials so that no-cutoff radius was employed (Harrison, 2003). Simulations were performed with a constant NVT (number, volume and time) ensemble corresponding to a pressure about 1 atm with classical

molecular mechanics MD program AMMP (Harrison & Weber, 1994; Chen *et al.*, 2004). The temperature was set at 310K. One thousand frames, one for each picosecond, were logged over 1-ns MD run. The *mode* structures which represent the most frequent position of each atom (Chen *et al.*, 2004) and the RMSDs of C $\alpha$ s of each residue were calculated over 1000 frames.

### 9.11. Chemicals

Chemicals used in my studies were purchased from Sigma (St. Louis, MO) unless otherwise stated. All chemicals were prepared as high concentration stocks in double distilled H<sub>2</sub>O or DMSO, and were diluted in the recording solution to experimental concentrations immediately before usage. In cases where DMSO was used, its concentration was maintained at less than 0.1% in the experimental solutions. This concentration of DMSO does not affect the Kir6.1/SUR2B channel. Isoproterenol, glibenclamide, pinacidil and forskolin were applied to cells using a perfusion system. Adrenergic  $\beta_1$  and  $\beta_2$  antagonists were administrated at least 2min prior to and during the isoproterenol exposure. PKA inhibitors RP-cAMP was included in the pipette solution (200 $\mu$ M) and added to the perfusion solution (100 $\mu$ M). PKA inhibitory peptide (PKI5-24, 10 $\mu$ M) was applied to the pipette solution. To avoid ATP degradation, all ATP containing solutions were made immediately before experiments and used no longer than 4hrs.

### **9.12. Data analysis**

Data are presented as means  $\pm$  s.e. Differences in means were tested with the ANOVA or Student t-test and were accepted as significant if  $P \leq 0.05$ .

## **10. Result 1: PKA Phosphorylation of SUR2B Subunit Underscores Vascular K<sub>ATP</sub> Channel Activation by B-Adrenergic Receptors**

Publication: Yun Shi, Zhongying Wu, Ningren Cui, Weiwei Shi, Yang Yang, Xiaoli Zhang, Asheebo Rojas, Binh T Ha, Chun Jiang. (2007) *Am J Physiol.* **293(3)**, R1205-14

Note: In this paper, Yun Shi did ~80% of the work, including experimental design, 90% of patch experiments, 60% of DNA mutations preparation, 80% of fusion-protein construction and purification, and *in vitro* phosphorylation. Zhongying Wu and Nga Ta (her name is in acknowledgement) helped prepare cDNAs and mutations. Dr. Ningren Cui did most of mesenteric ring experiments. Weiwei Shi, Yang Yang and Xiaoli Zhang helped in patch clamp experiments. Binh T Ha contributed to setting up protein purification system. Asheebo Rojas helped in setting-up *in vitro* phosphorylation assay.



## 10.1. Abstract

$K_{ATP}$  channels are activated by several vasodilating hormones and neurotransmitters through the PKA pathway. Here we show that phosphorylation at Ser1387 of the SUR2B subunit is critical for the channel activation. Experiments were performed in HEK293 cells expressing the cloned Kir6.1/SUR2B channel. In whole-cell patch, the Kir6.1/SUR2B channel activity was stimulated by isoproterenol via activation of  $\beta_2$  receptors. This effect was blocked in the presence of inhibitors for adenylyl cyclase or PKA. Similar channel activation was seen by exposing inside-out patches to the catalytic subunit of PKA. Since none of the previously suggested PKA phosphorylation sites accounted for the channel activation, we performed systematic mutational analysis on Kir6.1 and SUR2B. Two serine residues (Ser1351, Ser1387) located in the NBD<sub>2</sub> of SUR2B were critical for the channel activation. *In vitro* phosphorylation experiments showed that Ser1387 but not Ser1351 was phosphorylated by PKA. The PKA-dependent activation of cell-endogenous  $K_{ATP}$  channels was observed in acutely dissociated mesenteric smooth myocytes and isolated mesenteric artery rings where activation of these channels contributed drastically to the isoproterenol-induced vasodilation. Taken together, these results indicate that the Kir6.1/SUR2B channel is a target of  $\beta_2$  receptors, and the channel activation relies on PKA phosphorylation of SUR2B at Ser1387 in HEK cells.

## 10.2. Introduction

Vascular  $K_{ATP}$  channels are activated by PKA. Blockade of PKA signaling pathway eliminates the channel modulation by several vasodilators such as adenosine, calcitonin gene-related peptide and epoxyeicosatrienoic acids and abolishes their effects on vascular tone (Quayle *et al.*, 1994; Kleppisch & Nelson, 1995; Wellman *et al.*, 1998; Ye *et al.*, 2005). It is known that the PKA signaling pathway can be activated by  $\beta$  adrenergic receptors ( $\beta$ -ARs). Stimulation of  $\beta$ -ARs hyperpolarizes VSMs leading to vasodilation (Guimaraes & Moura, 2001; Johnson, 2006).

Although both  $\beta$ 1 and  $\beta$ 2 receptors may be involved, the  $\beta$ 2 receptor is known to play a major role. Mice with  $\beta$ 2 receptor gene knockout develop hypertension during exercise or with adrenaline challenges (Chruscinski *et al.*, 1999). Abnormalities in  $\beta$  adrenergic responses are seen in rats with spontaneous hypertension (Goto *et al.*, 2001). In humans, single nucleotide polymorphisms in the  $\beta$ 2 receptor gene are associated with increased vasoconstrictor sensitivity and stage-2 hypertension (Dishy *et al.*, 2004; Ge *et al.*, 2005). Pharmacologic studies have suggested that  $K^+$  channels contribute to the  $\beta$ -AR mediated vasodilation, especially  $K_{ATP}$  channels (Randall & McCulloch, 1995; Chang, 1997; Ming *et al.*, 1997; Sheridan *et al.*, 1997; Hein *et al.*, 2004).

Although the PKA-dependent activation of vascular  $K_{ATP}$  channels has been shown in previous studies, questions as to how PKA stimulation leads to the channel activation, whether the channels are directly phosphorylated by PKA, whether the Kir6.1, SUR2B or both are targets of PKA phosphorylation are still unclear. To address these questions, we performed studies on cloned Kir6.1/SUR2B channel. Our data suggest that

phosphorylation of SUR2B subunit underscores the channel activation by  $\beta$  adrenergic receptor signaling pathway in HEK cells.

### 10.3. Results

#### 10.3.1. Baseline Kir6.1/SUR2B channel activity

The Kir6.1/SUR2B channel was transiently co-expressed with GFP in HEK293 cells. Whole-cell voltage clamp was performed on GFP-positive cells. Without any treatment the currents remained small or were slightly increased in a period of 8-10min. Strong current activation was seen when the cell was exposed to 10 $\mu$ M pinacidil. The pinacidil-activated currents were strongly inhibited by 10 $\mu$ M glibenclamide. The currents did not show clear inward rectification (Fig. 10-1A). These characteristics are consistent with the Kir6.1/SUR2B currents reported previously (Yamada *et al.*, 1997; Satoh *et al.*, 1998). HEK293 cells transfected with the expression vector alone were used as negative control, in which small inward rectifier currents were seen. The currents were insensitive to pinacidil and glibenclamide (Fig. 10-1B).

#### Figure 10-1. Kir6.1/SUR2B channels expressed in HEK293 cells.

**A.** Whole-cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Symmetric concentrations of K<sup>+</sup> (145mM) were applied to both sides of cell membranes. The cell was held at 0mV, and pulse voltages from -120 to 80mV with a 20mV increment were applied. The current amplitude increased in response to isoproterenol (Isop, 100nM). The isoproterenol-activated currents were further activated by pinacidil (Pin, 10 $\mu$ M) and inhibited by glibenclamide (Glib, 10 $\mu$ M). **B.** Currents recorded from another cell transfected with the expression vector alone were insensitive to isoproterenol, pinacidil and glibenclamide. **C.** The time course for the Kir6.1/SUR2B channel modulation. Whole-cell currents were recorded with a holding potential at

0mV and command pulses of  $-80\text{mV}$  in every 3 seconds. After whole-cell configuration was formed, the cell was perfused with extracellular solution for a 4-6min baseline recording. Note that the baseline record was shortened in the figure. The currents were strongly activated by isoproterenol, and the maximum activation was reached in 3-4min of the exposure. The currents were inhibited by glibenclamide ( $10\mu\text{M}$ ) and further activated by pinacidil ( $10\mu\text{M}$ ). The lower panel shows individual currents produced by a single command pulse. **D.** In the presence of glibenclamide, isoproterenol failed to activate the Kir6.1/SUR2B channel.

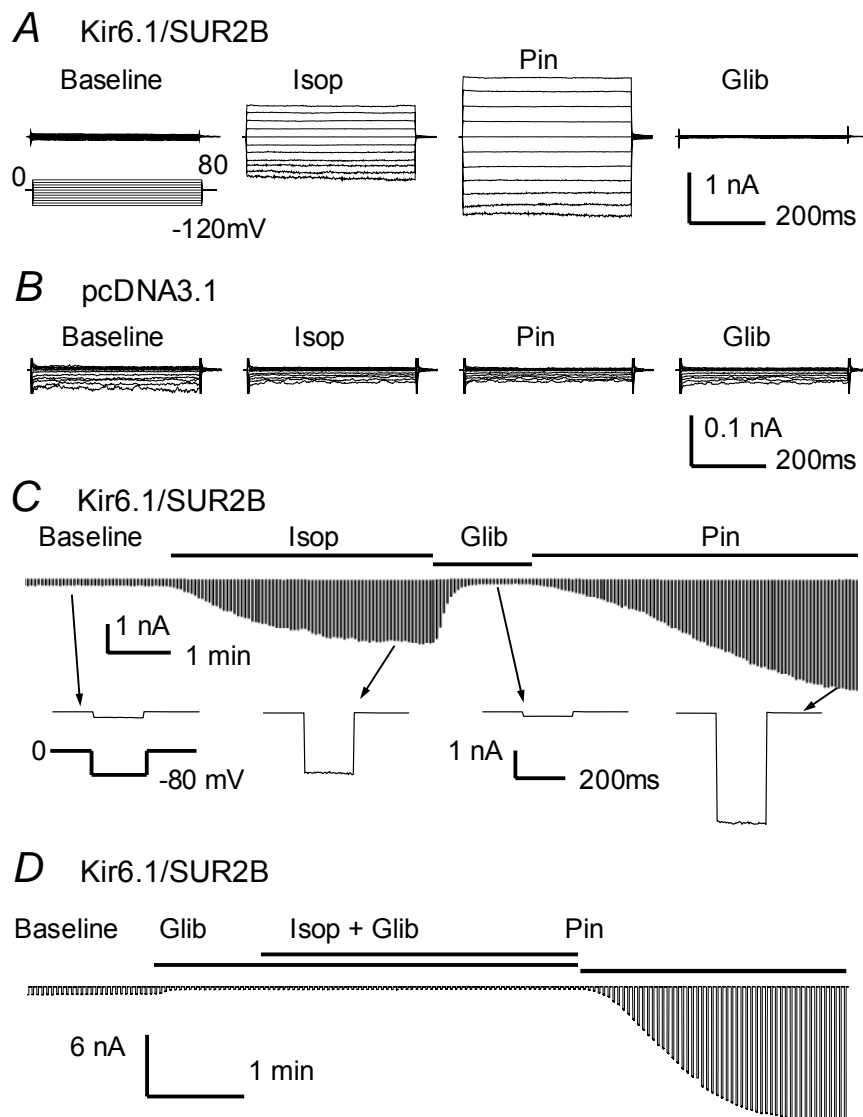
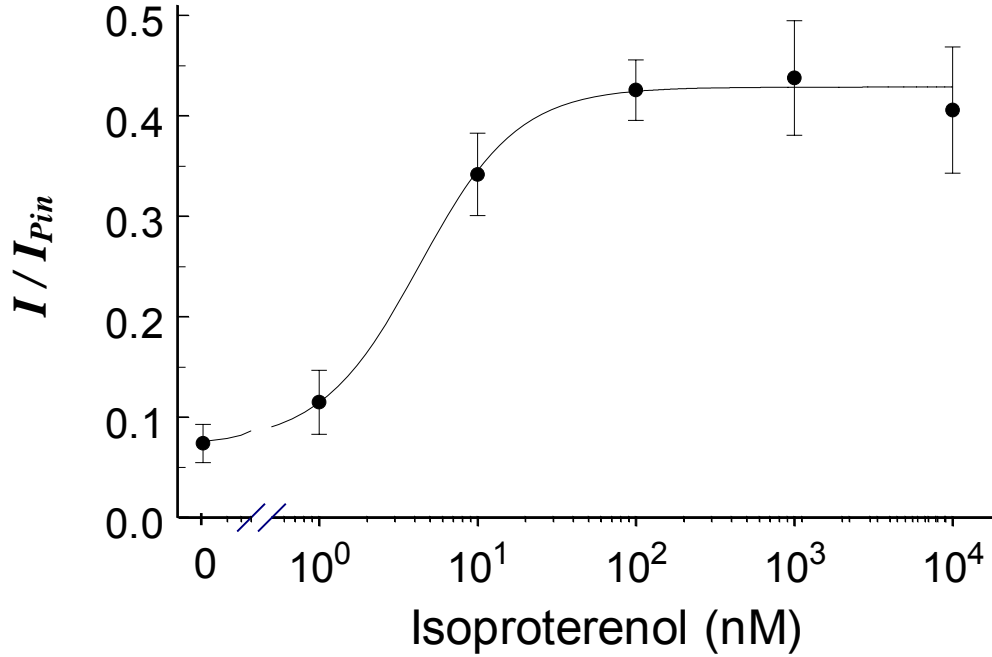


Figure 10-1

### 10.3.2. Activation of the Kir6.1/SUR2B channel by $\beta_2$ receptor stimulation

The  $\beta_2$ -AR was over-expressed in HEK293 cells together with Kir6.1 and SUR2B. The  $\beta$ -AR agonist isoproterenol was applied to the cell following stabilization of the baseline currents for 4-6 min. The isoproterenol exposure activated  $K^+$  currents that were sensitive to both pinacidil and glibenclamide. We found that without  $\beta_2$ -AR transfection, the Kir6.1/SUR2B currents were still activated by isoproterenol. This observation is consistent with the previous finding that  $\beta_2$ -AR is endogenously expressed in HEK293 cells (Daaka *et al.*, 1997; Smith *et al.*, 2002). Therefore, further experiments were conducted in the HEK293 cells without exogenous  $\beta$ -ARs. The currents activated by isoproterenol were further activated by pinacidil and inhibited by glibenclamide (Fig. 10-1A, C). The channel activation was totally eliminated in the presence of 10 $\mu$ M glibenclamide (Fig. 10-1D) and not seen in cells transfected with the expression vector alone (Fig. 10-1B).

After currents were normalized between maximum channel inhibition by 10 $\mu$ M glibenclamide and maximum activation by 10 $\mu$ M pinacidil, the baseline currents averaged  $6.9 \pm 1.5\%$  ( $n=27$ ) of the maximum channel activity. Isoproterenol (100nM) increased the currents to  $42.6 \pm 3.0\%$  ( $n=8$ ). The effect showed clear concentration dependence (Fig. 10-2). Evident current activation occurred with 1nM isoproterenol, and the maximum effect was reached with 100nM. The  $EC_{50}$  was 4.3nM with a Hill coefficient of 1.4 (Fig. 10-2).



**Figure 10-2. Concentration-dependent activation of Kir6.1/SUR2B currents by isoproterenol.**

The effect of isoproterenol was measured and normalized between the maximum channel inhibition by 10 $\mu$ M glibenclamide and maximum channel activation by 10 $\mu$ M pinacidil. Baseline currents with no isoproterenol were  $7.4 \pm 1.9\%$  ( $n=18$ ) of full channel activation by pinacidil. Evident activation of the Kir6.1/SUR2B currents was seen with 1nM isoproterenol ( $11.5 \pm 3.2\%$ ,  $n=4$ ), 10nM ( $34.2 \pm 4.1\%$ ,  $n=6$ ) and the maximum activation was reached with 100nM ( $42.6 \pm 3.0\%$ ,  $n=8$ ). Further increase in isoproterenol concentration had no further activation, 1 $\mu$ M ( $43.8 \pm 5.7\%$ ,  $n=6$ ) and 10 $\mu$ M ( $39.4 \pm 5.7\%$ ,  $n=7$ ). The concentration-current relationship was described using the Hill equation  $y = 0.074 + 0.355 / (1 + (EC_{50} / [Isop])^h)$ , where  $y$  is normalized Kir6.1/SUR2B currents,  $[Isop]$  is isoproterenol concentration,  $EC_{50}$  (4.3nM) is the Isop concentration for 50% channel activation, and  $h$  (1.4) is the Hill coefficient.

To identify the receptor subtype involved we applied specific  $\beta$ -AR antagonists to the cells 2min prior and during the administration of isoproterenol. The isoproterenol effect was almost completely blocked by  $\beta_2$ -AR antagonist ICI-118551 (100nM), whereas  $\beta_1$ -AR antagonist atenolol (1 $\mu$ M) had no effect (Fig. 10-3A-C). These results thus indicate that isoproterenol activates the Kir6.1/SUR2B channel through endogenous  $\beta_2$ -AR in HEK293 cells.

### 10.3.3. PKA dependence

The Kir6.1/SUR2B activation by isoproterenol was blocked when 8-(4-chlorophenylthio) adenosine-3', 5'-cyclic monophosphorothioate RP-isomer (RP-cAMP), a PKA inhibitor, was applied in both the pipette solution (200 $\mu$ M) and the perfusion solution (100 $\mu$ M) (Fig. 10-3C, D). Similar blockade was seen in the presence of the specific PKA inhibitory peptide (PKI5-24, 10 $\mu$ M) added in pipette solution, suggesting that PKA is involved in the activation of the channel by isoproterenol (Fig. 10-3C, E).

#### **Figure 10-3. Dissection of signal pathway for Kir6.1/SUR2B channel activation by isoproterenol.**

**A.** In the presence of  $\beta_2$  receptor antagonist ICI-118551 (100nM), isoproterenol had very little effect on the Kir6.1/SUR2B currents. **B.** With  $\beta_1$  antagonist atenolol (1 $\mu$ M), isoproterenol remained to activate the Kir6.1/SUR2B currents. Note that  $\beta$ -AR antagonists were perfused to cells 5min before and during the isoproterenol exposure. **C.** Currents were normalized to pinacidil and glibenclamide effects. Open bar, baseline current; black bar, isoproterenol; hatched bar, forskolin (FSK). 2Deox-ATP, 2',5'-dideoxyadenosine-3'-triphosphate; PTX, pertusis toxin. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  ( $n = 5$  to 14). **D.** RP-cAMP, a potent PKA inhibitor, was applied in both pipette solution (200 $\mu$ M) and perfusion solution (100 $\mu$ M). The currents activation by isoproterenol was almost completely blocked. **E.** Similar blockage of the channel activation was

observed with a PKA inhibitory peptide (PKI5-24, 10 $\mu$ M) in the pipette solution. **F**. The effect of cAMP (100 $\mu$ M) in pipette solution on the Kir6.1/SUR2B currents. After formation of whole-cell configuration, the current amplitude gradually increased and became plateaued at ~40% of the maximum activation by pinacidil in ~5min. Application of isoproterenol did not produce further activation of the Kir6.1/SUR2B currents.

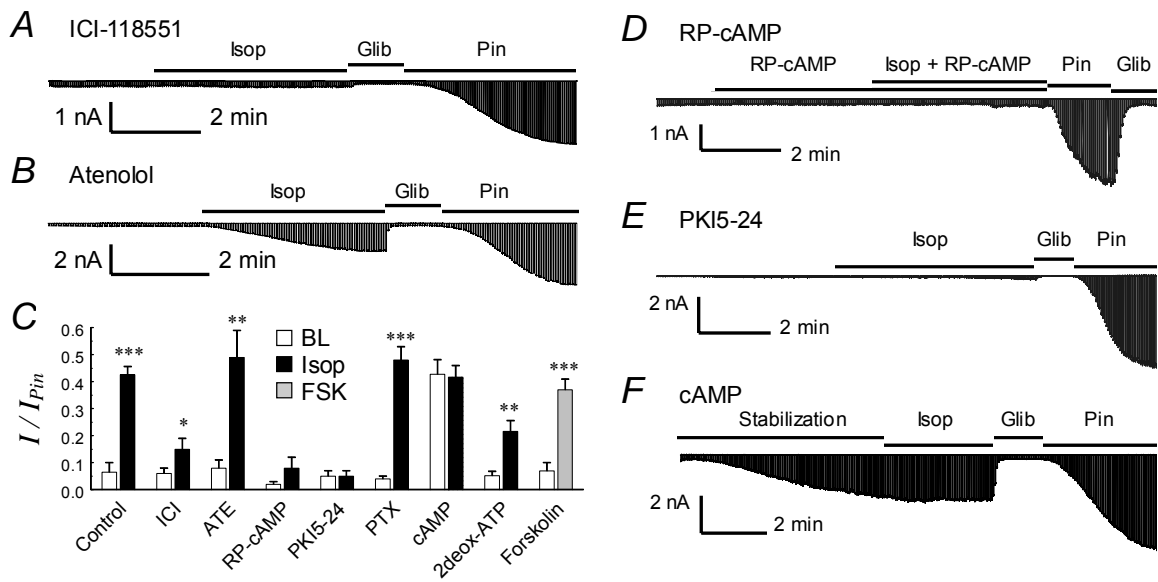


Figure 10-3

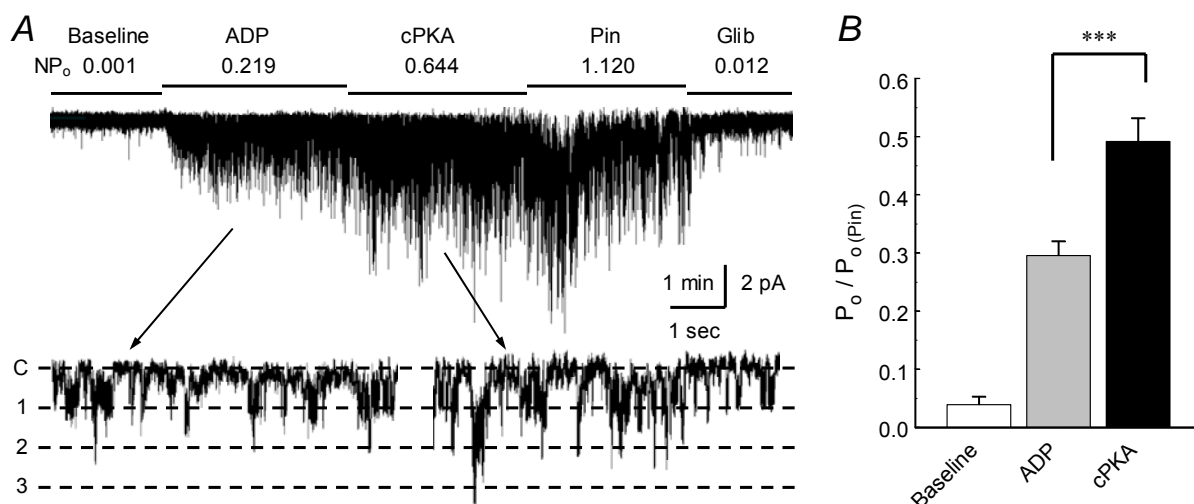


Since activation of  $G_s$  stimulates adenylyl cyclase, we studied the effect of forskolin, a potent adenylyl cyclase activator, on the Kir6.1/SUR2B currents. Exposure to 10 $\mu$ M forskolin activated the channel to almost the same degree as isoproterenol (Fig. 10-3C). The forskolin-activated currents also showed identical characteristics to those activated by isoproterenol. When the adenylyl cyclase inhibitor, 2',5'-dideoxyadenosine 3'-triphosphate (2deox-ATP) was included in the pipette solution, the channel activation by isoproterenol was diminished to  $21.3 \pm 7.2\%$  with 2 $\mu$ M (n=4) and  $21.9 \pm 4.9\%$  with 10 $\mu$ M (n=4), respectively. Since no difference was seen in these two concentrations, they were pooled together to compare with the experimental control. We found that these values were significantly lower than those of the control ( $P < 0.01$ , n=14; Fig. 10-3C), suggesting that  $G_s$ -activated adenylyl cyclase is involved.

The Kir6.1/SUR2B currents were gradually activated when 100 $\mu$ M cyclic AMP (cAMP) was included in pipette solution (Fig. 10-3C, F). The currents averaged  $42.8 \pm 5.4\%$  (n=5) of the total currents activated by 10 $\mu$ M pinacidil. Under such a condition, application of 100nM isoproterenol produced no further increase in the current amplitude (Fig. 10-3C, F).

It is known that persistent  $\beta_2$ -AR stimulation switches the intracellular signaling from a  $G_s$  cascade to  $G_i$ . To determine whether  $G_i$  also affects the Kir6.1/SUR2B channel activation by isoproterenol, we pretreated the cells overnight with pertussis toxin, a potent  $G_i$  inhibitor. Such a treatment had no effect on the Kir6.1/SUR2B channel activation by 10 $\mu$ M isoproterenol (Fig. 10-3C), indicating that  $G_i$  is not involved in the Kir6.1/SUR2B activation by isoproterenol.

To further demonstrate the PKA dependence, experiments were performed in excised inside-out patches. In the absence of nucleotide, the channels were mostly closed. Significant increase in the current amplitude was seen when the patches were exposed to perfusion solution containing  $Mg^{2+}$  and nucleotide, i.e., 1.0mM free  $Mg^{2+}$ , 0.5mM ADP and 1.0mM ATP (Fig. 10-4), consistent with previous reports (Sato *et al.*, 1998; Wang *et al.*, 2003). When the internal membrane of inside-out patches was exposed to the catalytic subunit of PKA in the presence of the same concentrations of  $Mg^{2+}$ , ADP and ATP, the Kir6.1/SUR2B channel was further activated (Fig. 10-4). Such channel activation was mediated by the augmentation of the open-state possibility ( $NP_o$ ) with no evident effect on the single-channel conductance. These data thus indicate that the channel activation is independent of other soluble cytosolic factors.



**Figure 10-4. Augmentation of Kir6.1/SUR2B channel activity by the catalytic subunit of PKA.**

**A.** The Kir6.1/SUR2B currents were recorded in an inside-out patch obtained from a HEK cell with a holding potential of -60mV and equal concentrations of  $K^+$  applied to both sides of the patch membranes. The channel activity was low in the baseline. Exposure of the internal patch membrane to 1.0mM ATP and 0.5mM ADP led to activation of the channels that showed a unitary conductance  $\sim 35$ pS (lower panel). The channels were further activated with an application of cPKA (100U/ml) to the internal solution in the presence of same concentrations of ATP and ADP. **B.** Summary of the experiment. Channel activity ( $NP_o$ ) was normalized to the level of pinacidil ( $P_o/P_o(Pin)$ ). The channel activity was rather low at baseline, increased markedly with ADP/ATP, and was further augmented with addition of cPKA. \*\*\*,  $P < 0.001$  (n=9 patches).

#### 10.3.4. PKA phosphorylation sites

A previous study suggested three PKA phosphorylation sites in the Kir6.1/SUR2B channel, i.e., Ser385 in the Kir6.1 subunit, and Thr633 and Ser1465 in the SUR2B (Quinn *et al.*, 2004). To further understand the  $K_{ATP}$  channel activation by  $\beta$ -ARs, we mutated these three residues either individually or jointly. The Kir6.1\_S385A/SUR2B mutant, in which the Ser385 was mutated to alanine, was activated by 100nM isoproterenol ( $48.7 \pm 3.0\%$ ,  $n=10$ ) and 10 $\mu$ M forskolin ( $39.2 \pm 2.3\%$ ,  $n=6$ ) to almost the same degree as the wild-type (WT) channel (Fig. 10-5A, D). The channel was still activated when the Ser385 was mutated to asparagine or glutamate, though to a less degree in the S385E mutation (Fig. 10-5D). A channel with all three residues mutated (Kir6.1\_S385A/SUR2B\_T633A\_S1465A or 3AA) responded to isoproterenol ( $44.6 \pm 4.3\%$ ,  $n=6$ ) and forskolin ( $42.0 \pm 3.0\%$ ,  $n=10$ ) and showed no significant difference from the WT channel under our experimental condition (Fig. 10-5B, D). Besides Ser385, Thr234 is another potential PKA site as suggested by its counterpart residue (Thr224) in Kir6.2 (Lin *et al.*, 2000). Neither Kir6.1\_T234A nor Kir6.1\_T234N mutation affected the channel activation by forskolin (Fig. 10-5C, D).

Since none of the PKA sites suggested by previous studies seems to be involved in the activation of the Kir6.1/SUR2B channel by isoproterenol, we performed systematic mutational analysis on both subunits. In the Kir6.1 subunit, nine other residues were predicted based on PKA consensus sequences (Blom *et al.*, 2004; Huang *et al.*, 2005). Site-specific mutations of eight of them had no effect on the channel activation by forskolin, while mutation of Thr190 to either alanine or asparagine failed to produce

pinacidil-sensitive currents (Fig. 10-5D), consistent with a previous observation that the T190A-mutant channel is non-functional (Thorneloe *et al.*, 2002).

**Figure 10-5. Mutation on potential PKA sites.**

**A.** With a mutation of Ser385 to alanine, the Kir6.1\_S385A/SUR2 currents were strongly activated by forskolin. **B.** The channel was still activated when three potential PKA sites, i.e., Ser385 in the Kir6.1, and Ser1465 and Thr633 in the SUR2B subunit, were all mutated to alanine. **C.** The Thr234 mutation on Kir6.1 did not abolish the channel activation by forskolin. **D.** Summary of mutagenesis analysis of potential PKA sites. All mutations were constructed on Kir6.1 except 3AA in which Ser385 in Kir6.1, and Ser1465 and Thr633 in SUR2B were mutated to alanine. \*,  $P < 0.05$

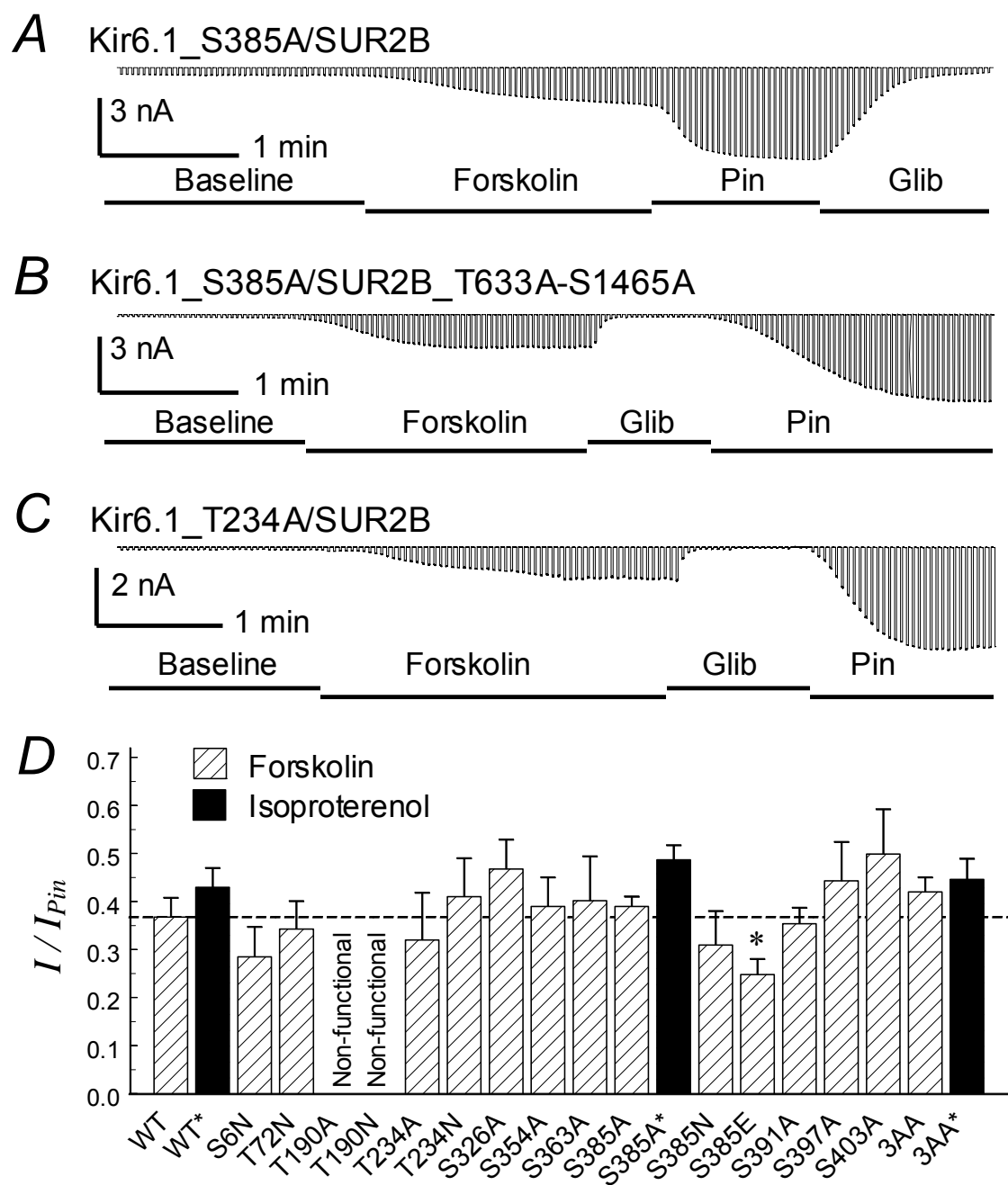


Figure 10-5

Subsequently, we performed systematic mutational analysis of the SUR2B subunit. Ten PKA consensus sequences were found in the NBD<sub>1</sub> and NBD<sub>2</sub>. Each site was mutated to alanine. All of these mutants expressed functional currents similar to the WT channel, i.e., small baseline currents stimulated by pinacidil and inhibited by glibenclamide (Table 10-1). Two residues (Ser1351, Ser1387) were found to be critical. Mutation of either residue to alanine caused severe disruption of the channel activation by isoproterenol and forskolin that was independent of the pinacidil effect (Fig. 10-6A, B, E and Table1). The effect of cAMP dialysis on Kir6.1/SUR2B\_S1387A was also tested (Fig. 10-6D, E). With the mutation, cAMP failed to activate the channel. Ser1351 is located in the NBD<sub>2</sub> immediately following the Walker A sequence, and 36 residues away lies the Ser1387. Two serine residues are found in the NBD<sub>1</sub> at corresponding locations, i.e., Ser710 and Ser748 (Fig. 10-6F). Mutation of either residue did not affect the channel activation by forskolin (Fig. 10-6C, E). Mutation of the remaining potential PKA sites had no effect on the channel sensitivity to forskolin either (Fig. 10-6E).

To address the question whether these two serine residues in SUR2B can be phosphorylated by PKA, we performed *in vitro* phosphorylation experiments on SUR2B peptides (containing residues 1308-1399 of SUR2B) with and without the S1351A and/or S1387A mutation. The peptides were fused to MBP, and expressed in bacteria. After purification with the amylose affinity column, these peptides were subjected to *in vitro* phosphorylation in the presence of the catalytic subunit of PKA and [<sup>32</sup>P-γ]-ATP. Strong phosphorylation was seen in the WT peptide and the peptide containing the S1351A

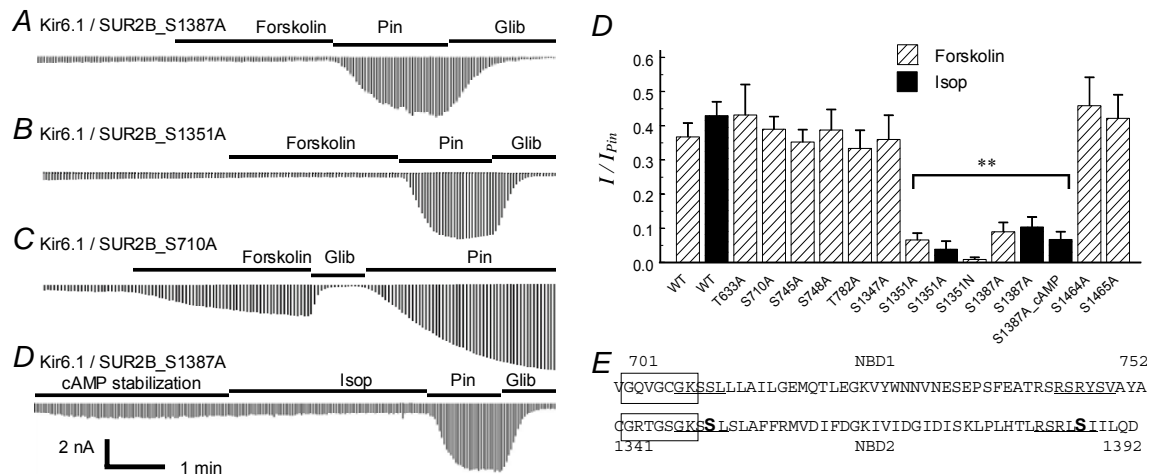
mutation. The peptides with the S1387A mutation either alone or jointly with the S1351A mutation were failed to be phosphorylated (Fig. 10-7A).

**Table 10-1. The effect of forskolin on Kir6.1/SUR2B and SUR2B mutants.**

	Baseline	Forskolin	Glib	Pinacidil	n
WT	0.64±0.34	1.89±0.60	0.39±0.11	4.08±0.98	7
T633A	0.69±0.27	3.58±1.59	0.34±0.11	6.63±2.71	5
S710A	0.76±0.12	3.65±0.82	0.22±0.05	9.04±1.96	5
S745A	0.64±0.09	1.59±0.30	0.33±0.07	3.92±0.71	9
S748A	0.57±0.10	2.41±0.54	0.24±0.09	5.76±0.66	5
T782A	0.78±0.26	1.79±0.70	0.34±0.05	4.50±1.67	5
S1347A	0.71±0.30	1.96±0.60	0.20±0.08	4.56±0.81	6
S1351A	0.37±0.15	0.48±0.15**	0.28±0.14	3.58±0.67	5
S1351N	0.29±0.12	0.25±0.11**	0.23±0.11	3.44±0.69	4
S1387A	0.26±0.07	0.48±0.16**	0.16±0.06	4.99±1.60	6
S1464A	0.88±0.46	5.88±1.91	0.50±0.24	11.26±2.49	5
S1465A	0.94±0.15	1.79±0.46	0.68±0.19	2.81±0.87	5

All of the mutants showed small baseline currents (nA) similar to the wilt-type (WT) Kir6.1/SUR2B channel. The currents were activated by pinacidil and inhibited by glibenclamide to a similar degree. The currents of the Ser1351 and Ser1387 mutants appeared to be smaller at the baseline, but they were not significantly different from the WT. After exposure to forskolin, the Ser1351 and Ser1387 mutants had smaller currents than the WT and other mutants (\*\* P<0.01). The normalized currents with forskolin exposure were shown in Fig. 10-6E.





**Figure 10-6. Identification of PKA phosphorylation sites in SUR2B.**

Mutants were co-expressed with WT Kir6.1 in HEK cells. **A,B.** Site-specific mutation of Ser1387 and Ser1351 in SUR2B abolishes the channel activation by 10 $\mu$ M forskolin. **C.** Similar mutation at Ser710 had no effect on forskolin sensitivity. **D.** Intracellular dialysis of cAMP (100 $\mu$ M in pipette solution) showed only modest stimulation of the S1387A currents, in sharp contrast to the WT channel shown in Fig. 10-3F. **E.** In comparison to WT, mutations of Ser1351 and Ser1387 caused a loss of channel activation by forskolin. The Ser1351 and Ser1387 mutants failed to be activated by isoproterenol either (\*\*,  $P < 0.01$ ,  $n = 4$  to 6). **F.** Alignment of amino acid sequences around Walker A in NBD<sub>1</sub> (upper) and NBD<sub>2</sub> (lower). Boxed are Walker A sequences. Ser1351 and Ser1387 are bold and the proposed PKA consensus sequence underlined. Similar sequences are seen in NBD<sub>1</sub>, while both Ser710 and Ser748 are not functional PKA sites.

**Figure 10-7. Characterization of Ser1387 in PKA phosphorylation.**

**A.** A short peptide in SUR2B (residues 1308 - 1399) was fused to C-terminus of MBP. Site-specific mutations of S1351A and/or S1387A were created on the peptide. After purification with amylose-affinitive beads, correct peptides were revealed in SDS-PAGE gel (left). A 50kDolton band is seen in Lanes 1 through 4 containing WT, S1351A, S1387A, and S1351A/S1387A double mutations, respectively. Lane 5, MBP protein. Another band of ~100kDolton is also seen suggesting dimerization of the fusion peptides. Autoradiograph with [<sup>32</sup>P-γ]-ATP after an 8-hour exposure (right) showed positive labeling of the WT and S1351A but not the S1387A, and S1351A/ S1387A peptides and the control MBP. **B, C.** In comparison to the WT channel, the S1387A mutant was slightly activated by the catalytic subunit of PKA (n=5). Note that the S1387A currents with cPKA exposure were smaller than the baseline level the WT channel.

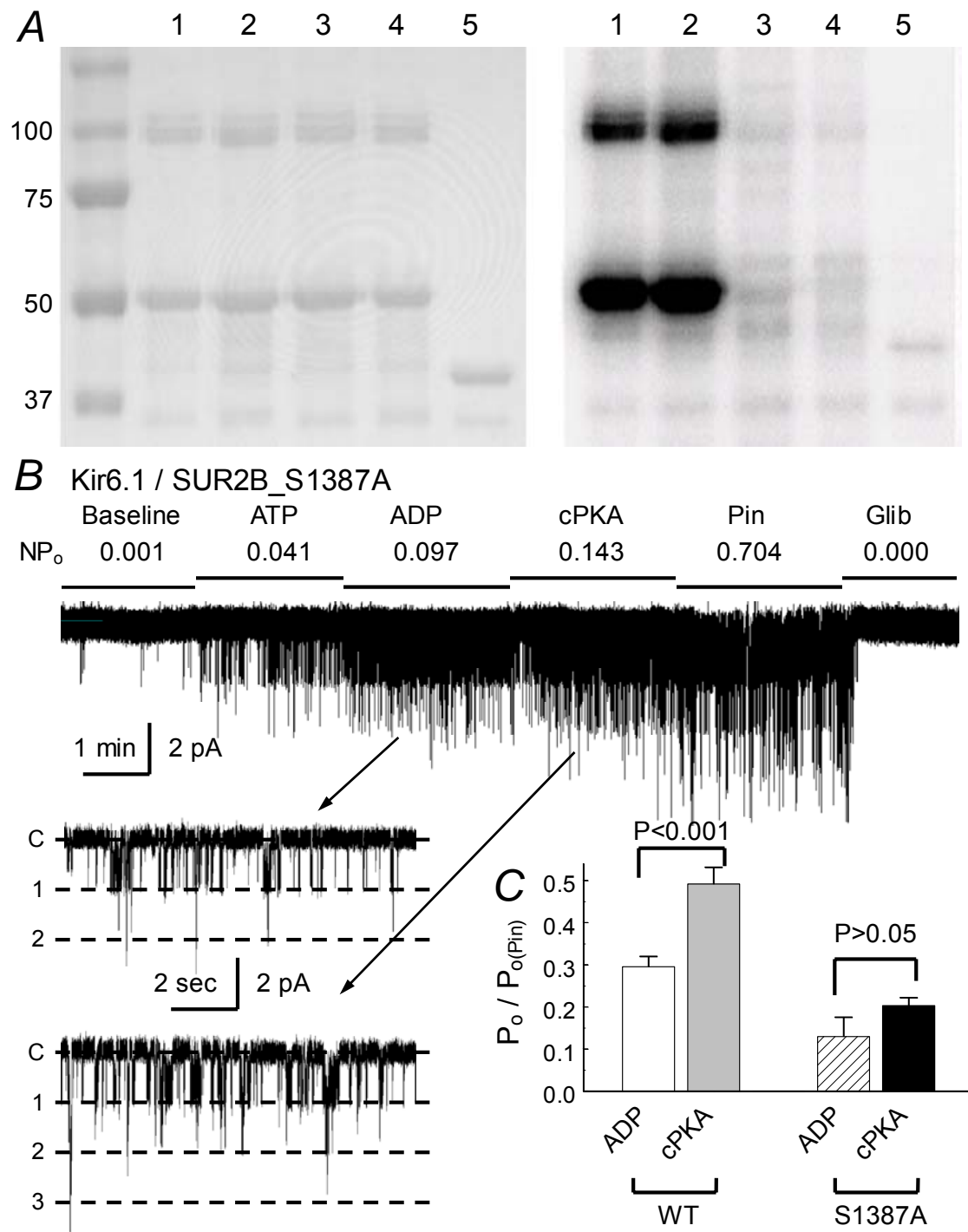
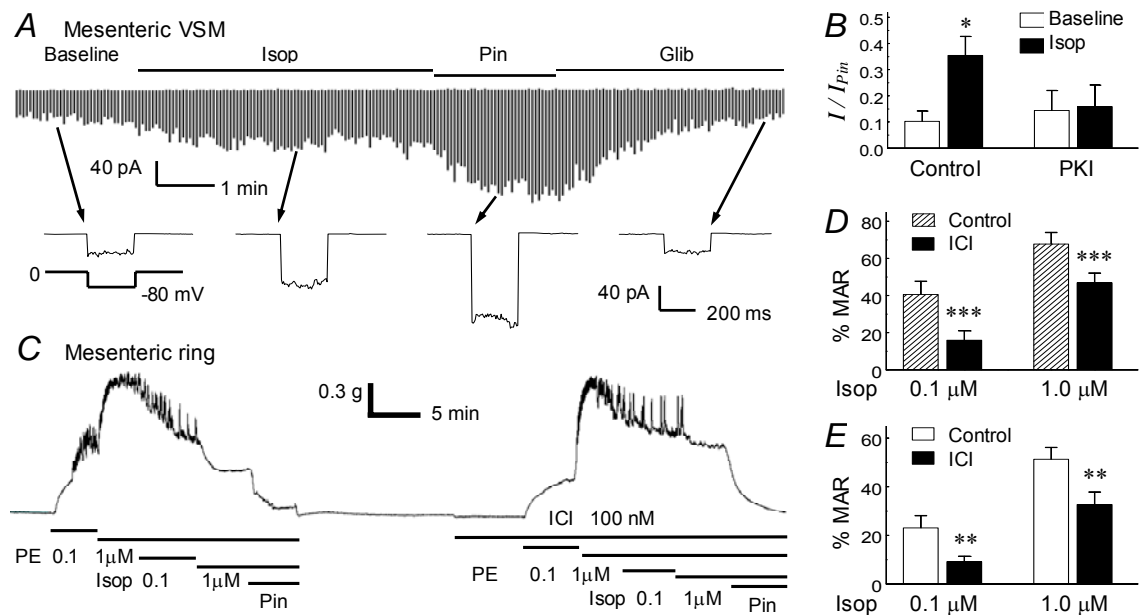


Figure 10-7

In excised patches, the S1387A channel had a rather low baseline activity. Channel activity increased with an exposure to 0.5mM ADP and 1.0mM ATP. Under this condition, the channel activity was slightly stimulated by the catalytic subunit of PKA (100U/ml) (Fig. 10-7B, C). Although the S1387A mutation did not completely eliminate the channel activation, the channel activity remained lower than the basal activity of the WT channel (Fig. 10-7C). These results are consistent with our observations in whole-cell recordings, indicating that the Ser1387 is likely to be a PKA site (Table 1 and Fig. 10-6A).

#### **10.3.5. Activation of vascular $K_{ATP}$ channels by isoproterenol**

In acutely dissociated VSMs obtained from rat mesenteric arteries, inward  $K^+$  currents were activated with an exposure to 100 nM isoproterenol (Fig. 10-8A). The isoproterenol-activated currents averaged  $35.4 \pm 7.3\%$  ( $n=6$ ) of the total currents activated by 10 $\mu$ M pinacidil, and showed a nearly identical pattern to the Kir6.1/SUR2B currents expressed in HEK cells (Fig. 10-8A, B). The same concentration of isoproterenol failed to produce significant current activation when the pipette solution contained PKI5-24 (Fig. 10-8B). These results consistent with those obtained from the Kir6.1/SUR2B channel suggest that the VMS-endogenous  $K_{ATP}$  channels are activated by PKA phosphorylation.



**Figure 10-8. Effects of isoproterenol on vascular smooth muscles.**

**A.** Whole-cell currents were recorded from a smooth myocyte acutely dissociated from the mesenteric artery. Exposure to 100nM isoproterenol activated the currents that were further activated by 10μM pinacidil. The lower panel shows current traces recorded with a single voltage protocol. **B.** The isoproterenol-activated currents were not seen in the presence of PKI5-24 (PKI, 10μM) in the pipette solution. **C.** In a mesenteric ring, phenylephrine (PE) produced vasoconstriction that was reversed by isoproterenol. Such vasorelaxation effects were markedly attenuated in the presence of a  $\beta_2$ -AR antagonist ICI-118551 (ICI). Note that pinacidil can completely relax the mesenteric ring. **D.** Summary of vasorelaxation effects of isoproterenol on the PE-induced vasoconstriction with and without ICI on endothelium-intact rings (n=7). **E.** Similar vasorelaxation was observed in endothelium-denuded rings (n=6). \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.

In endothelium-intact mesenteric rings, phenylephrine produced vasoconstriction. Such vasoconstriction could be dose-dependently reversed by isoproterenol, and was completely eliminated by pinacidil (Fig. 10-8C). The vasorelaxation effect of isoproterenol was significantly attenuated with a pretreatment with the  $\beta_2$ -AR antagonist ICI-118551 (100nM) (Fig. 10-8C, D). Similar phenomenon was found in endothelium-denuded mesenteric rings (Fig. 10-8E). These results suggest that the isoproterenol-induced vasodilation involves  $\beta_2$ -AR and requires the activation of VSM-endogenous  $K_{ATP}$  channels, consistent with our observations in the heterologous expression system and acutely dissociated vascular smooth myocytes.

## **10.4. Discussion**

### **10.4.1. PKA mediates Kir6.1/SUR2B channel activation by isoproterenol.**

Our results in HEK cells have shown that the Kir6.1/SUR2B channel is a downstream target of  $\beta_2$ -ARs. The channel regulation results from activation of the  $G_s$  – adenylyl cyclase – cAMP – PKA pathway. Two serine residues in the NBD<sub>2</sub> are critical for the channel activation by isoproterenol and forskolin. One of the residues indeed can be phosphorylated by PKA *in vitro*. PKA stimulation could explain activation of VSM-endogenous  $K_{ATP}$  channels and relaxation of mesenteric arteries by isoproterenol.

The Kir6.1/SUR2B channel activation by isoproterenol is mediated by the  $\beta_2$ -ARs– $G_s$ –adenylyl cyclase – cAMP – PKA pathway in HEK cells, and this transduction cascade may also exist in VSMs.  $\beta_2$ -AR regulation of  $K_{ATP}$  channels in VSMs is consistent with existing experimental evidence showing that the  $\beta_2$ -ARs are expressed in

vascular smooth muscles,  $\beta_2$ -AR antagonism affects vascular tone, and  $\beta_2$ -AR gene targeting causes disruption of vascular regulation and hypertension (Chruscinski *et al.*, 1999; Goto *et al.*, 2001; Guimaraes & Moura, 2001; Dishy *et al.*, 2004; Ge *et al.*, 2005; Johnson, 2006). In agreement with previous findings for cell-endogenous  $K_{ATP}$  channels (Chang, 1997; Wellman *et al.*, 1998; Ye *et al.*, 2005), Kir6.1/SUR2B channel activation by isoproterenol in HEK cells relies on PKA activity, as the PKA inhibitors RP-cAMP and PKI5-24 block channel activation. Activation of adenylyl cyclase is also necessary, since intracellular dialysis of cAMP and activation of adenylyl cyclase by forskolin augments the Kir6.1/SUR2B currents to the same extent as isoproterenol. It was previously shown that in HEK cells, the  $\beta_2$ -ARs can be phosphorylated by PKA leading to a switch to  $G_i$  cascade (Daaka *et al.*, 1997). Our results suggest that  $G_i$  is not a key player in Kir6.1/SUR2B activation by isoproterenol, as the channel activation remains following  $G_i$  inhibition by a pretreatment of HEK cells with pertussis toxin. Beside the PKA system, the exchange proteins directly activated by cAMP (Epacs) have been reported to mediate the inhibitory effects of cAMP on the pancreatic  $K_{ATP}$  isoform (Light *et al.*, 2002; Kang *et al.*, 2006). Glucagon-like peptide-1 raises cAMP concentrations that initiate binding of Epacs to SUR1, which inhibits the Kir6.2/SUR1 channel (Light *et al.*, 2002). Such a PKA-independent effect of cAMP does not seem to play a significant role in the activation of Kir6.1/SUR2B, as the channel activation is abolished by PKA inhibitors as well as mutation of the PKA site in SUR2B.

#### **10.4.2. Why can't we repeat the others' sites?**

A previous study has shown that the Kir6.1/SUR2B channel is modulated by PKA, and the channel activation was due to direct phosphorylation of the channel proteins at three sites (one in Kir6.1 and two in SUR2B) (Quinn *et al.*, 2004). We have examined these residues in the present study. However, our results suggest that in our system these three residues do not seem to play a role in the Kir6.1/SUR2B channel activation by PKA in the presence of physiological levels of nucleotides, as mutations of these residues did not show significant effect on the channel activation by isoproterenol and forskolin. The different observations are probably due to experimental conditions. The Kir6.1/SUR2B currents were recorded in the presence of 0.5mM UDP in the study by Quinn *et al.* (Quinn *et al.*, 2004) in comparison to 0.5mM ADP in the present study. Because the Kir6.1/SUR2B channel is strongly activated by UDP, forskolin only increased the whole-cell current amplitude by ~50% in their study (Quinn *et al.*, 2004), whereas the currents is augmented by ~500% by isoproterenol or forskolin in the present study. It is noted that cell lines from different sources may have different intracellular signaling cascades. Such a difference in HEK cells has been reported previously (Clark & Baro, 2007), contributing to different cellular responses to a give stimulus.

#### **10.4.3. What is the explanation for Ser1351 mutations?**

In the present study, we have systematically mutated all 11 consensus PKA sites in the Kir6.1 subunit. Ten of them do not seem to be functionally phosphorylated by PKA, as mutations to non-phosphorylatable residues do not affect the channel activation



by forskolin. The role of the other residue Thr190 remains uncertain as channels with a mutation at this position were non-functional (Thorneloe *et al.*, 2002) (Fig. 10-5D).

In the SUR2B subunit, our systematic mutational analysis revealed two serine residues, i.e., Ser1351 and Ser1387 that are both located in the NBD<sub>2</sub>. Mutation of either one abrogates Kir6.1/SUR2B channel activation by isoproterenol and forskolin. *In vitro* phosphorylation of a purified fusion peptide of SUR2B suggests that Ser1351 is not phosphorylated by PKA. A straightforward explanation of the results is that the Ser1351 may be involved in ADP binding on the Walker-A motif. Its mutation thus affects ADP binding. It is also possible that the isolated peptide may have lost its normal folding and failed to be phosphorylated *in vitro*. Interestingly, a corresponding serine is also found in SUR1 (Ser1387), its mutation (SUR1\_S1387F) and deletion (SUR1\_ΔS1387) have been found in patients with congenital hyperinsulinism (Aguilar-Bryan & Bryan, 1999; Thornton *et al.*, 2003; Stanley *et al.*, 2004). In the current study, a replacement of this serine residue (SUR2B\_S1351) with either non-polar alanine or polar asparagine causes disruption of the channel activation by PKA stimulation. Therefore, Ser1351 is an important site for channel regulation although it does not seem to be phosphorylated by PKA.

Why is it that mutation of the corresponding residue in NBD<sub>1</sub> (Ser710) does not affect channel activation by PKA? This may be related to the difference in the function of NBD<sub>1</sub> and NBD<sub>2</sub>. The NBD<sub>1</sub> in SURx hosts a Mg<sup>2+</sup>-independent high-affinity nucleotide-binding domain while the nucleotide-binding domain in NBD<sub>2</sub> is Mg<sup>2+</sup>-dependent and

low-affinity (Matsuo *et al.*, 2005). Thus mutation of the serine residue in NBD<sub>1</sub> may have little effect on nucleotide binding and channel activity.

#### **10.4.4. The Ser1387 is an important PKA site: its functional implication in SUR1 and SUR2A channels.**

Ser1387 is a phosphorylation site critical for the channel regulation by PKA, as shown in our pharmacological studies, mutagenesis analysis, *in vitro* phosphorylation assay and direct exposure to cPKA. This is a novel finding in comparison to previous studies in PKA regulation on K<sub>ATP</sub> channels. Lin *et al.* (Lin *et al.*, 2000) and Beguin *et al.* (Beguin *et al.*, 1999) have found that PKA activates Kir6.2/SUR1 through phosphorylation of the Kir6.2 subunit (Ser224 and Ser372, respectively). The SUR1 subunit has also been proposed as a target of PKA. Beguin *et al.* (Beguin *et al.*, 1999) reported that a human specific residue on SUR1 (Ser1571) has basal level phosphorylation. Light *et al.* (Light *et al.*, 2002) reported that the Kir6.2/SUR1 channel is inhibited by the glucagon-like peptide through PKA phosphorylation at Ser1448 of the SUR1 subunit. Both PKA sites on SUR1 subunit are located in the NBD<sub>2</sub> in which ADP binding takes place (Matsuo *et al.*, 2005). Thus, it is possible that PKA phosphorylation affects ADP sensitivity and thus the channel activity (Light *et al.*, 2002). These two sites are SUR1-specific, as the corresponding sites are not phosphorylatable residues or not in a consensus PKA sequence in SUR2B. In contrast, our newly identified PKA phosphorylation site Ser1387 in SUR2B is conserved among species and in all three SURx (Fig. 10-9A). It is of interest to know whether such a site plays a role in other isoforms of K<sub>ATP</sub> channels.

**Figure 10-9. The sites important for PKA regulation on K<sub>ATP</sub> channels.**

**A.** Alignment of NBD<sub>2</sub> of mouse SUR2A, SUR2B (residue 1339-end) and human SUR1 (residue 1376-end). The Nucleotide binding Walker A (W<sub>A</sub>) and Walker B (W<sub>B</sub>) are underlined. The S1351 that is important for PKA activation, the PKA phosphorylation site (S1387) in SUR2B and the conserved sites in SUR2A and SUR1 are highlighted in reverse form. The previously suggested PKA site (S1465) in SUR2B and sites (S1448 and S1571) in SUR1 are also highlighted. **B.** Schematic representation of Kir6.1 and SUR2B. The nucleotide binding domain 1 (NBD<sub>1</sub>) and 2 (NBD<sub>2</sub>) and Walker A (W<sub>A</sub>) and Walker B (W<sub>B</sub>) and N, C terminus are illustrated. The relative positions of important sites (Kir6.1\_Thr234, Ser385 and SUR2B\_Thr633, Ser1351, Ser1387, Ser1465) are marked. Note the Kir6.1\_Thr234 and Ser385 are also the corresponding sites of Kir6.2\_Thr224 and Ser372.

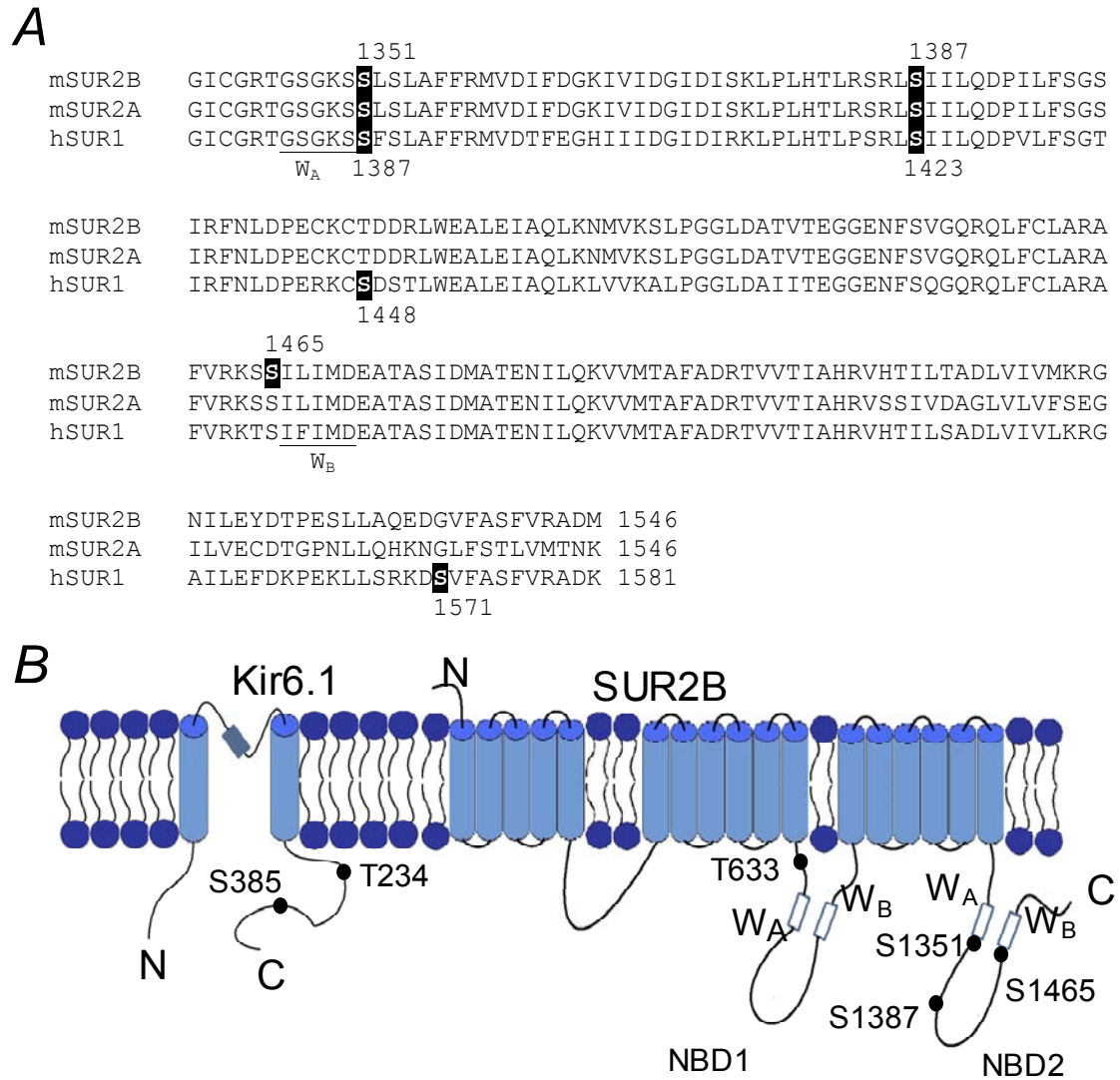


Figure 10-9

It should be noted that S1387 cannot explain the full effects of PKA, as cPKA can moderately activate the Kir6.1/SUR2B\_S1387A channel in the inside-out configuration (Fig. 6B). This may be due to the presence of other unconventional PKA sites that were not detected by our PKA consensus sequence screening. Besides, the possibility of phosphorylation on Ser1351 cannot be excluded as the isolated peptide may differ in protein folding, losing the capability to be phosphorylated. In addition, there may be other adaptor proteins and scaffolds absent in the *in vitro* phosphorylation assay, such as A-kinase anchoring proteins (Hayabuchi *et al.*, 2001a) and caveolae (Sampson *et al.*, 2004) that are important for PKA modulation of native vascular K<sub>ATP</sub> channels.

In conclusion, our results indicate that the Kir6.1/SUR2B channel can be a downstream effector of  $\beta_2$  receptors. In this case, channel activation involves G<sub>s</sub>, adenylyl cyclase, cAMP and PKA. Two serine residues (Ser1351 and Ser1387) in the SUR2B subunit are important for PKA activation and the channel protein is likely phosphorylated at Ser1387. The demonstration of an effector protein of  $\beta_2$ -ARs and intracellular signaling cascades may allow for the creation of therapeutical modalities. By targeting these molecules and their regulators, it may be possible to control vascular tone more effectively.

## **11. Result 2: PKA Phosphorylation Produces Interdomain Movement in SUR2B Suggesting a Gating mechanism for Activation of the Vascular $K_{ATP}$ Channel**

Manuscript submitted: Shi Y, Chen X, Wu Z, Shi W, Jiang C, Harrison R. (2007) PKA Phosphorylation produces interdomain movements in SUR2B leading to activation of vascular  $K_{ATP}$  channel.

Note: Yun Shi did ~80% of molecular construction and patch clamp experiments. Mr. Xianfeng Chen did molecular modeling. Ms. Zhongying Wu and Mr. Weiwei Shi helped in patch experiments. Generally, Shi Y and Chen X contribute to ~40% of the work. Therefore, Yun Shi and Xianfeng Chen are considered equal contributors to this work.

### 11.1. Abstract

Vascular  $K_{ATP}$  channels are activated by multiple vasodilating hormones and neurotransmitters via a cAMP-PKA signaling pathway. A critical PKA phosphorylation site (Ser1387) in NBD<sub>2</sub> of SUR2B subunit was revealed in our recent study. To understand how phosphorylation at Ser1387 leads to changes in channel activity, we used a newly crystallized ABC protein, SAV1866, to model the SUR2B core containing both nucleotide-binding domains (NBD<sub>1</sub>, NBD<sub>2</sub>) as well as two transmembrane domains (TMD<sub>1</sub>, TMD<sub>2</sub>). On the model, Ser1387 was located on the interface between TMD<sub>1</sub> and NBD<sub>2</sub> and physically interacted with an aromatic residue (Tyr506) in TMD<sub>1</sub>. A positively charged residue (Arg1462) in NBD<sub>2</sub> was also in the vicinity of Ser1387. Mutations of Arg1462 or Tyr506 abolished the PKA-dependent channel activation. Molecular dynamics simulations suggested Ser1387, Tyr506 and Arg1462 formed a compact triad upon phosphorylation at Ser1387. The simulations also suggested that the phosphorylation reshaped the NBD<sub>2</sub> interface and caused movement of TMD<sub>1</sub> (represented by a short intracellular linker segment, ICL<sub>2</sub>), strengthening the interaction between these two protein domains. Restriction of the hypothetical interdomain movement by introducing a disulfide bond between them prevented channel activation. The combination of modeling, molecular dynamics, and experimental protein engineering suggests a mechanism for the transfer of information of protein phosphorylation to channel activation.

## 11.2. Introduction

K<sub>ATP</sub> channels play a crucial role in vascular tone regulation (Quayle *et al.*, 1997; Rosenblum, 2003; Seino & Miki, 2003; Jackson, 2005). The channels are expressed in VSMs and activated by several vasodilating hormones most likely through a G<sub>s</sub>-cAMP-PKA signaling system (Quayle *et al.*, 1997; Shi *et al.*, 2007b; Quinn *et al.*, 2004). Critical phosphorylation sites have been found in the SUR2B subunit (Shi *et al.*, 2007b). Channel activation leads to hyperpolarization of VSM cells, a decrease in voltage-dependent Ca<sup>2+</sup> channel activity and relaxation of resistant arteries (Nelson *et al.*, 1995).

K<sub>ATP</sub> channels consist of 4 pore-forming Kir6 and 4 regulatory SUR subunits (Nichols, 2006). Kir6.1/SUR2B is the major isoform of K<sub>ATP</sub> channels in VSMs (Yamada *et al.*, 1997; Cui *et al.*, 2002; Cole & Clement-Chomienne, 2003; Li *et al.*, 2003). The channel does not open spontaneously at rest. Several groups of channel openers activate the channel, including pharmacological K<sub>ATP</sub> channel openers (KCOs, pinacidil, nicorandil) (Yamada & Kurachi, 2004; Moreau *et al.*, 2005), metabolites (MgADP, acidosis) (Matsushita *et al.*, 2002; Wang *et al.*, 2003), and hormonal vasodilators (calcitonin gene-related peptide, epoxyeicosatrienoic acids, beta-adrenergic receptor agonists and vasoactive intestinal peptide) (Wellman *et al.*, 1998; Lu *et al.*, 2001; Shi *et al.*, 2007b; Yang *et al.*, 2007). KCOs and Mg<sup>2+</sup>-nucleotides activate the Kir6.1/SUR2B channel via binding to the SUR subunit (Yamada & Kurachi, 2004; Moreau *et al.*, 2005; Nichols, 2006). Hormonal vasodilators activate the vascular K<sub>ATP</sub> channel through direct phosphorylation of the channel protein by PKA (Quinn *et al.*, 2004; Shi *et al.*, 2007b).



Our recent study has shown that Ser1387 in SUR2B is a key phosphorylation site (Shi *et al.*, 2007b).

It is unclear how phosphorylation at Ser1387 in SUR2B leads to channel activation. SURx belong to the family of ABC transporter proteins (Higgins, 1992; Moreau *et al.*, 2005). All ABC proteins have similar domain assemblies, i.e. two transmembrane domains (TMD<sub>1</sub> and TMD<sub>2</sub>) and two intracellular nucleotide-binding domains (NBD<sub>1</sub> and NBD<sub>2</sub>). In SURx, an additional transmembrane domain containing 5 helical segments termed TMD<sub>0</sub> is linked to TMD<sub>1</sub> with intracellular linker-0 (ICL<sub>0</sub>, Fig. 11-1A). The NBDs of several ABC proteins have been crystallized (Gaudet & Wiley, 2001; Smith *et al.*, 2002; Verdon *et al.*, 2003). They all show similar sandwich-like structures with nucleotides clamped between the signature sequence of one NBD and the Walker-A motif of the other NBD. The Walker-A (Gribble *et al.*, 1997; Yamada & Kurachi, 2004), Walker-B (Shyng *et al.*, 1997; Huopio *et al.*, 2000; Yamada *et al.*, 2004), and signature sequences (Matsuo *et al.*, 2002) are known to be important in nucleotide and/or KCO activation of K<sub>ATP</sub> channels, although how nucleotide/KCO-binding couples to channel activity is still not fully understood. Several lines of evidence suggest that SUR TMDs play a major role in interacting with Kir6 subunits (Schwappach *et al.*, 2000). Such interaction has been observed in the recombinant Kir6.2/SUR1 channel using electron microscopy (Mikhailov *et al.*, 2005). In addition, the TMD<sub>0</sub> and ICL<sub>0</sub> are involved in K<sub>ATP</sub> gating by channel modulators and nucleotides (Babenko & Bryan, 2003; Chan *et al.*, 2003). These data suggest that NBDs couple with TMDs during SUR-mediated channel gating.

Several ABC proteins have been recently crystallized (Locher *et al.*, 2002; Dawson & Locher, 2006; Hollenstein *et al.*, 2007; Pinkett *et al.*, 2007). Of them, SAV1866 (Dawson & Locher, 2006) shows highest homology to SUR2B. Using the SAV1866 crystal structure, we modeled SUR2B core paying particular attention to protein conformational changes following Ser1387 phosphorylation. Our combined studies with functional assays, mutagenesis analysis and molecular dynamics simulation suggest that conformational changes in two protein domains, NBD<sub>2</sub> and intracellular linker-2 (ICL<sub>2</sub>), underscore PKA-dependent channel activation.

**Figure 11-1. Alignment of SUR2B and SAV1866.**

TM-NBD<sub>1</sub> and TM-NBD<sub>2</sub> refer to residues 286-914 and 976-1546 of SUR2B. Residues in SUR2B identical and similar to SAV1866 were shaded in cyan and green respectively. Six transmembrane helices (TMH1-6) in SAV1866 are shown in bold and underlined, which correspond to TMH6-11 and TMH12-17 in SUR2B\_TMD<sub>1</sub> and TMD<sub>2</sub> respectively. Sequences between SAV1866 TMH2 and 3, and between SAV1866 TMH4 and 5 are intracellular linkers. Short helices that are critical for TMD-NBD interactions are shown in red. ICL<sub>1</sub>, ICL<sub>2</sub> are located in SAV1866 and SUR2B\_TMD<sub>1</sub> while ICL<sub>3</sub>, ICL<sub>4</sub> in SUR2B\_TMD<sub>2</sub>. Although the homology in TMDs between SAV1866 and SUR2B is low, the lengths of TMHs are the same. The lengths of intracellular and extracellular linkers are comparable. Therefore, when they are aligned, there are only 4 short gaps in TMD<sub>1</sub>, and 2 gaps in TMD<sub>2</sub>. The similar topology allowed us to model SUR2B TMDs with SAV1866 template. The homology in NBDs is higher. Critical motives such as Walker A (WA), Walker B (WB), Signature Sequence (SS), Q-loop and H-loop in NBDs are conserved (in red). The secondary structures in SAV1866\_NBD were shown in bold and underlined. The loop between  $\alpha$ 3 and  $\alpha$ 4 helices was named C-loop as Cys1408 was found in it. Tyr506, Ser1387 and Arg1462 in SUR2B are shown in bold and italic, and are critical for PKA activation.



### **11.3. Results**

#### **11.3.1. Alignment of SUR2B with SAV1866**

The sequences of SAV1866 were aligned with both halves of SUR2B core (TM-NBD<sub>1</sub> and TM-NBD<sub>2</sub>) using ClustalW (Thompson *et al.*, 1994). SAV1866 was 21.5% identical to TM-NBD<sub>1</sub> of SUR2B (46.5% similarity) and 21.3% to TM-NBD<sub>2</sub> (47.8% similarity). The NBDs were highly conserved, with 28.7% identity in NBD<sub>1</sub> and 33.5% in NBD<sub>2</sub>. The SAV1866 TMD was 16.0% and 12.8% identical to SUR2B\_TMD<sub>1</sub> and TMD<sub>2</sub> respectively (~35% when amino acids with similar side chain were considered). Although the homology in primary sequences in TMDs is low, we were still able to align them because their topology within each TMD was the same, i.e., 6 transmembrane helices (determined by hydrophobicity plots) in each domain with similar lengths. The sizes and position of extracellular and intracellular linker regions were also comparable. There were only a few short gaps in alignment (4 in TMD<sub>1</sub> and 2 in TMD<sub>2</sub>, Fig. 11-1). The similarity in the topology suggests the two proteins have the same structural arrangements, allowing modeling of SUR2B using SAV1866 structure.

#### **11.3.2. Modeling of the SUR2B\_core**

The dimerized SAV1866 was crystallized in ADP binding form (PDB: 2hyd). Based on this structure, we built a SUR2B core model (Fig. 11-2B), in which NBD<sub>1</sub> and NBD<sub>2</sub> formed a tight hetero-dimer (Fig. 11-2C). On the interface between NBD<sub>1</sub> and NBD<sub>2</sub> there were two nucleotide-binding pockets. A MgATP molecule was incorporated in the first nucleotide binding pocket and a MgADP in the second pocket. Different

nucleotides were used because the first pocket is likely to be a high-affinity ATP-binding site, and second to bind to MgADP (Bienengraeber *et al.*, 2000; Matsuo *et al.*, 2005). In the SUR2B\_core model, the TMDs interact with the NBDs via four short helices of the intracellular linkers (ICLs, Fig. 11-2C). ICL<sub>1</sub> and ICL<sub>3</sub> interacted with the NBD surface across the nucleotide-binding regions. ICL<sub>2</sub> from TMD<sub>1</sub> inserted deeply into NBD<sub>2</sub>, while ICL<sub>4</sub> from TMD<sub>2</sub> inserted into NBD<sub>1</sub> (Fig. 11-2C). The NBD<sub>2</sub> formed a spindle-like groove where a  $\beta$  sheet ( $\beta$ 5) formed the bottom while the Q-loop, C-loop and several  $\alpha$ -helices ( $\alpha$ 2,  $\alpha$ 3 and  $\alpha$ 6) lined the wall. Ser1387 was located on  $\beta$ 5 within the reach of ICL<sub>2</sub> (Fig. 11-2D). The side-chain of an aromatic residue (Tyr506) in ICL<sub>2</sub> was inserted deeply into the NBD<sub>2</sub> groove roughly  $\sim 4\text{\AA}$  from Ser1387 (Fig. 11-3A). Around the Ser1387 there was a positively charged residue (Arg1462) in NBD<sub>2</sub>,  $\sim 8\text{\AA}$  from Ser1387 (Fig. 11-3A).

**Figure 11-2. SUR2B core Model.**

**A.** A skeletal representation of  $K_{ATP}$  channel. SUR2B has 17 TMHs, forming three transmembrane domains (TMD<sub>0</sub>, TMD<sub>1</sub> and TMD<sub>2</sub>). The nucleotide binding domain-1 (NBD<sub>1</sub>) is located between TMD<sub>1</sub> and TMD<sub>2</sub>, and NBD<sub>2</sub> is located C-terminal to TMD<sub>2</sub>. The intracellular linkers (ICLs) between TMHs are indicated. SUR2B core is defined as TMD<sub>1</sub> to C-terminus. The region between residue 286 (arrow-1, a1) and 914 (a2) was taken as TM-NBD<sub>1</sub>. The sequence between 976 (a3) and 1546 (C-terminus) was taken as TM-NBD<sub>2</sub>. TM-NBD<sub>1</sub> and TM-NBD<sub>2</sub> were aligned to SAV1866 and used to generate the SUR2B core model. The sequence between a2 to a3 was omitted in SUR2B core model as it is not homologous to SAV1866. **B.** The overall structure of SUR2B core. TMD<sub>1</sub> (residues 286-617, green) was linked with NBD<sub>1</sub> (residues 666-913, blue) with 38 residues (loop in gray). TMD<sub>2</sub> (residues 976-1289, red) was linked with NBD<sub>2</sub> (residues 1310-1546, purple) with 20 residues. The linker regions between TMD<sub>1</sub> and NBD<sub>1</sub>, TMD<sub>2</sub> and NBD<sub>2</sub> were looped beside the NBD domains (gray). The first nucleotide binding pocket (formed by Walker A in the NBD<sub>1</sub> and signature sequence in the NBD<sub>2</sub>) was modeled with ATP (yellow). The second nucleotide binding pocket was modeled with ADP. **C.** Interaction of the intracellular linkers and NBDs. The ICL<sub>1</sub> and ICL<sub>3</sub> interacted with both NBD<sub>1</sub> and NBD<sub>2</sub> across the nucleotide-binding regions (yellow). ICL<sub>2</sub> inserted into a groove that is formed by the NBD<sub>2</sub> surface, and ICL<sub>4</sub> interacted with NBD<sub>1</sub>. **D.** Details of the interaction between ICL<sub>2</sub> and NBD<sub>2</sub>. The NBD<sub>2</sub> formed a big groove for binding of ICL<sub>2</sub>.  $\beta$ 5 of NBD<sub>2</sub> formed the bottom of the groove. Q-loop,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 6, and C-loop between  $\alpha$ 3 and  $\alpha$ 4 formed the wall of the groove. The phosphorylation site Ser1387 was located in the  $\beta$ 5 sheet and is shown in cyan. Critical residues like Tyr506 and Arg1462 are also shown.

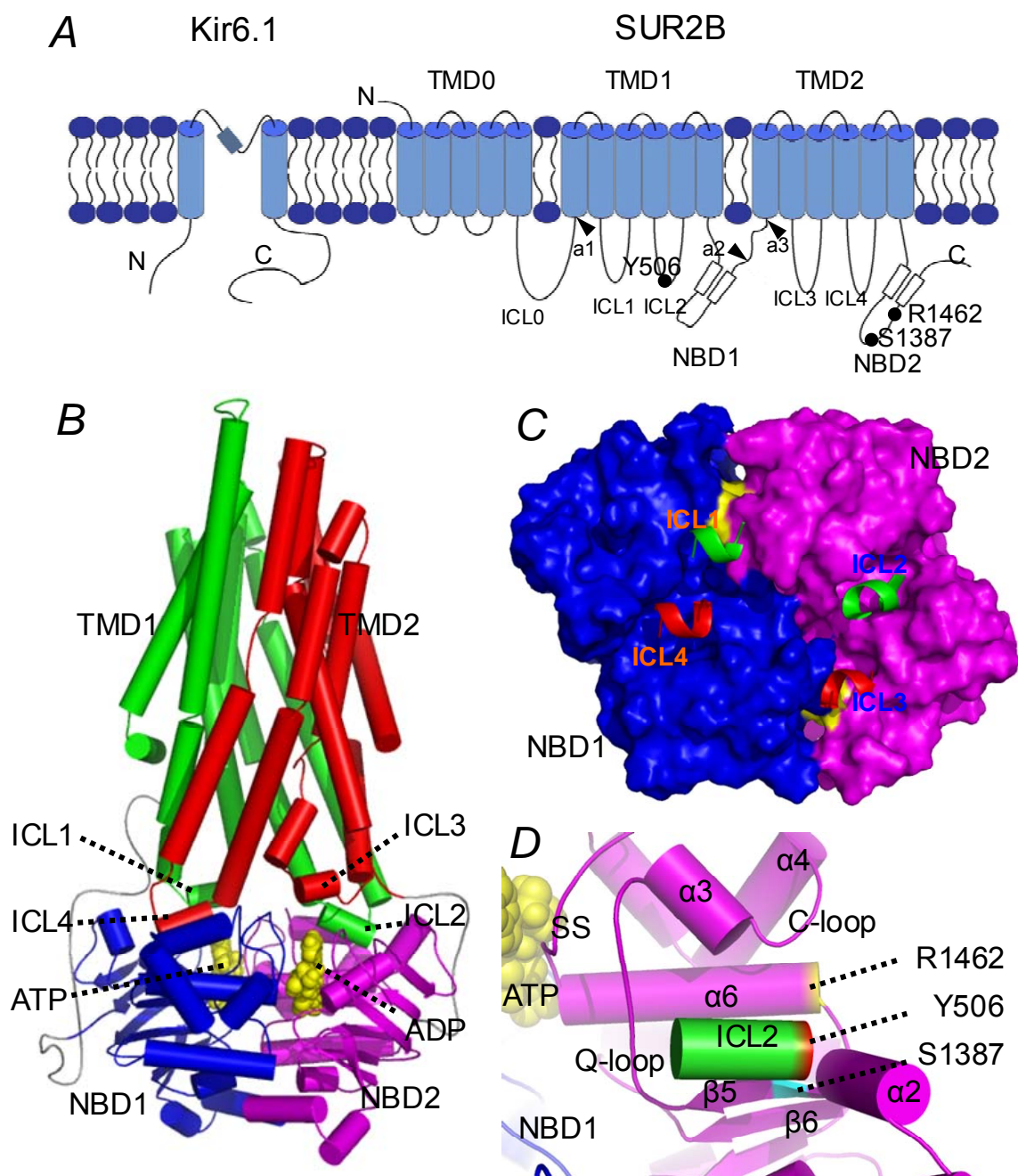
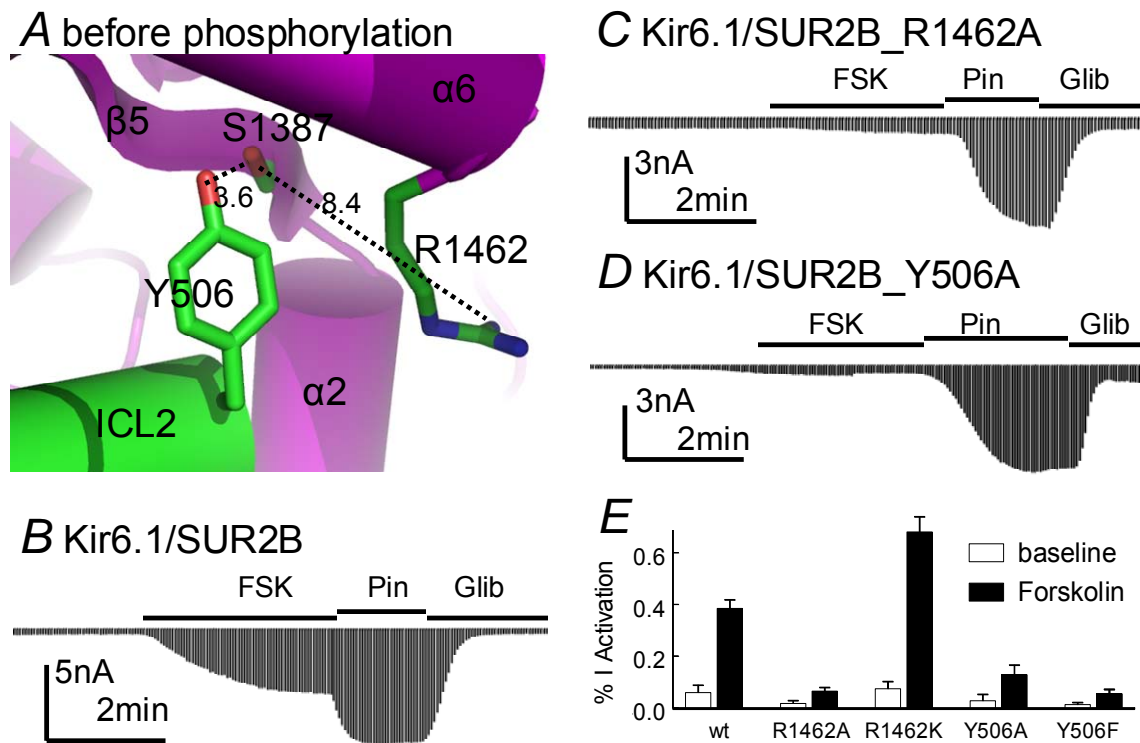


Figure 11-2



**Figure 11-3. Forskolin effects on wt and mutant Kir6.1/SUR2B.**

**A.** Ser1387 was close to Tyr506 and Arg1462. The atom  $O_g$  in Ser1387 side-chain was 3.6Å to atom  $O_h$  of Tyr506 and ~8Å to the positive charge of ArgR1462 (the middle of the two  $N_h$  atoms). **B.** Forskolin activated whole cell currents of Kir6.1/SUR2B, which were further activated by pinacidil and inhibited by glibenclamide. **C.** The channel with SUR2B\_R1462A mutant failed to be activated by forskolin. **D.** SUR2B\_Y506A mutant diminished forskolin activation of the channel. **E.** Summary of forskolin effects on wt and mutant Kir6.1/SUR2B channels. Note the Y506F mutant was not activated and the R1462K mutant was activated by forskolin.



### 11.3.3. Activation of the Kir6.1/SUR2B channel by forskolin

$\beta$ -Adrenergic receptor agonists activate vascular  $K_{ATP}$  channels in rat mesenteric artery, a response that can be reproduced in recombinant Kir6.1/SUR2B channel expressed in HEK293 cells. Forskolin (10 $\mu$ M) activates Kir6.1/SUR2B currents to 38.5 $\pm$ 3.0% of maximal activation by pinacidil in HEK cells (n=10, Fig. 11-3E). We therefore chose to study forskolin activation of recombinant Kir6.1/SUR2B expressed in HEK cells. We focused on the interaction of ICL<sub>2</sub> and NBD<sub>2</sub> as this is where PKA exerts its effects.

### 11.3.4. Elimination of PKA activation by mutation at Arg1462 or Tyr506

In our SUR2B\_core model, a positively charged residue Arg1462 was located in the vicinity of Ser1387. The distance of the charge of Arg1462 (measured at the center of two N<sub>h</sub> atoms) to Ser1387 side chain (O<sub>g</sub>) was  $\sim 8\text{\AA}$  (Fig. 11-3A). This small distance might allow an electronic attraction between the two amino acids when Ser1387 was phosphorylated. Mutation of Arg1462 to alanine indeed eliminated the forskolin-induced channel activation (Fig. 11-3C). Based on our model, positive charge at this site appears important. This prediction was verified by mutating the Arg1462 to lysine, and the R1462K mutant remained strongly activated by forskolin (68.5 $\pm$ 5.8%, n=5). The Arg1462 is located in a consensus PKA site (**RKSS**), although neither Ser1464 nor Ser1465 is a functional PKA phosphorylation site (Shi *et al.*, 2007b). Thus, a positively charged residue critical for the PKA-dependent channel activation is identified in the SUR2B\_core model, although it is far from Ser1387 in the primary sequence.

An aromatic residue, Tyr506 was in ICL<sub>2</sub>, deeply inserted its side chain into NBD<sub>2</sub> and physically interacted with Ser1387. When Tyr506 was mutated to alanine, the forskolin-induced channel activation was significantly diminished (Fig. 11-3D). A phenylalanine residue is found at the corresponding site in SAV1866 and SUR2B\_ICL4 (Fig. 11-1). The side chain of phenylalanine is similar to tyrosine but lacks a hydroxyl group. Interestingly, when Tyr506 was mutated to phenylalanine, the channel failed to be activated by forskolin (Fig. 11-3E). These data indicate that channel activation involves Tyr506.

#### **11.3.5. Triad formation by Arg1462, Tyr506 and Ser1387 following PKA phosphorylation**

Our mutational analysis suggests that PKA phosphorylation at Ser1387 induces conformational changes in the local area. In order to understand these changes, we used molecular dynamics simulations to reveal changes the peptide backbone. Our SUR2B core contained a large number of hydrophobic transmembrane segments that are technically difficult to run in molecular dynamics simulations. We therefore constructed two additional models consisting of NBDs and ICL<sub>2</sub> helix with and without phosphorylation at Ser1387. MD simulations were run at 310K and 1 atm. After 1000 runs, the ICL<sub>2</sub> still stayed in the groove formed by NBD<sub>2</sub>, suggesting the interaction between ICL<sub>2</sub> and NBD<sub>2</sub> is strong. The distances between the side chains of the three critical residues were monitored (Fig. 11-4A, B). Without phosphorylation, the average distance between Tyr506 and Ser1387 was 3.8Å, a distance that allows hydrogen bond formation between these two residues. The Tyr506-Arg1462 and Ser1387-Arg1462

distances were around 6-7Å (Fig. 11-4A, C), suggesting that there is no direct contact between Arg1462 and the other two residues before Ser1387 phosphorylation. When the Ser387 was phosphorylated, all the three distances were reduced to less than 4Å (Fig. 11-4C), indicating a stable triad formed following phosphorylation (Fig. 11-4D, E). The hydroxyl group of Tyr506 appeared to be critical for stabilization of the triad, which could form hydrogen bonds with oxygen atoms of the phosphate group of p-Ser1387 and N<sub>h</sub> atoms in Arg1462, consistent with our observation that forskolin failed to activate the Y506F mutant (Fig. 11-3E).

**Figure 11-4. Formation of a compact triad among Tyr506, Ser1387 and Arg1462 following phosphorylation.**

Molecular dynamic simulations were run on the ICL<sub>2</sub> with a short flanking sequence (residues 500 to 512) and NBDs. A phosphate group was linked to atom O<sub>g</sub> in Ser1387 representing post-phosphorylation. **A.** The distances between each two of the three residues were monitored in pre-phosphorylation form. Y-S was measured between Tyr506-O<sub>h</sub> and Ser1387-O<sub>g</sub>. Y-R was measured between Tyr506-O<sub>h</sub> and Arg1462-N<sub>h1</sub> or N<sub>h2</sub> depending on which one atom was closer to Tyr-O<sub>h</sub>. And so was S-R. **B.** The distances in post-phosphorylation form. pS represented one of the three O<sub>p</sub> atoms in phosphorylated Ser1387. Six distances were measured between p-Ser1387 and Arg1462, and three between Tyr506 and p-Ser1387, while only the closest distances were shown in the figure. **C.** The summary of the distances among the three residues pre- and post-phosphorylation. Since the molecules in the initial ~200 time frames might be unstable (see A and B), only the last 800 time frames were counted. All three distances were shorter in the post-phosphorylated form ( $P < 0.001$  for every distance). Y-S shortened from  $3.80 \pm 0.02 \text{Å}$  to  $3.13 \pm 0.02 \text{Å}$ . Y-R shortened from  $6.62 \pm 0.03 \text{Å}$  to  $3.61 \pm 0.01 \text{Å}$ . S-R shortened from  $6.43 \pm 0.02 \text{Å}$  to  $2.88 \pm 0.00 \text{Å}$ . Major changes were seen in distances between Ser1387 and Arg1462, and Tyr506 and Arg1462, suggesting the Arg1462 side chain moves toward Ser1387 and Tyr506 and forms a

stable triad. **D.** The *mode* structure of the post-phosphorylation form showing the triad. **E.** Enlargement of the boxed region in D. The numbers in the figure are the distances (in Å) between the interested residues. They are slightly, but not significantly, different from the average distances shown in C.

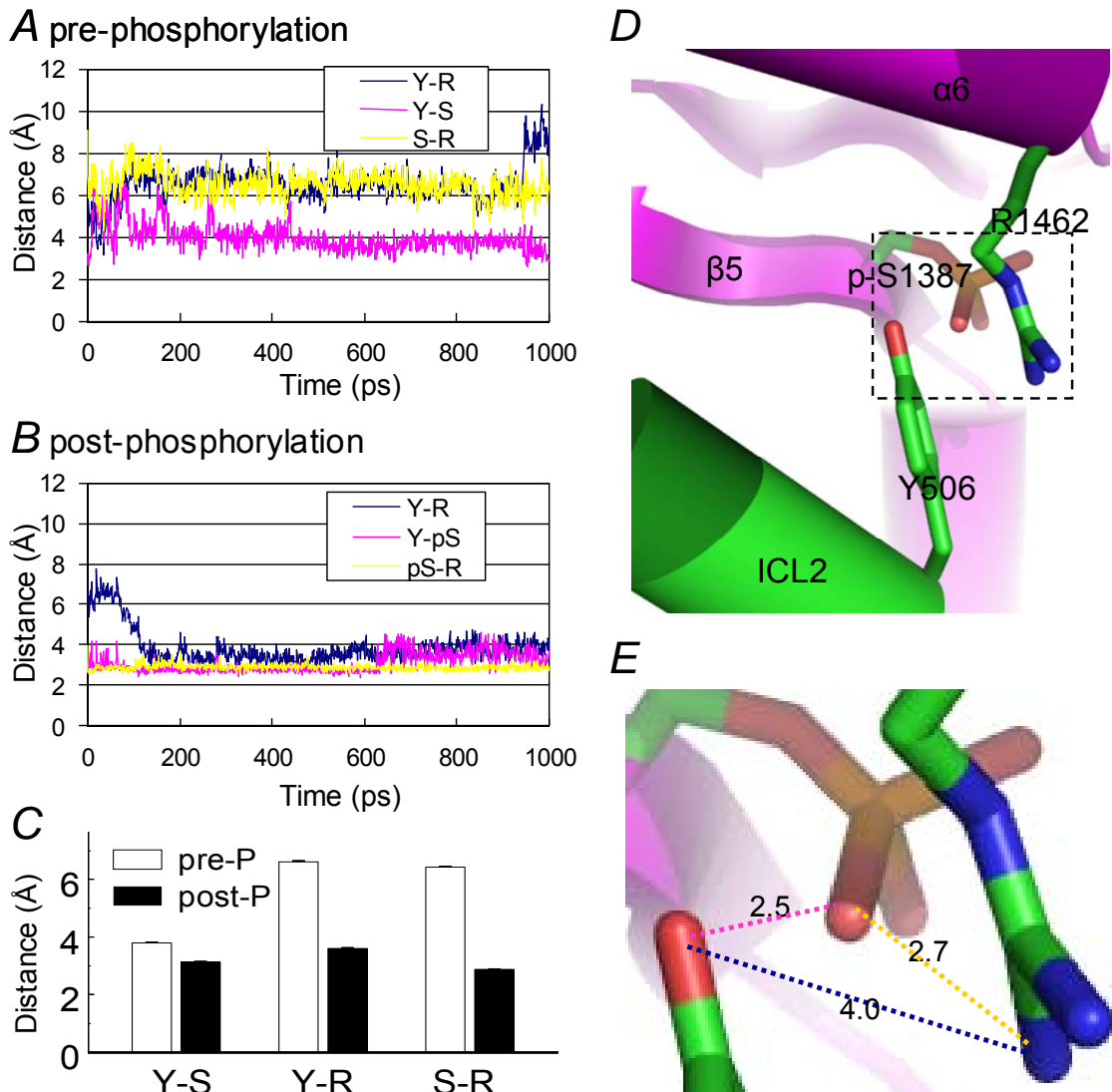
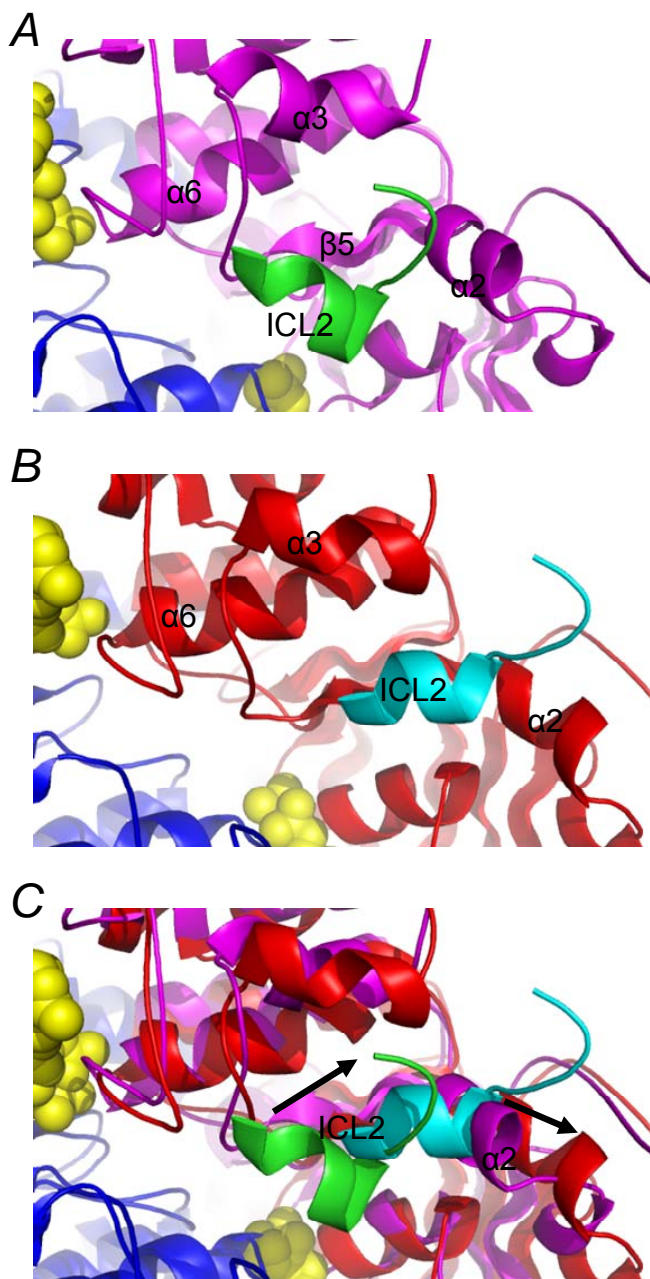


Figure 11-4

#### 11.3.6. The movement of ICL<sub>2</sub>

Following phosphorylation, the Tyr506 C<sub>α</sub> atoms (α carbon) moved by 9.2Å in the *mode* structures (see methods). The movement led to a horizontal shift of ICL<sub>2</sub> from a position close to the center of NBD<sub>2</sub> toward the edge of NBD<sub>2</sub> along the NBD<sub>2</sub> groove. Since the MD data were acquired after removing most TMDs, the restriction of ICL<sub>2</sub> by TMDs was not evaluated. Therefore, this ~10Å movement might be overestimated. However, the direction appeared reasonable. The Arg1462 was located at the end of α6 helix, close to the edge of NBD<sub>2</sub>, while Ser1387 was located on the bottom of the NBD<sub>2</sub> groove. With the triad formation, ICL<sub>2</sub> was pulled toward NBD<sub>2</sub> with lateral sliding along the groove. Meanwhile, the α2 helix in NBD<sub>2</sub> made a lateral move and left space for ICL<sub>2</sub> (Fig. 11-5).



**Figure 11-5. The movement of ICL<sub>2</sub> following phosphorylation.**

All pictures represent the *mode* structures in MD study. **A.B.C.** The top views of ICL<sub>2</sub> and NBD<sub>2</sub> in pre-phosphorylation, post-phosphorylation and overlay respectively. The NBD<sub>2</sub> was shown in purple and red in pre and post-phosphorylation forms. The ICL<sub>2</sub> was shown in green and cyan in pre and post phosphorylation forms. The NBD<sub>1</sub> and nucleotides were shown in blue and yellow respectively. Compared to the *mode* structure of pre-phosphorylated form, the ICL<sub>2</sub> helix moves horizontally from the center of NBDs toward NBD<sub>2</sub> edge with  $8.4 \pm 0.4 \text{ \AA}$  (measured at C $_{\alpha}$ s of residues 500-506). Accordingly, the  $\alpha$ 2 helix in NBD<sub>2</sub> moved to yield space for ICL<sub>2</sub>. The arrows in C. indicate the direction of movement of ICL<sub>2</sub> and  $\alpha$ 2 helices.

### 11.3.7. The NBD<sub>2</sub> groove is reshaped by phosphorylation

Studying the conformations of NBD<sub>2</sub> of pre- vs. post-phosphorylation, we found that the NBD<sub>2</sub> domain underwent major conformational changes at the interface with the ICL<sub>2</sub>. The groove for binding the ICL<sub>2</sub> consisted of residues from  $\beta$ 5,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 6, Q-loop and C-loop. The Arg1462 was the last residue of the long  $\alpha$ 6 helix, located close to the edge of NBD<sub>2</sub>. The  $\alpha$ 6 helix was expected to be stable because it was buried in the structure. Indeed, C $\alpha$ s in the corresponding segment in NBD<sub>1</sub> did not significantly change their positions ( $1.8 \pm 0.2 \text{ \AA}$ , Fig. 11-6D) in comparison to overall movements of C $\alpha$ s in NBDs ( $3.4 \pm 0.1 \text{ \AA}$ ). However, the phospho-Ser1387 (p-Ser1387) attracted the positively charged Arg1462, moving the residue by  $4.5 \text{ \AA}$  toward ICL<sub>2</sub> (measured at C $\alpha$ , Fig. 11-6C). This movement had major effects on the C-terminal half (tail) of  $\alpha$ 6 (residues 1455-1462) but not the N-terminal half (head) (residues 1448-1454). The average movement of C $\alpha$ s in the tail was  $4.2 \pm 0.3 \text{ \AA}$ , much greater than the head ( $2.2 \pm 0.3 \text{ \AA}$ ). Consequently, the  $\alpha$ 6 helix was slightly bent around Phe1454 and turned toward the ICL<sub>2</sub> with an angle  $\sim 10^\circ$ . The movement of the  $\alpha$ 6 helix appeared to cause the  $\alpha$ 3 helix to move toward ICL<sub>2</sub> about  $4.2 \pm 0.7 \text{ \AA}$ . Like the head of  $\alpha$ 6 helix, the Q-loop was quite stable ( $2.4 \pm 0.3 \text{ \AA}$ ). The C $\alpha$ s in  $\beta$ 5 sheet moved toward the ICL<sub>2</sub> with distance of  $3.0 \pm 0.4 \text{ \AA}$ . The  $\beta$ 5 movement was greater than the overall movements of  $\beta$ -sheets in the NBD<sub>2</sub> ( $2.3 \pm 0.2 \text{ \AA}$ ,  $P=0.11$ ) and the corresponding segment in the NBD<sub>1</sub> ( $2.4 \pm 0.2 \text{ \AA}$ ,  $P=0.07$ ). The movement of these segments toward ICL<sub>2</sub> suggested a stronger interaction between the ICL<sub>2</sub> and the NBD<sub>2</sub> following phosphorylation. We also observed a large movement ( $6.9 \pm 0.3 \text{ \AA}$ ) of the  $\alpha$ 2

helix. The  $\alpha 2$  helix appeared flexible in the SUR2B\_core model; it moved toward the edge of NBD<sub>2</sub> and yielded space for the ICL<sub>2</sub>.

**Figure 11-6. The conformational changes in NBD<sub>2</sub> interface.**

**A.B.C** The side views of NBD<sub>2</sub> interface in pre-phosphorylation, post-phosphorylation and overlay respectively. The backbone of the Arg1462 moved 4.46Å toward ICL<sub>2</sub>, which caused a movement of the  $\alpha 6$ -tail (residue 1455-1462). Major changes in position were also seen with  $\alpha 2$  and  $\alpha 3$  helices. The arrows in **C.** indicate the direction of movement of  $\alpha 6$ ,  $\alpha 3$  and  $\alpha 2$ . **D.** Analysis of critical residue and segments. Major changes were seen on NBD<sub>2</sub>- $\alpha 6$ -tail,  $\alpha 3$  and  $\alpha 2$ , compared to the corresponding segments in NBD<sub>1</sub> (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). The  $\alpha 6$  helix was long (15 residues) and buried in the NBDs, which were expected to be stable. Indeed, the  $\alpha 6$  helix in NBD<sub>1</sub> was very stable and moved less than the overall structure. The formation of the triad changed Arg1462 and moved the  $\alpha 6$ -tail  $4.2 \pm 0.3$ Å toward ICL<sub>2</sub>. The  $\alpha 3$  and its flanking sequence in C-loop were moved following the change in the  $\alpha 6$ -tail. The triad might also affect  $\beta 5$ , which appeared to change more than overall  $\beta$  sheets ( $2.3 \pm 0.2$ Å,  $P = 0.11$ ) in NBD<sub>2</sub> and the corresponding segment in NBD<sub>1</sub> ( $2.3 \pm 0.2$ Å,  $P = 0.07$ ). The  $\alpha 2$  helices in both NBDs changed large distance suggesting they are flexible, while the  $\alpha 2$  helix in the NBD<sub>2</sub> moved more to the edge of NBDs, giving space for ICL<sub>2</sub> (also see Fig. 11-5). On the other hand, the NBD<sub>2</sub>- $\alpha 6$ -head and the Q-loop did not significantly change position.



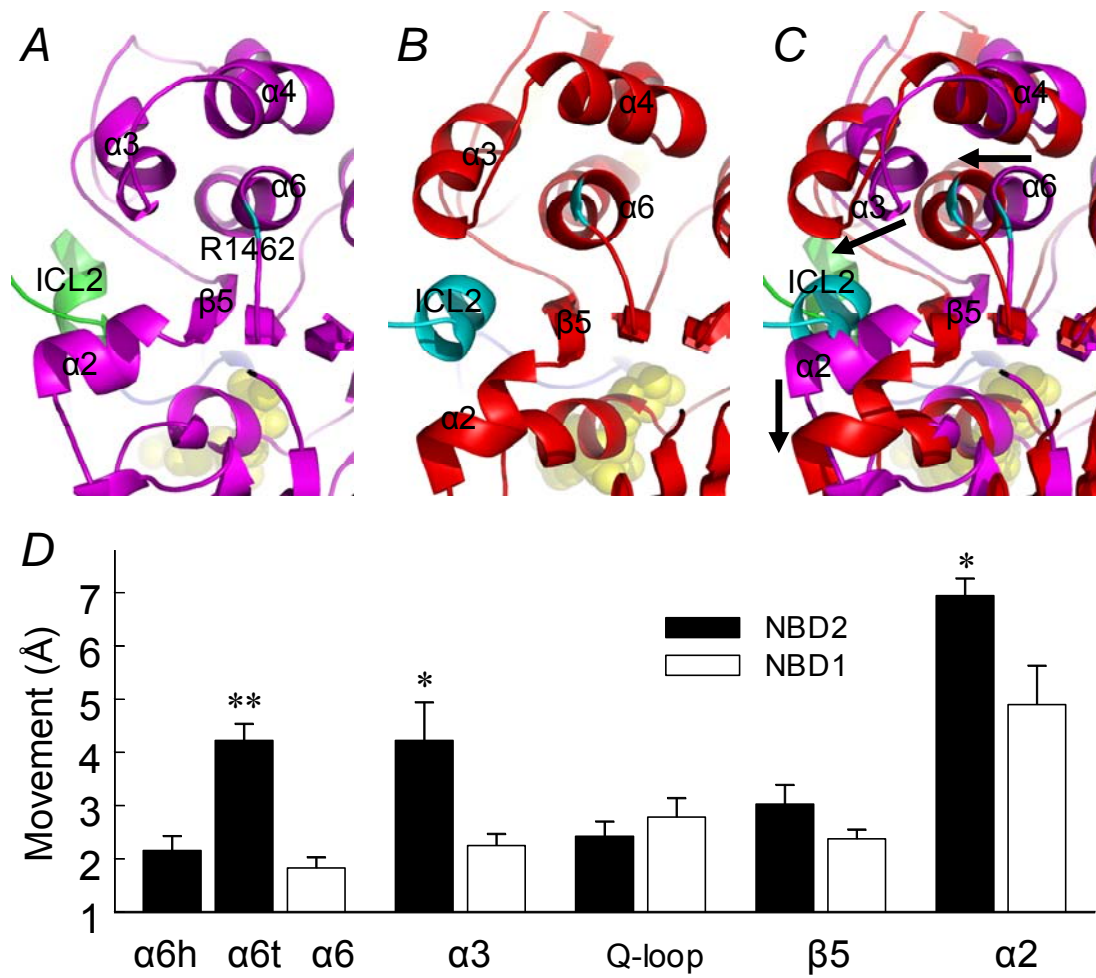


Figure 11-6

To make sure that these movements were not random events, the MD simulations were repeated twice for a total of three times with different starting velocities. We observed the same triad formation and protein domain movements in every runs. Therefore, even though there may be technical limitations in the MD simulations, we were able to conclude that the ICL<sub>2</sub> interacted with the NBD<sub>2</sub> rather loosely before phosphorylation, and the interaction of the two domains was strengthened with the Tyr506-pSer1387-Arg1462 triad formation after phosphorylation.

#### **11.3.8. Restriction of the interdomain movement by disulfide bond**

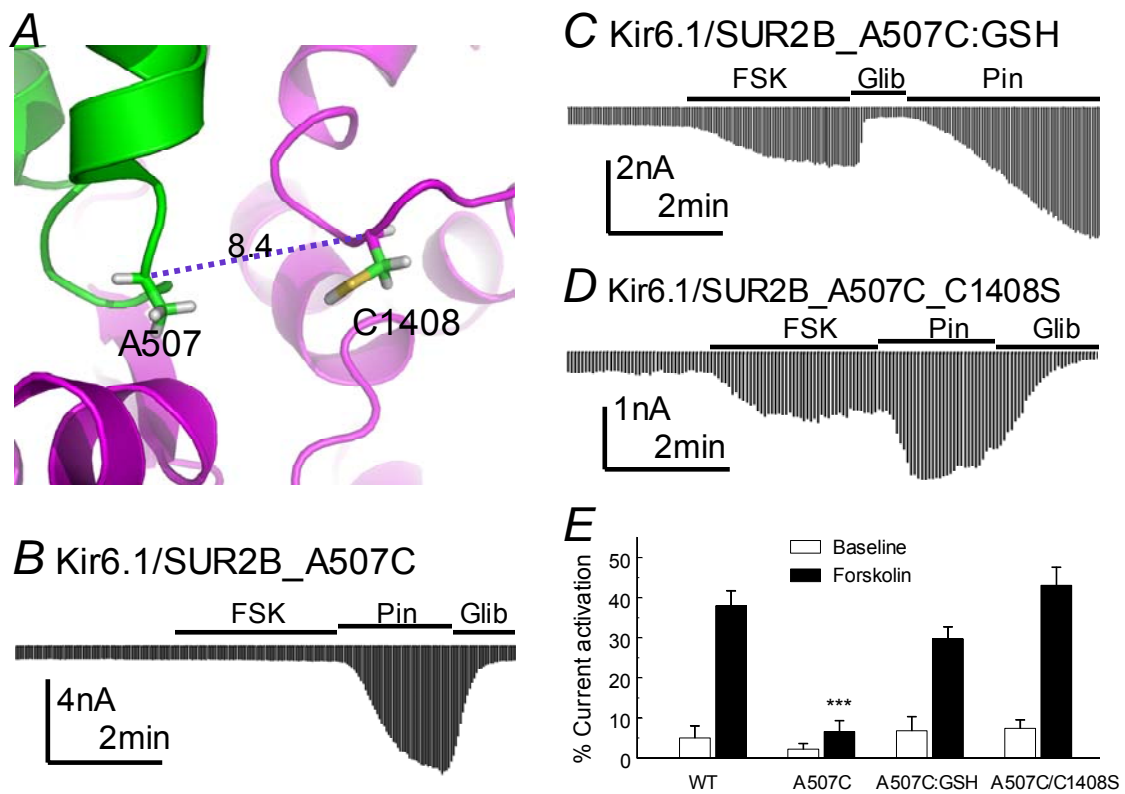
Mutations were engineered in the channel to test the hypothesis that interdomain motion plays a critical role in PKA-dependent channel activation. Forming a disulfide bond between the two domains can restrict the motion thus limiting the channel activation if the motion is critical for channel activation. Examining all residues in the NBD<sub>2</sub> groove and the ICL<sub>2</sub>, we found that a cysteine (Cys1408) on the wall of the NBD<sub>2</sub> groove was close to Ala507 in the ICL<sub>2</sub> (Fig. 11-7A). The distance between the C<sub>α</sub>s of the two residues was ~8Å. When the Ala507 was mutated to cysteine, the channel activation by forskolin was completely eliminated (Fig. 11-7B). Since the Ala507 is located next to Tyr506, the mutation itself instead of the disulfide bond formation could have affected the channel activation. To address this possibility, we included 3mM reduced glutathione in the pipette solution and found that the Kir6.1/SUR2B\_A507C channel activation by forskolin was nicely restored (29.7±3.0%, N=5, P>0.05 compared to wt, Fig. 11-7C). To show whether the introduced Cys507 indeed formed a disulfide bond with Cys1408 but not with another unidentified cysteine residue, we mutated the Cys1408 to serine, a

residue close to cysteine in side chain properties, but which is unable to form disulfide bonds. The mutant channel (Kir6.1/SUR2B\_A507C\_C1408S) was activated by forskolin to the same extent as wild type channel (Fig. 11-7D, E). Thus, the formation of an artificial disulfide bond between residues 507 and 1408 locked the channel in the pre-phosphorylation state and disrupted the channel activation by forskolin, supporting the hypothesis that a relative movement between the ICL<sub>2</sub> and NBD<sub>2</sub> is necessary for channel activation by PKA. We also tried to use disulfide bond to lock the channel in its open state without success.

## **11.4. Discussion**

### **11.4.1. The rational to choose SAV1866 to model SUR2B\_core.**

The K<sub>ATP</sub> channel regulatory subunit SURx (*abcc8* and *abcc9*) belongs to the ABC transporter superfamily. Four ABC proteins have been recently crystallized in full length with TMDs and NBDs. The NBDs are similar to each other in their overall structures and assemblies. The TMDs however, are much different. There are 10 TMHs in each TMD in HI1470/1 (Pinkett *et al.*, 2007) and BtuCD (Locher *et al.*, 2002) while 6 each in SAV1866 (Dawson & Locher, 2006) and ModBC (Hollenstein *et al.*, 2007). The primary sequence of the TMD in ModBC is much shorter than that of SUR2B and little homology exists between them, thus modeling SUR2B should not be based on ModBC structure. On the other hand, the TMD topology of SAV1866 is same as those of SUR2B (Fig. 11-1), allowing modeling of SUR2B\_core based on the SAV1866 template.



**Figure 11-7. Introduction of a disulfide bond between ICL<sub>2</sub> and NBD<sub>2</sub> blocked PKA effects.**

**A.** Close view at residue Ala507 and Cys1408. The distance between C $\alpha$ s of the two residues was  $\sim 8\text{\AA}$ , allowing the two residues to form a disulfide bond. **B.** When Ala507 on SUR2B was mutated to cysteine, the channel activation by forskolin was blocked. **C.** Including 3mM reduced glutathione in the pipette solution produced forskolin activation of the mutant channel. **D.** When the Cys1408 was mutated to serine, a similar residue that could not form disulfide bond, the Kir6.1/SUR2B\_A507C\_C1408S channel behaved like the wt channel. **E.** Summary of the forskolin effects on wt and mutants (\*\*\*,  $P < 0.001$ , compared to wt).

#### 11.4.2. What is new in our SUR2B\_core model?

In our SUR2B\_core model, NBD<sub>1</sub> and NBD<sub>2</sub> dimerize to form two nucleotide-binding pockets at their edges, consistent with the models of previous studies on SUR1 (Campbell *et al.*, 2003; Campbell *et al.*, 2004). A significant expansion of our model is to include TMDs and to show the interactions between TMDs and NBDs. SUR2B\_TMD<sub>1</sub> interacts NBDs via two short segments ICL<sub>1</sub> and ICL<sub>2</sub>. ICL<sub>1</sub> interacts with both NBDs crossing the first nucleotide-binding region while ICL<sub>2</sub> inserts deep into NBD<sub>2</sub> (see Fig. 11-1C). Therefore, TMD<sub>1</sub> mainly interacts with NBD<sub>2</sub>. A similar cross-interaction is found in TMD<sub>2</sub>-NBD<sub>1</sub>. Our disulfide bond experiment and functional analysis indicate that Cys1408 in NBD<sub>2</sub> does locate close to Ala507 in TMD<sub>1</sub>. Evidence supporting such a TMD-NBD cross-interaction also comes from a recent biochemical study (Zolnerciks *et al.*, 2007) on human multidrug transporter (*ABCB1*), a close homologue of SURx.

#### 11.4.3. The importance of defining TMD-NBD interaction in SURx.

The TMD-NBD interaction in SUR2B is not only important for PKA activation, but may also explain SUR-mediated channel gating by other ligands. For instance, it is well known that NBDs are important for Mg<sup>2+</sup>-nucleotide activation on K<sub>ATP</sub> channels (Gribble *et al.*, 1997; Shyng *et al.*, 1997; Matsuo *et al.*, 2002; Yamada & Kurachi, 2004). Although KCOs activate K<sub>ATP</sub> channels via binding to segments in TMDs (Moreau *et al.*, 2005), NBDs are also necessary for KCO effects (Gribble *et al.*, 1997; Shyng *et al.*, 1997; Yamada *et al.*, 2004), suggesting an allosteric modulation between these two distinct groups of channel activators. It is unclear how signals in NBDs are coupled to TMDs. Indeed, cis-interaction (TMD<sub>1</sub> interacts with NBD<sub>1</sub>, TMD<sub>2</sub> with NBD<sub>2</sub>) has been assumed

in SUR1 (Babenko & Bryan, 2003) and SUR2 (Yamada & Kurachi, 2004) based on the misassembled MSBA structure (Chang, 2003). Therefore, demonstration of the SAV1866-based NBD-TMD cross-interaction in SUR2B with experimental evidence contributes significantly to the understanding of the SUR-mediated gating of  $K_{ATP}$  channels.

#### **11.4.4. The mechanism of PKA activation.**

The SUR2B\_core modeling enhances our understanding of the mechanism underlying PKA activation on vascular  $K_{ATP}$  channel. The Ser1387 is a critical phosphorylation site for PKA-dependent activation of Kir6.1/SUR2B in HEK cells (Shi *et al.*, 2007b). Data from current study further support its role in the channel activation. Phosphorylation of the Ser1387 leads to a change in the NBD<sub>2</sub> conformation and strengthens the interaction of the NBD<sub>2</sub> with the ICL<sub>2</sub> through the formation of a triad by residues in different protein domains. Every residue of the triad is critical, as shown in our mutagenesis analysis in the current and previous studies (Shi *et al.*, 2007b). The interaction of p-Ser1387 and Arg1462 is apparently caused by the electronic attraction. The interactions of Tyr506 with the other two residues seem to rely on hydrogen bonds. Our simulation study indicates this triad is stable and might act as a primary force that changes the conformation of NBD<sub>2</sub> and ICL<sub>2</sub> at their interface. The ICL<sub>2</sub> appears to move toward NBD<sub>2</sub> while NBD<sub>2</sub> is reshaped and moves toward ICL<sub>2</sub>, especially the  $\alpha$ 6-tail and the  $\alpha$ 3 helix and probably the  $\beta$ 5 sheet as well. Thus the two domains interact with each other more tightly post phosphorylation. The NBDs thereby couple to the TMDs more effectively. Such a strong NBD-TMD coupling is likely to ensure the transfer of

mechanical forces and movements necessary for channel gating between these protein domains. Since such a coupling is weakened without Ser1387 phosphorylation, the mechanical forces and movements produced in NBDs may not be sufficient for channel gating. By enhancing the NBD-TMD interaction, therefore, vasodilators seem to be able to use the existing forces to augment the channel activity without producing additional mechanical work. It is noteworthy that the NBD-TMD force transfer depends on elaborate interactions of the triad, as restriction of these domains with disulfide bond does not lead to channel activation.

**Figure 11-8. Schematics of the PKA-dependent channel gating.**

**A.** Channel closure before Ser1387 phosphorylation. **B.** Binding ADP/ATP to NBDs changes the NBD conformation, which does not result in great channel activation without NBD-TMD tight interaction. **C.** Ser1387 phosphorylation enhances the NBD-TMD interaction, allows coupling between the BND and TMD, and leads to channel activation.

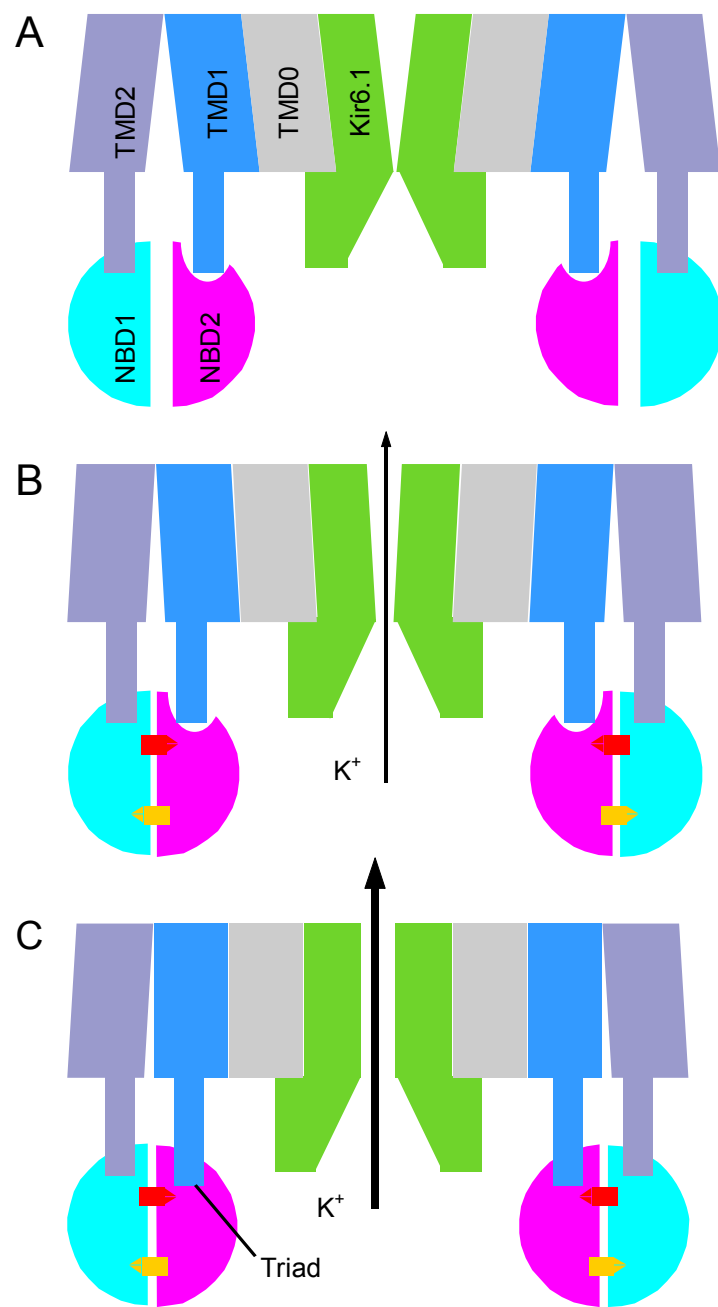


Figure 11-8



#### 11.4.5. Possible cooperation of PKA and nucleotide activators.

The SAV1866 is crystallized in ADP (PDB: 2hyd) binding forms, which represents a dimer in NBDs. The NBD dimerization has been seen in almost all the ABC proteins and seen in simulation studies in NBDs of SUR1 (Campbell *et al.*, 2003; Campbell *et al.*, 2004). In the SUR2B\_core model, the two NBDs form a hetero-dimer with two nucleotide-binding pockets at the interface. Such dimerization of NBD<sub>1</sub> and NBD<sub>2</sub> in SURx suggests a channel conformation that is in an 'active' state. Nevertheless, the channel activity remains low (less than 10% of maximal activity by pinacidil according to our observation in previous and current studies), probably because the interaction between TMDs and NBDs is loose. PKA phosphorylation enhances the interaction of TMD<sub>1</sub> (via ICL<sub>2</sub>) and NBD<sub>2</sub> and thus drastically activates the channel to ~40%. It is possible that PKA activation may rely on NBD dimerization caused by nucleotide binding. A study (Light *et al.*, 2002) on the Kir6.2/SUR1 channel indeed indicates ADP concentration determines whether the channel is activated or inhibited by PKA. Though we have not observed inhibition on Kir6.1/SUR2B, we do see decreasing ADP concentration reduces PKA effects (unpublished data). It is of interest to know whether PKA can activate the channel in the absence of nucleotides.

Demonstrating the molecular mechanism underlying PKA on channel activation is important in understanding the vasodilators effects on the K<sub>ATP</sub> channel and vascular tone regulation. Additionally, discovery of the interaction between TMDs and NBDs contributes to a significant step in understanding SUR subunit mediated gating in K<sub>ATP</sub> channels.

## **12. Result 3: A Unique Motif in Kir6.1 Consisting of 4 Phosphorylation Repeats Underlies the Vascular K<sub>ATP</sub> Channel Inhibition by Protein Kinase C**

Manuscript submitted: Shi Y, Cui N, Shi W, Jiang C. (2007) A unique motif in Kir6.1 consisting of 4 phosphorylation repeats underlies vascular K<sub>ATP</sub> channel inhibition by protein kinase C. *J Biol Chem, in press*

Note: The work was mostly done by Yun Shi (90%). Dr. Ningren Cui and Mr. Weiwei Shi did some patch clamp experiments.

### 12.1. Abstract

Vascular ATP-sensitive K<sup>+</sup> channels are inhibited by multiple vasoconstricting hormones via the PKC signaling pathway. However, the molecular substrates for PKC phosphorylation remain unknown. To identify the PKC sites, Kir6.1/SUR2B and Kir6.2/SUR2B were expressed in HEK293 cells. Following channel activation by pinacidil, the catalytic fragment of PKC inhibited Kir6.1/SUR2B but not Kir6.2/SUR2B currents. Phorbol 12-myristate 13-acetate (PMA, a PKC activator) had similar effects. Using Kir6.1-Kir6.2 chimeras two critical protein domains for PKC-dependent channel inhibition were identified. The proximal N-terminus of Kir6.1 was necessary for the channel inhibition. Since there was no PKC phosphorylation site in the N-terminus, our results suggest its potential involvement in channel gating. The distal C-terminus of Kir6.1 was also crucial, and it contains several consensus PKC sites. Mutation of Ser354, Ser379, Ser385, Ser391 or Ser397 to non-phosphorylatable alanine reduced PKC inhibition moderately but significantly. Combined mutations of these residues had greater effects. The channel inhibition was almost completely abolished when all five were jointly mutated. In-vitro phosphorylation assay showed that 4 of the serine residues were necessary for the PKC-dependent <sup>32</sup>P incorporation into the distal C-terminal peptides. Thus, we identified a motif containing 4 phosphorylation repeats in the Kir6.1 subunit that is likely to underlie PKC-dependent inhibition of the Kir6.1/SUR2B channel. The presence of the phosphorylation motif in Kir6.1 but not in its close relative Kir6.2, suggests that the vascular K<sub>ATP</sub> channel may have undergone evolutionary optimization

allowing it to be regulated by a variety of vasoconstricting hormones and neurotransmitters.

## **12.2. Introduction**

In chapter 10 and 11, we demonstrated the activation of vascular  $K_{ATP}$  channels by vasodilators. In a separate study in our laboratory (Shi *et al.*, 2007a), we have shown  $K_{ATP}$  channels are also inhibited by vasoconstrictors. The bidirectional modulation by vasoactive agents allows the channels to play a key role in controlling the VSMs membrane potential and regulating vascular tone. Experimental evidence suggests that vasoconstrictors act on the vascular  $K_{ATP}$  channel through the PKC signaling system; however, the mechanism is unknown.

Our previous studies have shown that the Kir6.1/SUR2B channel and its counterpart in VMS cells are inhibited by vasopressin, and the channel inhibition can be abolished by specific PKC blockers (Shi *et al.*, 2007a). Similar observations have been made by other groups with endothelin (Park *et al.*, 2005), muscarinic M3 receptor agonist (Quinn *et al.*, 2003) and angiotensin II (Hayabuchi *et al.*, 2001b). The effect of angiotensin II on vascular  $K_{ATP}$  channel requires translocation of  $PKC_{\epsilon}$  to plasma membranes (Hayabuchi *et al.*, 2001b; Sampson *et al.*, 2007). By comparing effects of acetylcholine M3-receptor on Kir6.1/SUR2B and Kir6.2/SUR2B channels, Quinn *et al.* (Quinn *et al.*, 2003) suggest that the Kir6.1/SUR2B channel inhibition is mediated via a direct effect of PKC rather the change in PIP2 concentrations. They also show evidence for Kir6.1 phosphorylation using an in-vitro biochemical assay (Quinn *et al.*, 2003).

Consistently, purified PKC inhibits the cloned Kir6.1/SUR2B and VMS-endogenous  $K_{ATP}$  channels in inside-out patches where cytosolic soluble components are absent (Cole *et al.*, 2000; Thorneloe *et al.*, 2002). Although these previous studies have significantly improved our understanding of the vascular  $K_{ATP}$  channel regulation by vasoconstrictors, the molecular substrate of PKC remain unknown. Therefore, we performed these studies to identify the critical protein domain and amino acid residues for PKC phosphorylation.

### **12.3. Results**

#### **12.3.1. PKC-dependent inhibition of the Kir6.1/SUR2B channel**

Kir6.1/SUR2B channels were expressed in HEK cells, and recordings were performed 2-5 days post-transfection. Kir6.1/SUR2B currents remained small during 8-10 min of baseline recording, and were strongly activated by 10 $\mu$ mol/L pinacidil and inhibited by 10 $\mu$ mol/L glibenclamide (Fig. 12-1A, B). At maximum activation, application of 100nmol/L PMA strongly inhibited the Kir6.1/SUR2B currents ( $77.5\pm3.7\%$ ,  $n=19$ ), which was blocked by PKCi, a specific PKC inhibitor (Fig. 12-1C, F). The inactive phorbol ester, 4 $\alpha$ -phorbol-12,13-didecanoate (4 $\alpha$ -PDD), did not affect Kir6.1/SUR2B currents (Fig. 12-1F).

In inside-out patches, channel activity was low in the absence of nucleotides. The Kir6.1/SUR2B channel was activated in the presence of MgADP/ATP, which was further augmented with pinacidil application (Fig. 12-2A). When the catalytic fragment of PKC (cPKC) was applied to the internal patch membranes, the Kir6.1/SUR2B currents were markedly inhibited ( $61.1\pm3.8\%$ ,  $n=6$ , Fig. 12-2A, C). These results thus indicate that the

Kir6.1/SUR2B channel is inhibited by PKC independently of cytosolic soluble components, consistent with previous reports (Thorneloe *et al.*, 2002). In contrast, PMA had no effects on Kir6.2/SUR2B channel in whole cell recordings (Fig. 12-1 D-F), neither the cPKC in inside-out patches (Fig. 12-2B, C), suggesting that the SUR2B subunit is not critical.

**Figure 12-1. Kir6.1/SUR2B and Kir6.2/SUR2B channels expressed in HEK293 cells.**

**A.** Whole-cell currents were recorded from a cell transfected with Kir6.1/SUR2B. Symmetric concentrations of  $K^+$  (145mmol/L) were applied to the pipette and bath solutions. The cell was held at 0mV, and pulse voltages from -120 to 80mV with a 20mV increment were applied. The current amplitude increased in response to pinacidil (Pin, 10 $\mu$ mol/L). The pinacidil-induced currents were strongly inhibited by PMA (100nmol/L) and completely inhibited by glibenclamide (Glib, 10 $\mu$ mol/L). **B.** The time course for Kir6.1/SUR2B channel modulation. Whole-cell currents were recorded with a holding potential at 0mV and command pulses of -80mV every 3 seconds. After the whole-cell configuration was formed, the cell was perfused with extracellular solution for a ~2 min baseline recording. The currents were strongly activated by pinacidil (10 $\mu$ mol/L), and the maximum activation was reached after 3-4 min of the exposure. The currents were maximally inhibited by PMA (100nmol/L) by 4-5min and could be further inhibited by glibenclamide. The lower panel shows individual currents produced by a single command pulse. **C.** Kir6.1/SUR2B currents were recorded with PKCi (10 $\mu$ mol/L) in the pipette solution. PKCi almost completely blocked Kir6.1/SUR2B current inhibition by PMA. **D. E.** Kir6.2/SUR2B currents were recorded from a transfected HEK293 cell with the same treatment as for the Kir6.1/SUR2B. PMA did not affect the pinacidil-activated Kir6.2/SUR2B currents. **F.** Summary of PMA effects on Kir6.1/SUR2B and Kir6.2/SUR2B currents activated by pinacidil. PMA inhibited Kir6.1/SUR2B currents strongly. The non-active PMA analog, 4 $\alpha$ -phorbol 12, 13-didecanoate (4 $\alpha$ -PDD, 100nmol/L), had little effects on the Kir6.1/SUR2B currents. In the presence of PKCi, the PMA effect was reduced. PMA had no inhibitory effects on Kir6.2/SUR2B currents.

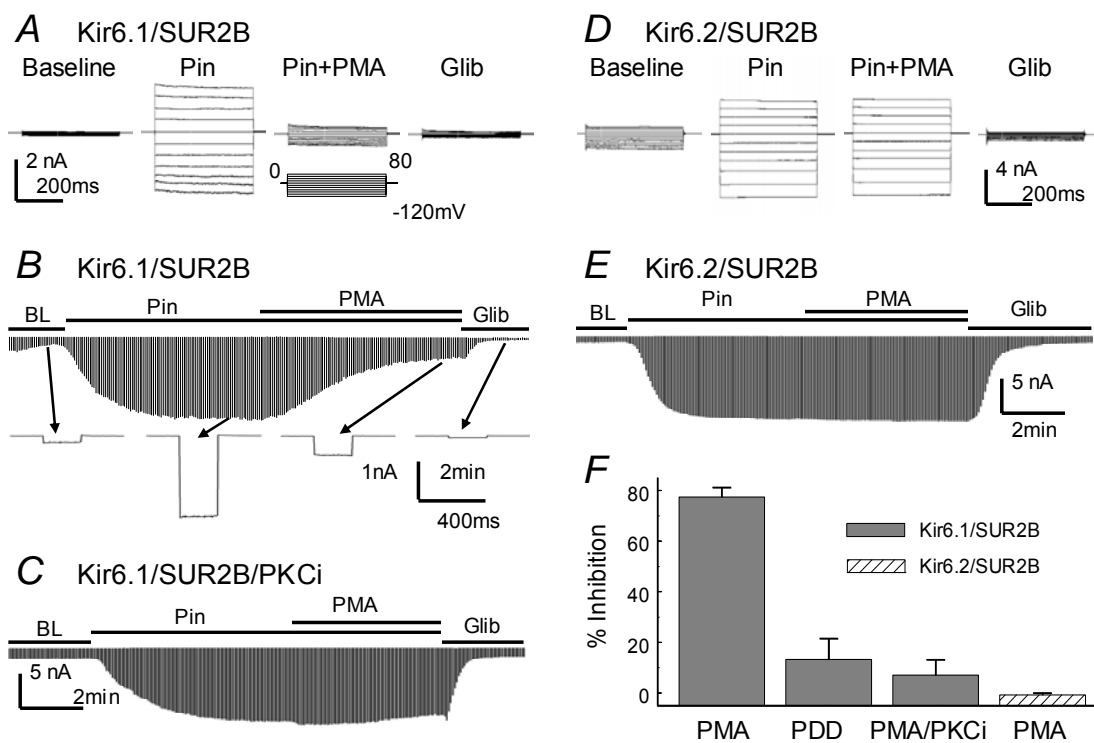
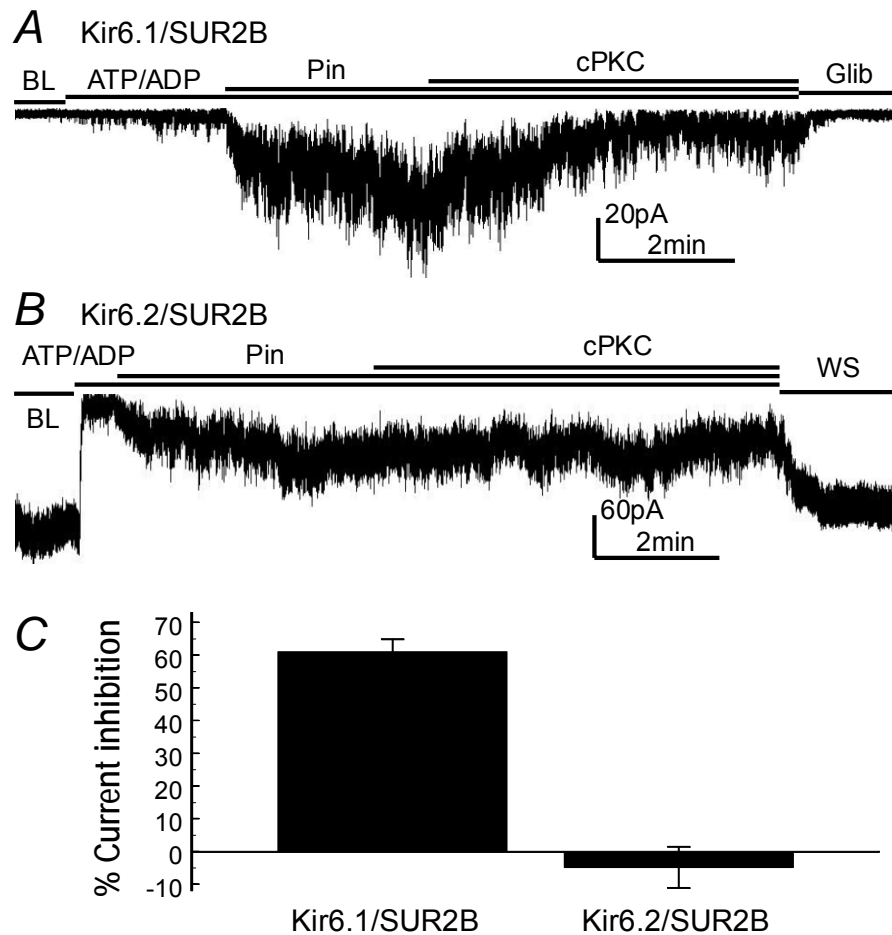


Figure 12-1



**Figure 12-2. Effects of the recombinant catalytic fragment of PKC on Kir6.1/SUR2B and Kir6.2/SUR2B currents in inside-out patches.**

**A.** At baseline (BL) without nucleotides, the Kir6.1/SUR2B channels were closed. Application of 1mmol/L ATP / 0.5mmol/L ADP to the internal patch membrane slightly activated the currents, which were strongly activated by pinacidil (Pin, 10 $\mu$ mol/L). Application of cPKC inhibited the Kir6.1/SUR2B currents. **B.** Without nucleotides, the Kir6.2/SUR2B channel was open. The channel was inhibited by 1mmol/L ATP / 0.5mmol/L ADP and activated by pinacidil. Unlike Kir6.1/SUR2B, cPKC has no effects on Kir6.2/SUR2B currents under the same experimental condition. **C.** Summary of cPKC effects on Kir6.1/SUR2B and Kir6.2/SUR2B currents that were activated by pinacidil. The Kir6.1/SUR2B currents were significantly inhibited by cPKC, while the Kir6.2/SURB currents were not affected.



### 12.3.2. Critical protein domains for the PKC-dependent channel inhibition

The differential PKC sensitivity of the Kir6.1/SUR2B from Kir6.2/SUR2B suggests that critical protein domains for PKC regulation are likely to be located on the Kir subunit. Thus, we constructed chimeric channels between Kir6.1 and Kir6.2 and expressed them with SUR2B in HEK293 cells. The Kir6.1 and Kir6.2 were divided into three regions: the N-terminus, the core sequence containing two transmembrane domains and the pore loop, and the C-terminus (Fig. 12-3). Six chimeras were obtained, all of which showed functional channels and were sensitive to pinacidil and glibenclamide. When the N-terminus of Kir6.1 was replaced with that of Kir6.2 (we named it 211), the channel inhibition by PMA was greatly diminished (Fig. 12-3A, D). A similar result was observed in the 112 chimera whose C-terminus derived from Kir6.2 (Fig. 12-3B, D), suggesting that both N- and C-termini are necessary for channel inhibition. Consistent with this, replacement of either the N-terminus or the C-terminus of Kir6.2 (122 and 221 respectively) did not confer the PMA sensitivity to the chimeric channels (Fig. 12-3D). However, when both the N-terminus and the C-terminus of Kir6.2 were exchanged for their Kir6.1 counterparts, the 121 chimera responded to PMA just like Kir6.1 (Fig. 12-3C, D). Therefore, both N-terminus and C-terminus appear critical for PKC-dependent channel inhibition.

#### **Figure 12-3. Responses of Kir6.1-Kir6.2 chimeras to PMA.**

All chimeric channels were expressed with SUR2B. **A.** When the N-terminus of Kir6.1 was replaced with that of Kir6.2, PMA failed to inhibit the 211 channel. **B.** When the C-terminus of Kir6.1 was replaced with that of Kir6.2, the 112 channel lost its response to PMA. **C.**

Construction of a chimera with both Kir6.1 N- and C-termini on the Kir6.2 core resulted in PMA sensitivity as wt Kir6.1/SUR2B. **D.** Summary of PMA inhibition of chimeras. Kir6.1 N-terminus, core and C-terminus refer to residues 1-71, residues 72-186, and residues 187-424, respectively. Kir6.2 N-terminus, core and C-terminus refer to residues 1-70, residues 71-176, and residues 177-390.

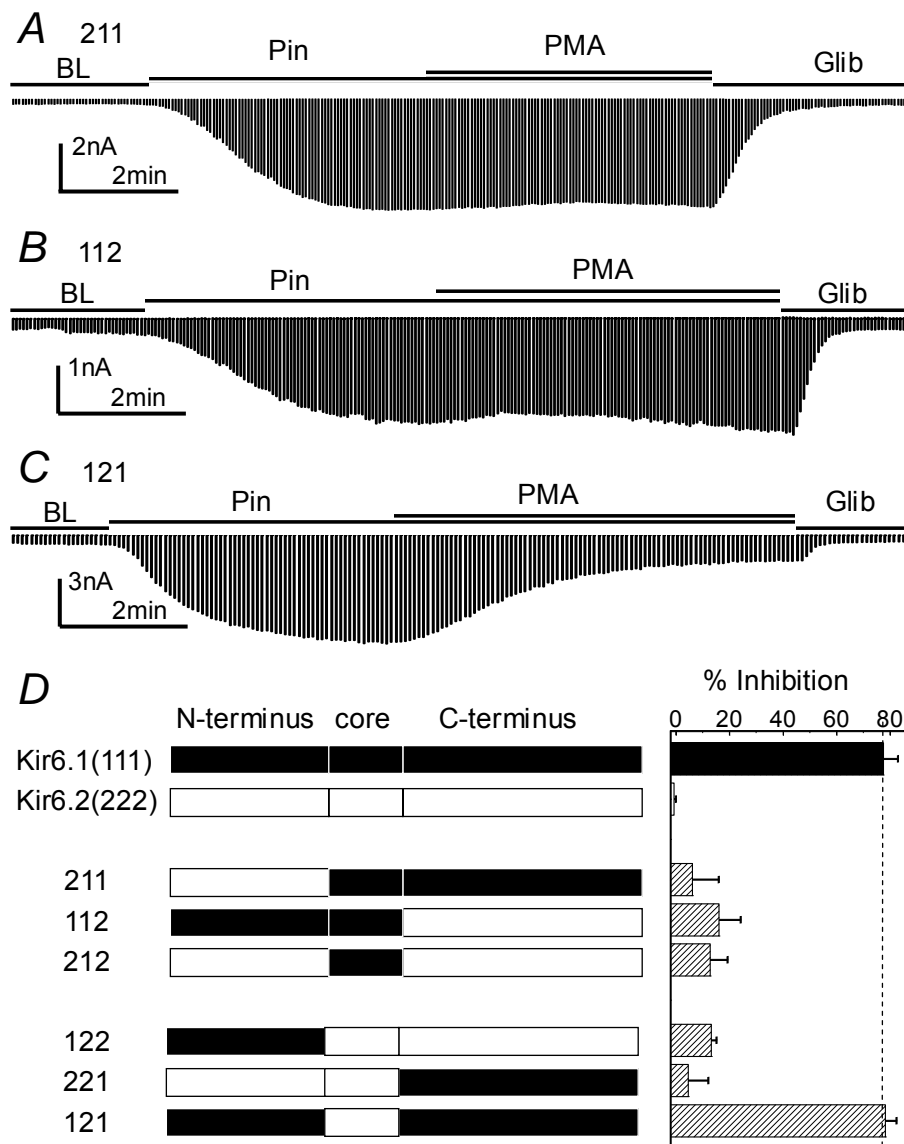


Figure 12-3

We further divided the N-terminus into two segments and the C-terminus into three. All chimeras showed similar responses to pinacidil and glibenclamide as the wt channels. When the distal N-terminus was replaced the Kir6.1-21\_111 channel responded to PMA to the same extent ( $71.1 \pm 4.2\%$ ,  $n=5$ , Fig. 12-4A) as Kir6.1. Replacement of the proximal N-terminus, however, partially but significantly eliminated PMA effects ( $36.7 \pm 7.8\%$ ,  $n=5$ , Fig. 12-4B). A serine residue (Ser40) was found in the proximal N-terminus of Kir6.1 but not in Kir6.2. When Ser40 was mutated to the corresponding site (Lys39) in Kir6.2, the channel Kir6.1-S40K was still inhibited as the wt Kir6.1, suggesting there is no PKC site in this segment. When the proximal C-terminus was replaced, the Kir6.1-11\_211 responded to PMA as the same as the wt channel (Fig. 12-4C). When either the middle or distal segment swapped, the Kir6.1-11\_121 and Kir6.1-11\_112 channels' responses to PMA were significantly diminished (Fig. 12-4D, E). PMA inhibition was further decreased in the chimera in which both the middle and the distal segments were derived from Kir6.2 (Kir6.1-11\_122, Fig. 12-4F), although none of these chimeras showed complete block of PMA effects.

**Figure 12-4. Dissection of critical protein domains for the PKC-dependent channel inhibition.**

The N-terminus was further divided into two segments at residue 35 in Kir6.1 and residue 34 in Kir6.2. The C-terminus was divided into 3 segments at residue 275 and 363 in Kir6.1 as well as residue 265 and 354 in Kir6.2. **A.** Kir6.1-21\_111, the distal N-terminus of Kir6.1 replaced with that of Kir6.2, was inhibited by PMA as much as the wt Kir6.1/SUR2B channel. **B.** The PMA effect was disrupted in Kir6.1-12\_111, where the proximal N-terminus of Kir6.1 was replaced. **C.** Replacement of the proximal C-terminus of Kir6.1 did not affect PMA sensitivity.

**D.-F.** Replacement the middle segment of the C-terminus, the distal C-terminus or both disrupted channel inhibition to various degrees. **G.-I.** Construction of necessary protein segments in Kir6.2 core. **G.** The distal C-terminus conferred partial PMA effects to the Kir6.2-core channel. **H.** When the middle and distal segments were switched, the channel displayed PMA inhibition with no significant difference from the wt Kir6.1 channel. **I.** In Kir6.2-21\_221' the distal C-segment was elongated from kir6.1 residues 364-terminus to 346-terminus, PMA fully inhibited the channel. **J.** Summary of the effect of N- and C-terminal segments on the channel inhibition. Data were obtained from 4-6 cells in each construct. The amino acid sequences of the distal C-termini (Kir6.1-346-424 and corresponding sequence in Kir6.2) are shown in the lower panel with putative PKC phosphorylation sites highlighted.

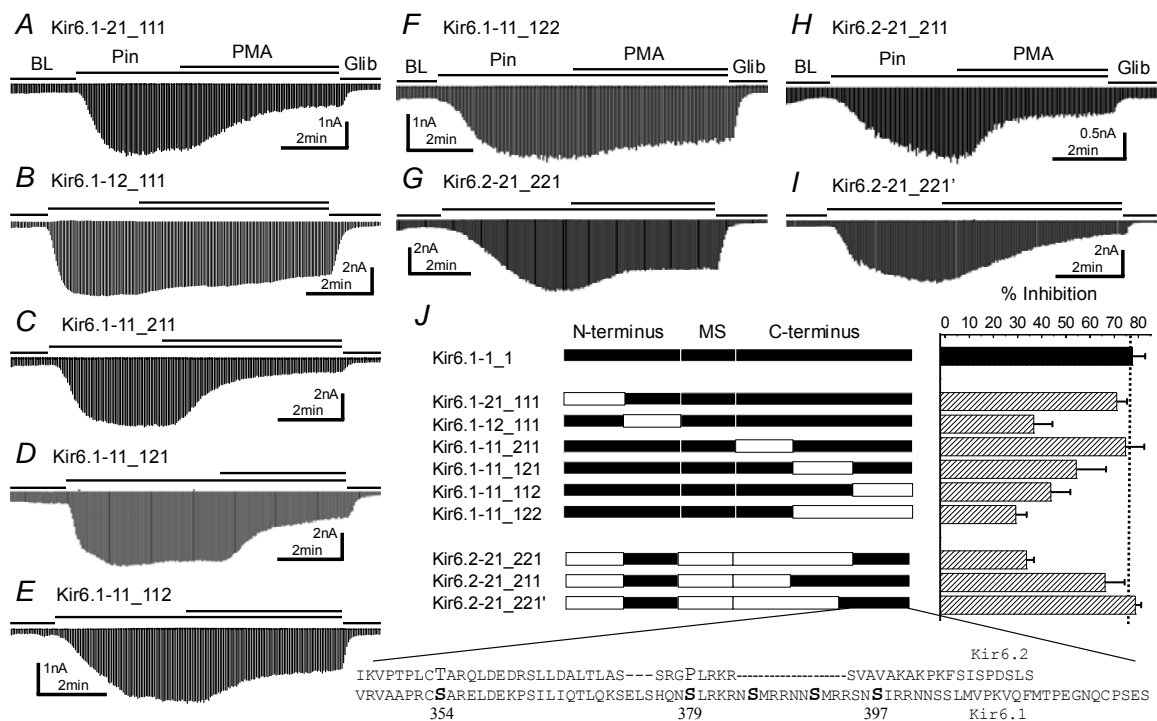


Figure 12-4

We then used chimeras with a Kir6.2 core and necessary N-terminal segment to search for the minimal requirement in the C-terminus for the PMA effect. Kir6.2-21\_211 showed full PMA sensitivity ( $69.9 \pm 6.7\%$ ,  $P > 0.05$  compared Kir6.1,  $n=4$ , Fig. 12-4H). Kir6.2-21\_221 showed partial PMA sensitivity ( $33.7 \pm 3.1\%$ ,  $P < 0.001$  compared to Kir6.1 and Kir6.2,  $n=5$ , Fig. 12-4G). By elongating the distal segment (Kir6.2-21\_221'), the channel had full PMA response ( $78.8 \pm 2.5\%$ ,  $P > 0.05$  compared to Kir6.1,  $n=5$ , Fig. 12-4I), suggesting that the distal segment appears to contain the essential C-terminus elements for PKC phosphorylation.

### 12.3.3. Phosphorylation of a stretch of serine repeats in the C terminus by PKC

There are multiple consensus PKC sequences in the distal C-terminus from residue 346 to the end residue, 424. Particularly remarkable are 4 SXRR/KXN repeats at residues 379, 385, 391 and 397. We thus performed alanine mutational screening for all consensus PKC sites. Mutation of any individual produced moderate but significant reduction of the PKC-dependent inhibition (Fig. 12-5B, E). Mutation of Ser354 in the same protein domain showed a similar effect. Combined mutations of 3 or 4 had greater effects (Kir6.1-3A, Kir6.1-4A, Fig. 12-5C, E). When all of these 5 serine residues are jointly mutated (Kir6.1-5A, Fig. 12-5D, E), the PKC-dependent channel inhibition was almost completely eliminated (Fig. 12-5A-E).

#### Figure 12-5. Mutational analysis of potential PKC phosphorylation sites.

**A.** Mutation Ser403 of Kir6.1 to alanine did not affect PMA inhibition. **B.** Mutation of Ser385 decreased PMA effects. **C,D.** When 4 and 5 potential PKC sites were mutated to alanine simultaneously, channel inhibition was largely eliminated. **E.** Summary of the mutations. 3A,

Kir6.1-S385A/Ser391A/S397A; 4A, Kir6.1-S379A/S385A/Ser391A/S397A; 5A, Kir6.1-S354A/S379A/S385A/Ser391A/S397A. Data were obtained from 4-9 patches. White bars,  $P>0.05$ ; Black bars,  $P<0.05$ . Application of chemicals was the same as in panel A.

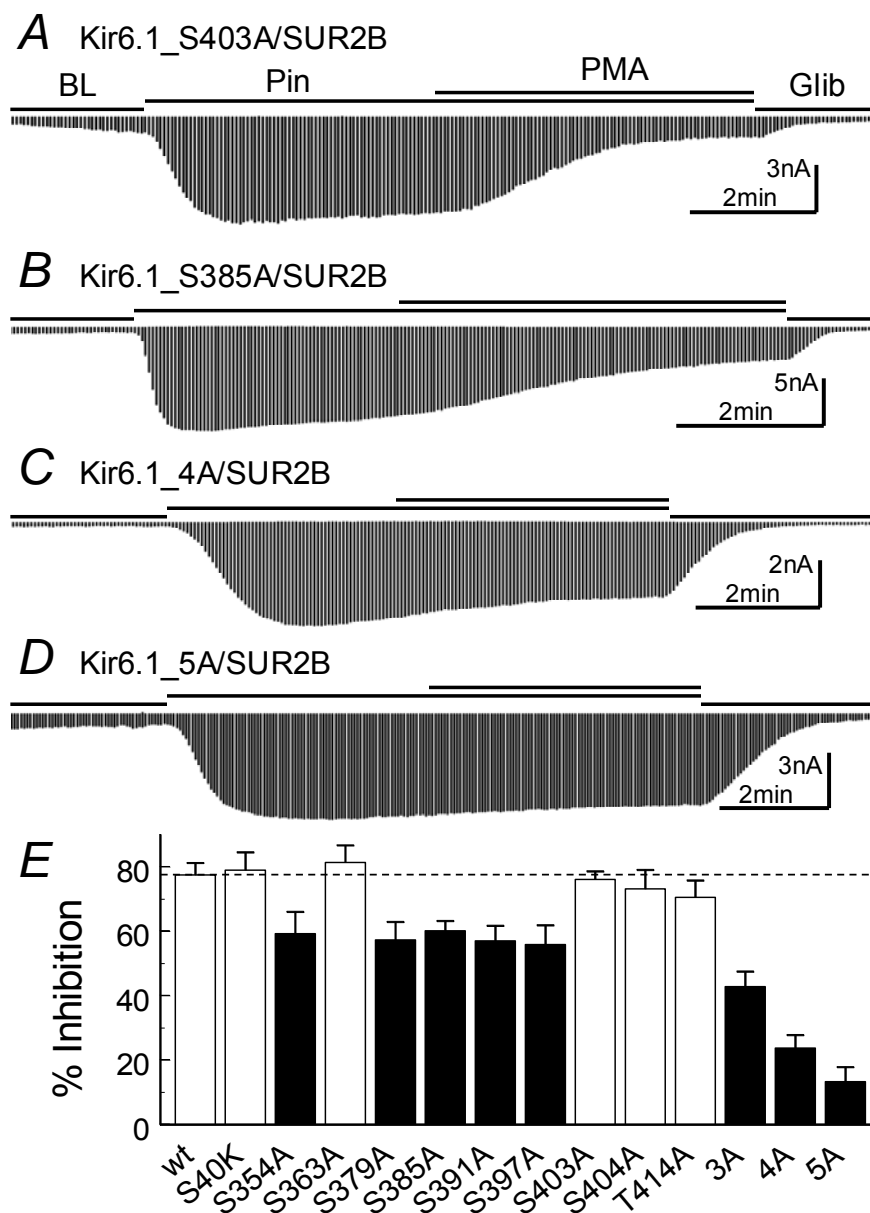


Figure 12-5

To confirm that these serine residues are indeed phosphorylated, we generated a few C-terminal peptides of Kir6.1 (residues 346-424) with and without mutations on the serine residues. After extraction and purification, the peptides were subjected to SDS-page separation, western blot, and *in vitro* phosphorylation. In Coomassie blue stain and Western blot, an ~52kd band was detected that was consistent with the peptide size (Fig. 12-6B), while there was another ~49kd band indicating a degraded protein fragment. *In vitro* phosphorylation experiment showed strong  $^{32}\text{P}$  incorporation in both bands of the wt peptide (Fig. 12-6 C), consistent with functional assay. The strength of  $^{32}\text{P}$  incorporation was significantly reduced in the Kir6.1-3A peptide. The Kir6.1-4A and Kir6.1-5A peptides were weakly phosphorylated. Comparing the phosphorylation patterns, we found that Ser354 was not phosphorylated (See analysis in Fig. 12-6 legend). The Kir6.1-4A and Kir6.1-5A peptides remained to be phosphorylated to a very low degree, suggesting the presence of other unidentified PKC sites more distal to Ser397. Nevertheless,  $^{32}\text{P}$  incorporation to the Kir6.1-4A and Kir6.1-5A peptides was rather weak in comparison to the wt, suggesting that the Ser379, Ser385, Ser391, Ser397 are the major players in the PKC-dependent channel inhibition.

**Figure 12-6. *In vitro* phosphorylation on MBP-fusion peptides.**

**A.** Four MBP-fusion proteins were constructed by linking Kir6.1 distal C fragment (residues 346-424) to MBP with or without mutations on critical serine residues. The sequence underlined is the antigen for Western detection. **B.** All the constructs showed two bands with Western blot. The band around 52kd represented the intact fusion proteins (arrow), while the band of ~49kd was a degraded protein fragment (arrowhead). These bands were weakly detected in 3A, 4A and 5A by Western because mutations were made in antigen region. Lower panel

showed protein input colored with Coomassie blue. The heavy bands shown in Coomassie blue staining indicated the stronger degradation. **C.** *In vitro* phosphorylation showed strong 32P incorporation in both 52kd and 49kd bands in wt. The 32P incorporation was markedly reduced in 3A. The 4A and 5A were only weakly phosphorylated in the 52kd band. By comparing 3A and 4A, the phosphorylation of 49kd band on 3A must occur on Ser379. The 49kd of 4A was not phosphorylated also suggested that Ser354 was not phosphorylated.

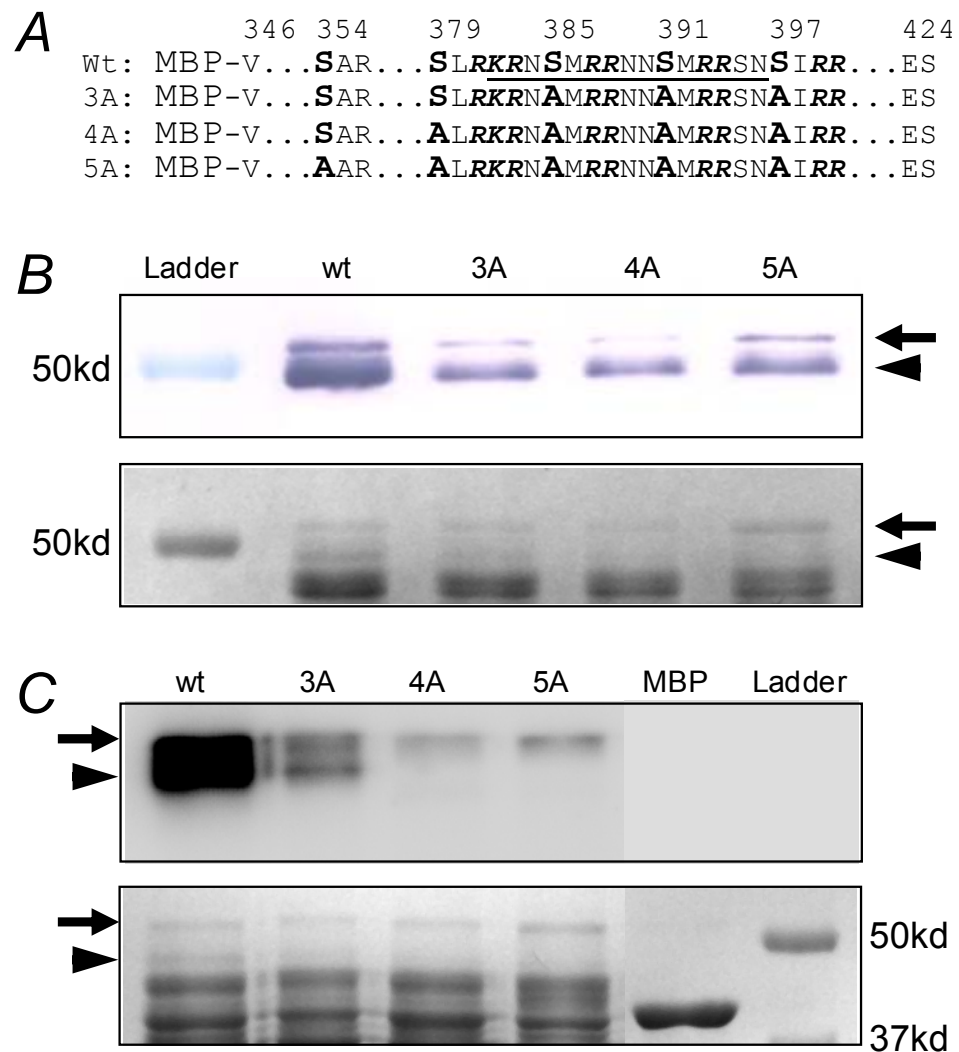


Figure 12-6



## 12.4. Discussion

The present study considers the molecular mechanism underlying the Kir6.1/SUR2B channel inhibition by PKC. The channel inhibition relies on a short motif consisting of 4 phosphorylation repeats in the Kir6.1 subunit. Graded channel inhibitions are produced by phosphorylation of different numbers of serine residues. The presence of the phosphorylation motif in the Kir6.1 but not in its close relative Kir6.2 suggests that the vascular  $K_{ATP}$  channel may have undergone evolutionary optimization allowing it to be regulated by a variety of vasoconstricting hormones and neurotransmitters.

### 12.4.1. PKC inhibition is unique in Kir6.1/SUR2B channels

PKC acts on several isoforms of  $K_{ATP}$  channels. Previous studies indicate that PKC activation leads to inhibition of the vascular Kir6.1/SUR2B channel, and this effect is likely to be mediated by direct phosphorylation of the channel protein (Cole *et al.*, 2000; Thorneloe *et al.*, 2002). PKC activates the pancreatic isoform (Kir6.2/SUR1) and striated muscular isoform (Kir6.2/SUR2A) of  $K_{ATP}$  channels (Light *et al.*, 2002). Expressed with SUR2B, the Kir6.2 channel is activated by PKC or shows no response, depending on recording conditions (Thorneloe *et al.*, 2002; Quinn *et al.*, 2003). A critical PKC phosphorylation site (Thr180) has been found in Kir6.2 mediating the fast channel activation (Light *et al.*, 2002). It is unclear however whether a corresponding site in Kir6.1 (Thr190) plays a similar role, as mutation of the residue leads to a non-functional channel. PKC also causes the Kir6.2/SUR1 and Kir6.2/SUR2A channel internalization. Since the effect of PKC on the Kir6.1/SUR2B (inhibition) is clearly in contrast to Kir6.2-

containing channels (activation or no effect), the Kir subunit is likely to be targeted by PKC regulation. The differential responses of the Kir6.1 and Kir6.2 to PKC allowed dissection of the critical protein domains using a chimeric strategy. Our studies on the Kir6.1-Kir6.2 chimeras have shown that the proximal N-terminus and distal C-terminus of Kir6.1 are critical for the PKC-dependent channel inhibition.

#### **12.4.2. The N-terminus might be involved in channel gating**

Interestingly only 7 residues are different between Kir6.1 and Kir6.2 proximal N-termini, none of which can be phosphorylated. The lack of PKC phosphorylation sites in the protein domain suggests that it is likely to be involved in the channel gating or that it interacts with other domains to confer PKC sensitivity. Previous studies have shown that the proximal N-terminus is important for Kir6.2 channel gating by multiple channel regulators such as ATP and PIP2 (Cukras *et al.*, 2002; Schulze *et al.*, 2003a). A notable difference between Kir6.1 and Kir6.2 channels is that in the absence of nucleotide the Kir6.2 channels are spontaneously open while the Kir6.1 channels are completely closed (Kondo *et al.*, 1998), a phenomenon that is also explained as fast inactivation of the Kir6.1 channels (Babenko & Bryan, 2001). Using Kir6.1-Kir6.2 chimeras, Kondo *et al.* found that both the N- and C-termini are important for this difference and that 5 residues in proximal N-terminus are largely responsible for the difference (Kondo *et al.*, 1998). The Kir6.2 channels are also activated by intracellular H<sup>+</sup> (Xu *et al.*, 2001a). The pH-dependent channel gating requires both N- and C-termini although there is only one protonation site (His175) located in the C-terminus (Piao *et al.*, 2001; Xu *et al.*, 2001b). The involvement of the proximal N-terminus in modulating channel activity has been

studied with different ligands, indicating that this region is indeed involved in Kir6.2 channel gating rather than ligand binding (Wang *et al.*, 2006; Wang *et al.*, 2007). In addition to Kir6.2, the Kir2.3 channel is gated by acidic pH in an N-terminus dependent manner, where there is no protonation site (Qu *et al.*, 2000). The requirement of N-terminus for the PKC-dependent channel inhibition found in our current studies is thus consistent with these previous reports, suggesting that this protein domain is likely to be involved in interaction with other protein domain(s) in channel gating.

#### **12.4.3. The C-terminus contains a PKC motif with phosphorylation repeats**

The distal C-terminus (residues 346-424) of Kir6.1 is critical for PKC-dependent channel inhibition. This protein domain is a direct target of PKC as multiple PKC phosphorylation sites are found in this narrow region. Our functional analysis and *in vitro* phosphorylation assay indicate that there are at least four phosphorylation sites in this region, i.e., residues 379, 385, 391 and 397. Our patch clamp study suggests Ser354 is another, a result that is not supported by our *in vitro* phosphorylation assay. Therefore it is unclear whether Ser354 can be phosphorylated *in vivo*. Of them Ser397 in the human Kir6.1 has been previously reported to be phosphorylated by an unknown kinase in a global phosphorylation screening study (Olsen *et al.*, 2006). None of the rest has been studied previously. Interestingly, the effects of these residue phosphorylations are additive or cumulative. Mutation of each individual reduces PKC effects by ~20%. Combined mutations of multiple residues have greater effects. Channel inhibition is almost completely abolished with mutations of all five serine residues. Multiple phosphorylation sites often results in sequential phosphorylation, which does not seem to

be the case in the Kir6.1/SUR2B, as our results suggest none of the residues shows a dominant effect over others. Of the 5 putative phosphorylation sites, 4 serine residues appear in a clear pattern of repeats (SXRR/KXN). To our knowledge, this is the first demonstration regarding such a phosphorylation motif in ion channels.

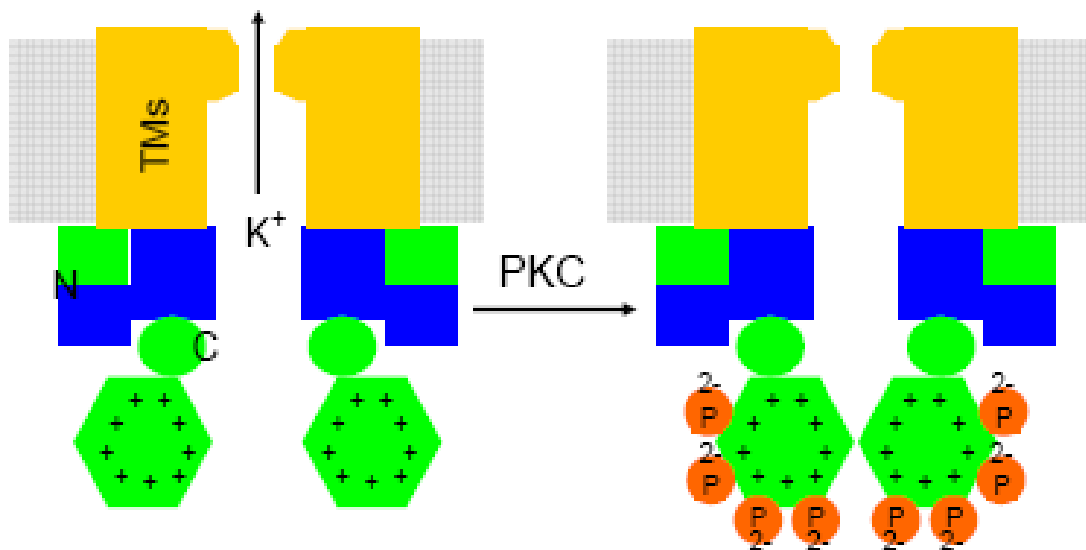
#### **12.4.4. A hypothetic model for gating mechanisms of PKC phosphorylation**

The distal C-termini of inward rectifier K<sup>+</sup> channels play a critical role in channel trafficking and thus control the number of functional channels on the cell membrane (Ma *et al.*, 2002; Yoo *et al.*, 2004; Grishin *et al.*, 2006; Pearson *et al.*, 2006). There is an ER retention sequence (RKR, residues 381-383 in Kir6.1 and 369-371 in Kir6.2) (Zerangue *et al.*, 1999), which warrants the Kir6 subunit targeting membrane together with the SUR subunit. A dileucine motif (Leu355, Leu356) in Kir6.2 is involved in channel endocytosis (Hu *et al.*, 2003). Our results indicate that the distal C-terminus of Kir6.1 is also involved in controlling channel activity. In the narrow C-terminal region where last 4 PKC sites are found, there are 9 alkaline residues including the 'RKR' ER retention signal, making this region extremely positively charged. The positive charges appear to favor the open state in the presence of pinacidil or other channel openers. These charges are largely neutralized following phosphorylation of the serine repeats, which may attenuate the channel opening (Fig. 12-7).

#### **12.4.5. The formation of multiple PKC sites may undergo evolutionary selection.**

Why does the PKC phosphorylation motif exist in Kir6.1 but not in Kir6.2? The phosphorylation motif renders 16 phosphorylation sites in a K<sub>ATP</sub> channel with four Kir6.1 subunits. This as well as the cumulative nature of each phosphorylation, may

allow the channel to be elaborately modulated according to the levels of PKC activation. This is consistent with the functional needs of the vascular  $K_{ATP}$  channel targeted by a variety of vasoconstricting hormones and neurotransmitters. The presence of the phosphorylation motif in the Kir6.1 but not in its close relative Kir6.2 suggests that the vascular  $K_{ATP}$  channel may have undergone evolutionary optimization serving for the vascular regulation under various physiological and pathophysiological conditions.



**Figure 12-7. A hypothetical model of PKC inhibition on Kir6.1/SUR2B channel.**

Two subunits of Kir6.1 are shown with SUR2B omitted. The Kir6.1 is shown in an open state representing that channel is pre-activated by pinacidil. The transmembrane segments are shown in yellow. The segments critical for PKC inhibition are shown in green including proximal N-terminus (tangle), the segment of residues 346-363 (circle) and the distal C-terminal segment (residues 363-424, hex-angle). The distal segment contains 9 positive charges; PKC phosphorylation (red) neutralizes the charges and leads to channel closure.

## 13. General Discussion

### 13.1. Comparison of different working patterns in PKA and PKC phosphorylations

The studies in this thesis have demonstrated the modulation of vascular  $K_{ATP}$  channels by two protein kinases that are activated by several vasoregulatory hormones. How do the phosphorylations of the same channel protein lead to completely different consequences in terms of channel activity? Comparison in the working patterns of PKA and PKC phosphorylation may provide some answers to the question.

All PKC sites are located on the Kir6 subunits where ATP binds. It is possible that phosphorylation by PKC alters the channel sensitivity to ATP leading to channel inhibition. The ATP sensitivity of vascular  $K_{ATP}$  channels reported by different groups varies considerably (Quayle *et al.*, 1997). Although the Kir6.1/SUR2B channel in hetero-expression systems (Yamada *et al.*, 1997) shows rather poor sensitivity to ATP as the  $K_{NDP}$  channels found in smooth muscle cells isolated from rat portal veins (Zhang & Bolton, 1995; Zhang & Bolton, 1996), other reports show that vascular  $K_{ATP}$  channels are sensitive to ATP (Quayle *et al.*, 1997). Considering that the recording conditions in different research groups may affect their results, a parallel study on Kir6.1/SUR2B and native  $K_{ATP}$  channels would provide more convincing information. Indeed, Cole *et al.* (Cole *et al.*) have compared ATP sensitivity of the vessel-native  $K_{ATP}$  channels with recombinant Kir6.1/SUR2B channels simultaneously, and found that the native channels are clearly inhibited by  $<1\text{mM}$  ATP while Kir6.1/SUR2B can only be inhibited by  $>5\text{mM}$  ATP. Thereby they concluded that the Kir6.1/SUR2B does not represent all properties of

native  $K_{ATP}$  channels. In our lab, we have observed ATP inhibition of both native channels and recombinant Kir6.1/SUR2B channels (Wang *et al.*, 2003). The discrepancy in ATP sensitivity is not well understood. One possibility is that there are unknown factors missing in expression systems, which may have affected channel sensitivity to ATP. Another possibility is that the  $K_{ATP}$  channels with different phosphorylation states may have different ATP sensitivity through the change of electrostatic charges in local protein domains. The multiple phosphorylation sites identified in the current studies seem to support the second explanation as phosphorylations of some of serine residues may occur with the level of PKC activation that can vary with different physiological status, affecting the ATP sensitivity. Since different cells have different signal transduction cascades, the same receptor in different cell-types can produce different responses (Clark & Baro, 2007). Clearly, further studies are needed to fully understand the channel gating mechanisms resulting from the PKC phosphorylation.

In contrast to PKC, the functional PKA site is located on SUR2B. There is only one in each subunit, although several other serine residues are also important for the PKA-dependent channel activation. How can the phosphorylation of a single residue in the SUR subunit remote from the ion-conductive pore have such a potent effect on the channel activity? As shown in our modeling study, the interdomain movement caused by phosphorylation is dramatic and widespread. The TMD<sub>1</sub> moves toward NBD<sub>2</sub> through ICL<sub>2</sub>, while the NBD<sub>2</sub> also moves toward TMD<sub>1</sub>. In addition, significant conformational changes are seen in  $\alpha 6$  helix and  $\alpha 3$  helix. Even the  $\beta 5$  sheet ( $\beta$  sheets are most stable in the structure) moves toward TMD<sub>1</sub> by  $\sim 3\text{\AA}$ . Together the two domains show  $\sim 10\text{\AA}$

movement following Ser1387 phosphorylation by PKA. Such a large scale of movements can certainly produce the necessary mechanical forces for channel gating, providing plausible explanations why phosphorylation of a single site in auxiliary  $\beta$  subunit is adequate for activation of the Kir6.1/SURB channel that is normally inhibited by phosphorylation of multiple residues in the pore-lining Kir6.1 subunit by PKC. In addition, it is possible that the PKA phosphorylation has cooperative effects on ADP binding through an allosteric mechanism, leading to enhanced channel activity by several fold (>500%, see discussion in chapter 12.4.5).

### **13.2. Unique bi-directional regulation of the vascular $K_{ATP}$ channels**

Although all  $K_{ATP}$  channels are similar in their basic structure, channel modulation by intracellular nucleotides, extracellular messengers and intracellular signal pathways are quite different. Channel regulation by hormones and transmitters is particularly important for vascular  $K_{ATP}$  channels, while the pancreatic and cardiac  $K_{ATP}$  channels are prominently regulated by metabolites. There are reports of Kir6.2/SUR1 (pancreatic) and Kir6.2/SUR2A (cardiac) channel regulation by hormones and associated intracellular signaling pathways; however, the data are contradictory and the pictures of the effects are elusive (Table 13-1,2).



**Table 13-1. Summary of PKA effects on recombinant K<sub>ATP</sub> channels.**

K <sub>ATP</sub> channel	Effects	Phosphorylation sites	References
Kir6.2/SUR1	<b>Activation</b>	Kir6.2 Thr224	Lin, 2000
	<b>Activation</b>	Kir6.2 Ser372	Beguin, 1999
	<b>Inhibition</b>	SUR1 Ser1448	Light, 2002
Kir6.2/SUR2A	<b>Activation</b>	Kir6.2 Thr224	Lin, 2000
Kir6.2ΔC36	<b>Activation</b>	Kir6.2 Thr224	Lin, 2000
Kir6.1/SUR2B	<b>Activation</b>	Kir6.1 Ser385 SUR2B Thr633 & Ser1465	Quinn, 2004
	<b>Activation</b>	SUR2B Ser1387	Shi, 2007b

The effects of Kir6.1/SUR1 channel regulation presented in literature. Both activation and inhibition have been reported. The PKA phosphorylation sites on Kir6.2/SUR1 channel are debatable. PKA only activates Kir6.1/SUR2B channel, consistent with native vascular K<sub>ATP</sub> channel. Concerning Kir6.1/SUR2B channels, a previous report is inconsistent with our results. Possible reasons are discussed in the text in Chapter 10.

**Table 13-2. Summary of PKC effects on recombinant K<sub>ATP</sub> channels.**

K <sub>ATP</sub> channel	Effects	Phosphorylation sites	References
Kir6.2/SUR1	<b>Activation</b>	T180	Light, 2000
Kir6.2/SUR2A	<b>Activation</b>	T180	Light, 2000
Kir6.2ΔC26	<b>Activation</b>	T180	Light, 2000
Kir6.1/SUR2B	<b>Inhibition</b>	NA	Quinn, 2003; Thorneloe, 2002
	<b>Inhibition</b>	Kir6.1 Ser379, Ser385, Ser391 & Ser397	Shi, et al. 2008
Kir6.2/SUR2B	<b>Activation</b>	NA	Thorneloe 2002
	<b>No effects</b> in the presence of pinacidil		Quinn 2003; Data in this thesis.

PKC activates Kir6.2 channels but not in the presence of pinacidil. We observed that Kir6.1/SUR2B channel is inhibited by PKC, consistent with previous observations. We also demonstrate PKC phosphorylation sites in Kir6.1 subunit.

The reported PKA effects on Kir6.1/SUR1 channels found in the pancreas and in brain are contradictory. PKA appears to activate the channels via phosphorylation at Kir6.2\_Thr224 (Lin *et al.*, 2000) or Ser372 (Beguin *et al.*, 1999). A human specific site on SUR1 is also proposed to have baseline phosphorylation (Beguin *et al.*, 1999). Another study however shows the  $K_{ATP}$  channels in the pancreas are likely inhibited by glucagon-like peptide that activates the cAMP-PKA pathway (Light *et al.*, 2002). A phosphorylation site (Ser1448) in SUR1 seems to mediate the inhibitory effects. The inhibition of PKA can turn to activation with different ADP concentrations (Light *et al.*, 2002). While, the discrepancy of the PKA effects on Kir6.2/SUR1 channel is not fully understood, there seems to be PKA-independent channel inhibition mediated by a cAMP-EPAC pathway (Kang *et al.*, 2006), making the situation more complicated. The activation of the cAMP-PKA pathway causes insulin release from the  $\beta$ -cells probably via  $K_{ATP}$ -independent pathways (Miura & Matsui, 2003).

Activation of the  $G_q$  pathway by membrane receptors causes PIP2 degradation and PKC activation. All Kir6.2 channels are activated by PKC (Light *et al.*, 1995; Light *et al.*, 2000). When the channels are pre-activated by pinacidil, PKC does not have further effects probably because the channels have reached saturating activity (Quinn *et al.*, 2003), consistent with the observation in this thesis. On the other hand, activation of  $\alpha$ -adrenergic receptors causes inhibition on cardiac  $K_{ATP}$  channel (Kir6.2/SUR2A) via break-down of PIP2 (Xie *et al.*, 1999; Haruna *et al.*, 2002). Quinn has also proposed that activation of M-receptors leads to inhibition of Kir6.2/SUR2B channel via decrease of PIP2 concentration (Quinn *et al.*, 2003). Therefore, it is debatable whether Kir6.2

channels are activated or inhibited following activation of  $G_q$  signaling system by membrane receptors.

In contrast, vascular  $K_{ATP}$  channel regulation by hormones and intracellular signaling systems is consistent in the literature. Activation of the  $G_s$  pathway clearly leads to channel activation mediated through PKA phosphorylation as shown in the current studies. Activation of the  $G_q$  pathway only results in channel inhibition via PKC phosphorylation, while PIP2 concentration change in the physiological range has little effects on the channel activity (Quinn *et al.*, 2003). The bi-directional effects by protein kinases seem to fulfill the functional needs of the channels in regulating vascular tone regulation under multiple physiological conditions where the vessels are exposed to vasodilating and vasoconstricting hormones.

### **13.3. Functional impacts of the PKA and PKC phosphorylation.**

Although only  $\beta$ -adrenergic receptors have been studied in this thesis, the molecular mechanisms revealed in these studies for PKA activation and PKC inhibition can be applied to explain the action of the vasoactive agents in multiple physiological and pathophysiological situations. I generally refer those factors as to hormones in this thesis. However, the vasoactive agents are clearly from all kinds of resources that we can think of, including circulating hormones, neurotransmitters, secreted from vascular endothelial cells or metabolites released by local tissues.

### **13.3.1. Circulating hormones**

Norepinephrine and epinephrine have bi-directional effects on vascular tone. They activate  $K_{ATP}$  channels through the PKA pathway in the coronary, cerebral and skeletal muscular arteries where  $\beta$ -adrenergic receptors are dominant. The channels are inhibited via the PKC pathway in arteries of skin and gastrointestinal tract through the  $\alpha$ -adrenergic receptors (Bonev & Nelson, 1996). Therefore, the PKA and PKC phosphorylations of vascular  $K_{ATP}$  channels are the likely molecular basis for the vascular effects of epinephrine and norepinephrine. Another two vasoactive hormones, angiotensin II and vasopressin, can constrict arterials partially via inhibition of vascular  $K_{ATP}$  channels in a PKC-dependent manner (Miyoshi & Nakaya, 1991; Kubo *et al.*, 1997; Hayabuchi *et al.*, 2001b; Shi *et al.*, 2007a). In addition, angiotensin II may reduce  $K_{ATP}$  channel activity by inhibition of PKA (Hayabuchi *et al.*, 2001b).

### **13.3.2. Other factors released from nerve terminals**

Calcitonin gene-related peptide (CGRP) and vasoactive intestine polypeptide (VIP) are strong vasodilators found in the sensory nerve terminals in vascular smooth muscles of cerebral arteries (Edvinsson, 2001). They are released when the tissues are subject to noxious stimuli (Quayle *et al.*, 1997). Over-release of the neuropeptides causes over-dilation of cerebral arteries which may be related to headache and migraine (Edvinsson, 2001). The CGRP (Nelson *et al.*, 1990a; Quayle *et al.*, 1994; Wellman *et al.*, 1998) and VIP (Yang *et al.*, 2007) have been shown to activate vascular  $K_{ATP}$  channels via the PKA pathway. Since the PKA phosphorylation of Kir6.1/SUR2B is critically

involved in the pathology, it is possible to develop new therapies by interference with the PKA pathway or phosphorylation process.

#### **13.3.3. Endothelium-derived vasoregulators**

Vascular endothelial cells can release endothelium-dependent relaxation and contraction factors regulating blood flow in local tissues. NO released from the endothelium cells causes vasodilation, where  $K_{ATP}$  channels may be involved. The intracellular pathway for NO-induced  $K_{ATP}$  activation is unclear but possibly via PKA signaling pathway (Murphy & Brayden, 1995; Brayden, 2002). The endothelium-derived hyperpolarization factors are recently found to be epoxyeicosatrienoic acids (Michaelis & Fleming, 2006). The epoxyeicosatrienoic acids have been shown to activate vascular  $K_{ATP}$  channels in mesenteric artery via the PKA pathway (Ye *et al.*, 2005; Ye *et al.*, 2006). Endothelium also releases vasoconstrictors such as endothelin (Marasciulo *et al.*, 2006). Its vasoconstrictive effect is partially caused by inhibition on  $K_{ATP}$  channels via PKC (Miyoshi *et al.*, 1992; Park *et al.*, 2005). Therefore, PKA and PKC phosphorylations of Kir6.1/SUR2B channels are some of the important molecular mechanisms mediating endothelium-mediated regulation of regional blood flows.

#### **13.3.4. Local mediators released from damaged tissues**

The PKA action on vascular  $K_{ATP}$  channels can also explain the channel protective effects on local tissues when they are subjected to metabolic stress such as hypoxia and ischemia. Hypoxia and ischemia are not only metabolic challenges but also lead to release of variable vasodilatory signal molecules including NO,  $K^+$ ,  $H^+$  and adenosine by damaged local tissues (Obata, 2002). The vasodilatory effect through

adenosine is remarkable as blockade of adenosine receptors reduces about 50% of ischemia reperfusion-induced vessel dilation in cerebral arteries (Rudolphi *et al.*, 1992; Nakhostine & Lamontagne, 1993). The adenosine effects are partially mediated via activation of the  $K_{ATP}$  channels, as glibenclamide diminishes significantly the adenosine-induced vasodilation, and adenosine is known to activate the PKA system (Kleppisch & Nelson, 1995). Thus, PKA phosphorylation of the Kir6.1/SUR2B channel appears to underlie the adenosine-induced vasodilation.

#### **13.3.5. Channel regulation by anesthetics.**

In addition to endogenous vasoregulators, several clinically used anesthetics also act on the  $K_{ATP}$  channels. For example, isoflurane seems to activate vascular  $K_{ATP}$  channels via PKA (Tanaka *et al.*, 2007). This effect depends on SUR2B subunit (Fujita *et al.*, 2006), consistent with our current findings that PKA sites are located on SUR2B.

#### **13.4. The vascular $K_{ATP}$ channel as a key integration molecule in vascular tone regulation**

Intracellular ATP, ADP, pH and phospholipids modulate activity of  $K_{ATP}$  channels. Such modulations allow  $K_{ATP}$  channels to function as a linker between the change of the intracellular metabolic states and membrane potentials (Aguilar-Bryan & Bryan, 1999). Intracellular and extracellular pH levels decrease in local tissues seen in heavy exercise, fasting, diabetes and in various types of metabolic stress including hypercapnia, hypoxia and ischemia. Previous studies in our laboratory have shown that pancreatic and cardiac  $K_{ATP}$  channels are strongly activated by hypercapnia and

intracellular acidosis (Xu *et al.*, 2001a; Wu *et al.*, 2002b; Li *et al.*, 2005). Thus, pH is another potent regulator of  $K_{ATP}$  channels in addition to ATP, ADP and phospholipids. The modulations of  $K_{ATP}$  channel activity by protons, ATP and ADP are via direct binding on Kir6.x or SURx as shown in numerous previous studies (Matsuo *et al.*, 2000; Xu *et al.*, 2001a; Dabrowski *et al.*, 2004). The modulation of the metabolites leads to the change in channel activity, allowing  $K_{ATP}$  channels to sense and integrate metabolic signals under different physiological conditions. The capacity of vascular  $K_{ATP}$  channels to detect the intracellular ATP/ADP ratio (Ashcroft & Gribble, 1998) and pH changes (Wang *et al.*, 2003) allow autoregulation of regional blood flows according to activity and energy consumption in local tissues.

Experimental data in this thesis suggest that channel regulation by protein kinases is another important regulatory mechanism. Such a regulatory mechanism enables vascular  $K_{ATP}$  channels to control vessel diameters by multiple extracellular vasoactive molecules (Fig. 13-1). Some of the vasoactive substances are released during hypoxic ischemia, leading to stronger vasodilation and a greater increase in local blood flow than metabolic regulation alone.

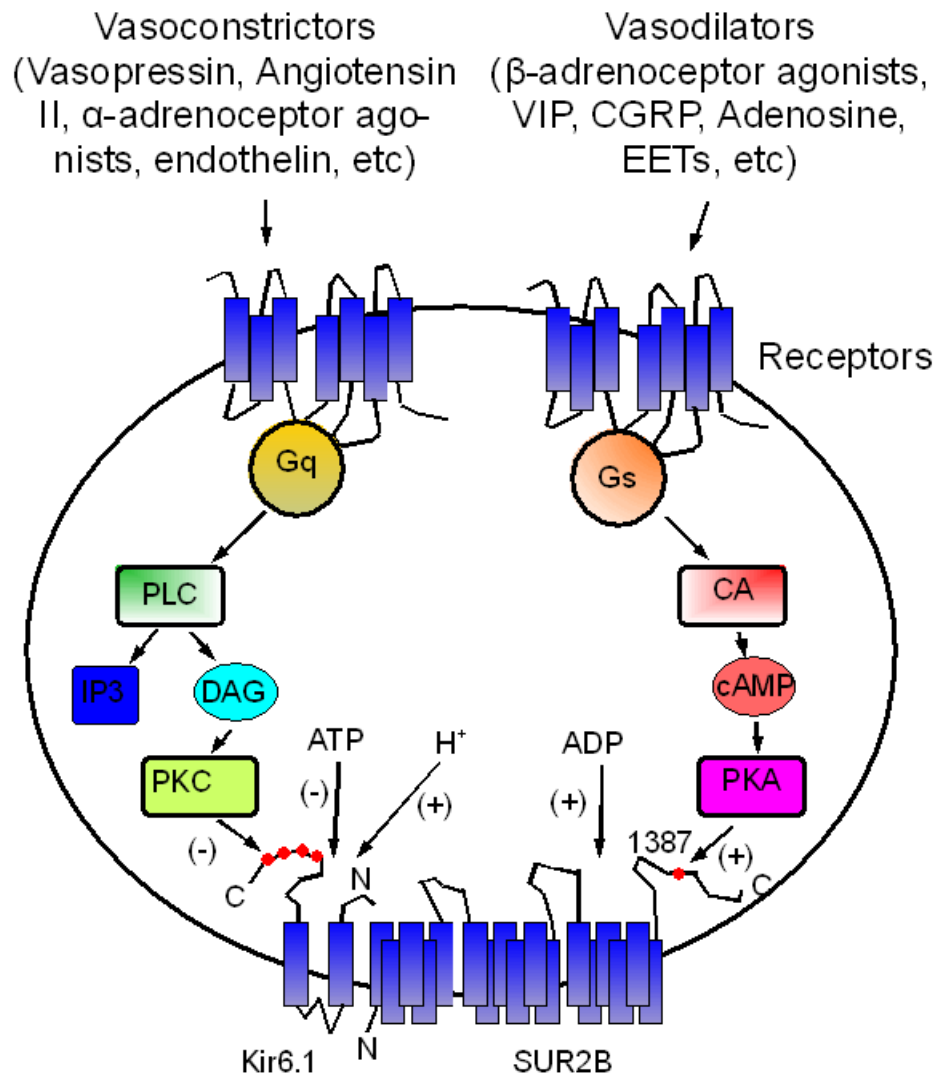


Figure 13-1. Regulation of vascular  $K_{ATP}$  channels by metabolites and vasoactive hormones.



Channel regulation by vasoconstricting hormones is also strong and may be predominant over metabolic regulation in some situations. Hypertensive circulating hormones such as adrenaline, vasopressin and angiotensin II dictate blood supply to local tissues according to systemic needs by shutting off  $K_{ATP}$  channel activity through activation of PKC, even though the cellular metabolic activity may demand more blood in local tissues. Taken together, the vascular  $K_{ATP}$  channels integrate signals of the complex vasoregulation network into seemingly well-organized regulation of VSM contractility. Channel modulation by different kinases can maintain the systemic blood pressure. On the other hand, blood supply can be dynamically regulated according to the functional needs of the tissues via metabolites, local vasoactive mediators, circulating hormones and neurotransmitters under various physiological conditions.

The capability of  $K_{ATP}$  channels to regulate blood flow may play a role in pathological conditions. For example, although appropriate release of CGRP causes dilation of cerebral arteries that would increase blood flow according to nerve activity, strong activation of  $K_{ATP}$  channels by CGRP leads to over-dilation of the cerebral arteries causing primary headache (Edvinsson, 2001). A widespread over-activation of  $K_{ATP}$  channels in a large number of arteries may produce systemic hypotension seen in sepsis (Landry & Oliver, 1992).

The capability of  $K_{ATP}$  channels in vascular tone regulation suggests potential therapeutics for the control of abnormal vascular contractility. Channel openers have been used in treatment of hypertension and vasospasm (Edwards & Weston, 1993). The channel inhibitor glibenclamide may help to maintain blood pressures in sepsis (Landry

& Oliver, 1992). All of these rely on the understanding how the  $K_{ATP}$  channels regulate vascular tone and of the molecular mechanisms that regulate  $K_{ATP}$  channel activity. In this regard, this thesis provides information about the channel regulation at molecular level, which may eventually help develop therapeutic strategies.

In conclusion, vascular  $K_{ATP}$  channels are activated and inhibited by PKA and PKC signaling systems, respectively. These effects rely on intrinsic properties of the channel protein that are present in Kir6.1 and SUR2B but not in their close relatives. By targeting on these intrinsic molecular and biophysical properties, vasodilators and vasoconstrictors can modulate the channel activity as well as vascular tones. The understanding of these intrinsic molecular and biophysical properties therefore provides information of cardiovascular physiology and helps the design of effective therapeutical approaches for cardiovascular diseases.

## 14. Reference List

- Aguilar-Bryan L & Bryan J. (1999). Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* **20**, 101-135.
- Aguilar-Bryan L, Clement JP, Gonzalez G, Kunjilwar K, Babenko A & Bryan J. (1998). Toward understanding the assembly and structure of K<sub>ATP</sub> channels. *Physiol Rev* **78**, 227-245.
- Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd AE, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J & Nelson DA. (1995). Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**, 423-426.
- Akatsuka Y, Egashira K, Katsuda Y, Narishige T, Ueno H, Shimokawa H & Takeshita A. (1994). ATP sensitive potassium channels are involved in adenosine A2 receptor mediated coronary vasodilatation in the dog. *Cardiovasc Res* **28**, 906-911.
- Amoroso S, Schmid-Antomarchi H, Fosset M & Lazdunski M. (1990). Glucose, sulfonylureas, and neurotransmitter release: role of ATP-sensitive K<sup>+</sup> channels. *Science* **247**, 852-854.
- Andersson R. (1973). Cyclic AMP as a mediator of the relaxing action of papaverine, nitroglycerine, diazoxide and hydralazine in intestinal and vascular smooth muscle. *Acta Pharmacol Toxicol (Copenh)* **32**, 321-336.
- Antcliff JF, Haider S, Proks P, Sansom MSP & Ashcroft FM. (2005). Functional analysis of a structural model of the ATP-binding site of the K<sub>ATP</sub> channel Kir6.2 subunit. *Embo J* **24**, 229-239.
- Ashcroft FM. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Annu Rev Neurosci* **11**, 97-118.
- Ashcroft FM & Gribble FM. (1998). Correlating structure and function in ATP-sensitive K<sup>+</sup> channels. *Trends Neurosci* **21**, 288-294.
- Ashcroft FM & Gribble FM. (2000). Tissue-specific effects of sulfonylureas: lessons from studies of cloned K<sub>ATP</sub> channels. *J Diabetes Complications* **14**, 192-196.
- Ashcroft FM, Harrison DE & Ashcroft SJ. (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* **312**, 446-448.

- Ashford ML, Sturgess NC, Trout NJ, Gardner NJ & Hales CN. (1988). Adenosine-5'-triphosphate-sensitive ion channels in neonatal rat cultured central neurones. *Pflugers Arch* **412**, 297-304.
- Babenko AP & Bryan J. (2001). A conserved inhibitory and differential stimulatory action of nucleotides on K(IR)6.0/SUR complexes is essential for excitation-metabolism coupling by K<sub>ATP</sub> channels. *J Biol Chem* **276**, 49083-49092.
- Babenko AP & Bryan J. (2003). Sur domains that associate with and gate K<sub>ATP</sub> pores define a novel gatekeeper. *J Biol Chem* **278**, 41577-41580.
- Babenko AP, Gonzalez G & Bryan J. (2000a). Pharmacology-topology of sulfonylurea receptors. Separate domains of the regulatory subunits of K<sub>ATP</sub> channel isoforms are required for selective interaction with K<sup>+</sup> channel openers. *J Biol Chem* **275**, 717-720.
- Babenko AP, Gonzalez GC & Bryan J. (2000b). Hetero-concatemeric KIR6.X4/SUR14 channels display distinct conductivities but uniform ATP inhibition. *J Biol Chem* **275**, 31563-31566.
- Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg JP & Fakler B. (1998). PIP<sub>2</sub> and PIP as determinants for ATP inhibition of K<sub>ATP</sub> channels. *Science* **282**, 1141-1144.
- Beech DJ, Muraki K & Flemming R. (2004). Non-selective cationic channels of smooth muscle and the mammalian homologues of Drosophila TRP. *J Physiol* **559**, 685-706.
- Beech DJ, Zhang H, Nakao K & Bolton TB. (1993). K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells. *Br J Pharmacol* **110**, 573-582.
- Beguin P, Nagashima K, Nishimura M, Gono T & Seino S. (1999). PKA-mediated phosphorylation of the human K<sub>ATP</sub> channel: separate roles of Kir6.2 and SUR1 subunit phosphorylation. *Embo J* **18**, 4722-4732.
- Belloni FL & Hintze TH. (1991). Glibenclamide attenuates adenosine-induced bradycardia and coronary vasodilatation. *Am J Physiol* **261**, H720-727.
- Bernardi H, De Weille JR, Epelbaum J, Mourre C, Amoroso S, Slama A, Fosset M & Lazdunski M. (1993). ATP-modulated K<sup>+</sup> channels sensitive to antidiabetic sulfonylureas are present in adenohypophysis and are involved in growth hormone release. *Proc Natl Acad Sci U S A* **90**, 1340-1344.

- Bienengraeber M, Alekseev AE, Abraham MR, Carrasco AJ, Moreau C, Vivaudou M, Dzeja PP & Terzic A. (2000). ATPase activity of the sulfonylurea receptor: a catalytic function for the K<sub>ATP</sub> channel complex. *FASEB J* **14**, 1943-1952.
- Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S & Brunak S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**, 1633-1649.
- Bonev AD & Nelson MT. (1996). Vasoconstrictors inhibit ATP-sensitive K<sup>+</sup> channels in arterial smooth muscle through protein kinase C. *J Gen Physiol* **108**, 315-323.
- Brayden JE. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am J Physiol* **259**, H668-673.
- Brayden JE. (2002). Functional roles of K<sub>ATP</sub> channels in vascular smooth muscle. *Clin Exp Pharmacol Physiol* **29**, 312-316.
- Bryan J & Aguilar-Bryan L. (1999). Sulfonylurea receptors: ABC transporters that regulate ATP-sensitive K<sup>+</sup> channels. *Biochim Biophys Acta* **1461**, 285-303.
- Campbell JD, Proks P, Lippiat JD, Sansom MSP & Ashcroft FM. (2004). Identification of a functionally important negatively charged residue within the second catalytic site of the SUR1 nucleotide-binding domains. *Diabetes* **53 Suppl 3**, S123-127.
- Campbell JD, Sansom MSP & Ashcroft FM. (2003). Potassium channel regulation. *EMBO Rep* **4**, 1038-1042.
- Chan KW, Zhang H & Logothetis DE. (2003). N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits. *Embo J* **22**, 3833-3843.
- Chang G. (2003). Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *J Mol Biol* **330**, 419-430.
- Chang HY. (1997). The involvement of ATP-sensitive potassium channels in beta 2-adrenoceptor agonist-induced vasodilatation on rat diaphragmatic microcirculation. *Br J Pharmacol* **121**, 1024-1030.
- Chen X, Weber IT & Harrison RW. (2004). Molecular dynamics simulations of 14 HIV protease mutants in complexes with indinavir. *J Mol Model* **10**, 373-381.
- Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D & Kobilka BK. (1999). Targeted disruption of the beta2 adrenergic receptor gene. *J Biol Chem* **274**, 16694-16700.

- Chutkow WA, Samuel V, Hansen PA, Pu J, Valdivia CR, Makielski JC & Burant CF. (2001). Disruption of Sur2-containing K<sub>ATP</sub> channels enhances insulin-stimulated glucose uptake in skeletal muscle. *Proc Natl Acad Sci U S A* **98**, 11760-11764.
- Clark MC & Baro DJ. (2007). Arthropod D2 receptors positively couple with cAMP through the Gi/o protein family. *Comp Biochem Physiol B Biochem Mol Biol* **146**, 9-19.
- Clement JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L & Bryan J. (1997). Association and stoichiometry of K<sub>ATP</sub> channel subunits. *Neuron* **18**, 827-838.
- Cole WC & Clement-Chomienne O. (2003). ATP-sensitive K<sup>+</sup> channels of vascular smooth muscle cells. *J Cardiovasc Electrophysiol* **14**, 94-103.
- Cole WC, Malcolm T, Walsh MP & Light PE. (2000). Inhibition by protein kinase C of the K(NDP) subtype of vascular smooth muscle ATP-sensitive potassium channel. *Circ Res* **87**, 112-117.
- Cook DL & Hales CN. (1984). Intracellular ATP directly blocks K<sup>+</sup> channels in pancreatic B-cells. *Nature* **311**, 271-273.
- Cui N, Wu J, Xu H, Wang R, Rojas A, Piao H, Mao J, Abdulkadir L, Li L & Jiang C. (2003). A threonine residue (Thr71) at the intracellular end of the M1 helix plays a critical role in the gating of Kir6.2 channels by intracellular ATP and protons. *J Membr Biol* **192**, 111-122.
- Cui Y, Gliblin JP, Clapp LH & Tinker A. (2001). A mechanism for ATP-sensitive potassium channel diversity: Functional coassembly of two pore-forming subunits. *Proc Natl Acad Sci U S A* **98**, 729-734.
- Cui Y, Tran S, Tinker A & Clapp LH. (2002). The molecular composition of K<sub>ATP</sub> channels in human pulmonary artery smooth muscle cells and their modulation by growth. *Am J Respir Cell Mol Biol* **26**, 135-143.
- Cukras CA, Jeliaskova I & Nichols CG. (2002). The role of NH2-terminal positive charges in the activity of inward rectifier K<sub>ATP</sub> channels. *J Gen Physiol* **120**, 437-446.
- Daaka Y, Luttrell LM & Lefkowitz RJ. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**, 88-91.

- Dabrowski M, Tarasov A & Ashcroft FM. (2004). Mapping the architecture of the ATP-binding site of the K<sub>ATP</sub> channel subunit Kir6.2. *J Physiol* **557**, 347-354.
- Dawson RJ & Locher KP. (2006). Structure of a bacterial multidrug ABC transporter. *Nature* **443**, 180-185.
- Dishy V, Landau R, Sofowora GG, Xie HG, Smiley RM, Kim RB, Byrne DW, Wood AJ & Stein CM. (2004). Beta2-adrenoceptor Thr164Ile polymorphism is associated with markedly decreased vasodilator and increased vasoconstrictor sensitivity in vivo. *Pharmacogenetics* **14**, 517-522.
- Doyle DA, Morais Cabral J, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT & MacKinnon R. (1998). The structure of the potassium channel: molecular basis of K<sup>+</sup> conduction and selectivity. *Science* **280**, 69-77.
- Drain P, Li L & Wang J. (1998). K<sub>ATP</sub> channel inhibition by ATP requires distinct functional domains of the cytoplasmic C terminus of the pore-forming subunit. *Proc Natl Acad Sci U S A* **95**, 13953-13958.
- Edvinsson L. (2001). Calcitonin gene-related peptide (CGRP) and the pathophysiology of headache: therapeutic implications. *CNS Drugs* **15**, 745-753.
- Edwards G & Weston AH. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu Rev Pharmacol Toxicol* **33**, 597-637.
- Fajmut A, Brumen M & Schuster S. (2005). Theoretical model of the interactions between Ca<sup>2+</sup>, calmodulin and myosin light chain kinase. *FEBS Lett* **579**, 4361-4366.
- Fan Z & Makielski JC. (1997). Anionic phospholipids activate ATP-sensitive potassium channels. *J Biol Chem* **272**, 5388-5395.
- Fujita H, Ogura T, Tamagawa M, Uemura H, Sato T, Ishida A, Imamaki M, Kimura F, Miyazaki M & Nakaya H. (2006). A key role for the subunit SUR2B in the preferential activation of vascular K<sub>ATP</sub> channels by isoflurane. *Br J Pharmacol* **149**, 573-580.
- Ganitkevich V & Isenberg G. (1990). Contribution of two types of calcium channels to membrane conductance of single myocytes from guinea-pig coronary artery. *J Physiol* **426**, 19-42.
- Gao M, Xue H, Wang Y & Wang H. (2005). Iptakalim, opener of K<sub>ATP</sub>, reverses the enhanced expression of genes encoding K<sub>ATP</sub> subunits in spontaneously hypertensive rats. *Life Sci* **77**, 2743-2751.

- Garland JG & McPherson GA. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br J Pharmacol* **105**, 429-435.
- Gaudet R & Wiley DC. (2001). Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. *Embo J* **20**, 4964-4972.
- Ge D, Huang J, He J, Li B, Duan X, Chen R & Gu D. (2005). beta2-Adrenergic receptor gene variations associated with stage-2 hypertension in northern Han Chinese. *Ann Hum Genet* **69**, 36-44.
- Giblin JP, Cui Y, Clapp LH & Tinker A. (2002). Assembly limits the pharmacological complexity of ATP-sensitive potassium channels. *J Biol Chem* **277**, 13717-13723.
- Giblin JP, Leaney JL & Tinker A. (1999). The molecular assembly of ATP-sensitive potassium channels. Determinants on the pore forming subunit. *J Biol Chem* **274**, 22652-22659.
- Gloyn AL, Hashim Y, Ashcroft SJ, Ashfield R, Wiltshire S & Turner RC. (2001). Association studies of variants in promoter and coding regions of beta-cell ATP-sensitive K-channel genes SUR1 and Kir6.2 with Type 2 diabetes mellitus (UKPDS 53). *Diabet Med* **18**, 206-212.
- Goto K, Fujii K & Abe I. (2001). Impaired beta-adrenergic hyperpolarization in arteries from prehypertensive spontaneously hypertensive rats. *Hypertension* **37**, 609-613.
- Gribble FM, Tucker SJ & Ashcroft FM. (1997). The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *Embo J* **16**, 1145-1152.
- Grishin A, Li H, Levitan ES & Zaks-Makhina E. (2006). Identification of gamma-aminobutyric acid receptor-interacting factor 1 (TRAK2) as a trafficking factor for the K<sup>+</sup> channel Kir2.1. *J Biol Chem* **281**, 30104-30111.
- Guimaraes S & Moura D. (2001). Vascular adrenoceptors: an update. *Pharmacol Rev* **53**, 319-356.
- Haider S, Tarasov AI, Craig TJ, Sansom MSP & Ashcroft FM. (2007). Identification of the PIP2-binding site on Kir6.2 by molecular modelling and functional analysis. *Embo J* **26**, 3749-3759.



- Hambrock A, Preisig-Muller R, Russ U, Piehl A, Hanley PJ, Ray J, Daut J, Quast U & Derst C. (2002). Four novel splice variants of sulfonylurea receptor 1. *Am J Physiol Cell Physiol* **283**, C587-598.
- Harrison RW. (2003). Amortized fast multipole algorithm for molecular modeling. In *Proceedings of the International Conference on Computer Science and its Applications*, pp. 77–81. Gatton TM (eds) Dey PP, Amin MN.
- Harrison RW & Weber IT. (1994). Molecular dynamics simulations of HIV-1 protease with peptide substrate. *Protein Eng* **7**, 1353-1363.
- Haruna T, Yoshida H, Nakamura TY, Xie LH, Otani H, Ninomiya T, Takano M, Coetzee WA & Horie M. (2002). Alpha1-adrenoceptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate inhibits pinacidil-activated ATP-sensitive  $K^+$  currents in rat ventricular myocytes. *Circ Res* **91**, 232-239.
- Hayabuchi Y, Dart C & Standen NB. (2001a). Evidence for involvement of A-kinase anchoring protein in activation of rat arterial  $K_{ATP}$  channels by protein kinase A. *J Physiol* **536**, 421-427.
- Hayabuchi Y, Davies NW & Standen NB. (2001b). Angiotensin II inhibits rat arterial  $K_{ATP}$  channels by inhibiting steady-state protein kinase A activity and activating protein kinase C. *J Physiol* **530**, 193-205.
- Hein TW, Zhang C, Wang W & Kuo L. (2004). Heterogeneous beta2-adrenoceptor expression and dilation in coronary arterioles across the left ventricular wall. *Circulation* **110**, 2708-2712.
- Higgins CF. (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**, 67-113.
- Hilgemann DW & Ball R. (1996). Regulation of cardiac  $Na^+$ ,  $Ca^{2+}$  exchange and  $K_{ATP}$  potassium channels by PIP2. *Science* **273**, 956-959.
- Hisayama T, Takayanagi I & Okamoto Y. (1990). Ryanodine reveals multiple contractile and relaxant mechanisms in vascular smooth muscle: simultaneous measurements of mechanical activity and of cytoplasmic free  $Ca^{2+}$  level with fura-2. *Br J Pharmacol* **100**, 677-684.
- Hollenstein K, Frei DC & Locher KP. (2007). Structure of an ABC transporter in complex with its binding protein. *Nature* **446**, 213-216.
- Hu K, Huang CS, Jan YN & Jan LY. (2003). ATP-sensitive potassium channel traffic regulation by adenosine and protein kinase C. *Neuron* **38**, 417-432.

- Huang HD, Lee TY, Tzeng SW & Horng JT. (2005). KinasePhos: a web tool for identifying protein kinase-specific phosphorylation sites. *Nucleic Acids Res* **33**, W226-229.
- Huopio H, Reimann F, Ashfield R, Komulainen J, Lenko HL, Rahier J, Vauhkonen I, Kere J, Laakso M, Ashcroft F & Otonkoski T. (2000). Dominantly inherited hyperinsulinism caused by a mutation in the sulfonylurea receptor type 1. *J Clin Invest* **106**, 897-906.
- Inagaki N, Gono T, Clement JP, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S & Bryan J. (1995a). Reconstitution of  $IK_{ATP}$ : an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**, 1166-1170.
- Inagaki N, Gono T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J & Seino S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive  $K^+$  channels. *Neuron* **16**, 1011-1017.
- Inagaki N, Gono T & Seino S. (1997). Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive  $K^+$  channel. *FEBS Lett* **409**, 232-236.
- Inagaki N, Tsuura Y, Namba N, Masuda K, Gono T, Horie M, Seino Y, Mizuta M & Seino S. (1995b). Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. *J Biol Chem* **270**, 5691-5694.
- Insuk SO, Chae MR, Choi JW, Yang DK, Sim JH & Lee SW. (2003). Molecular basis and characteristics of  $K_{ATP}$  channel in human corporal smooth muscle cells. *Int J Impot Res* **15**, 258-266.
- Ishibashi Y, Duncker DJ, Zhang J & Bache RJ. (1998). ATP-sensitive  $K^+$  channels, adenosine, and nitric oxide-mediated mechanisms account for coronary vasodilation during exercise. *Circ Res* **82**, 346-359.
- Isomoto S, Kondo C, Yamada M, Matsumoto S, Higashiguchi O, Horio Y, Matsuzawa Y & Kurachi Y. (1996). A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive  $K^+$  channel. *J Biol Chem* **271**, 24321-24324.
- Isomoto S, Yamada M, Horio Y & Kurachi Y. (1997). Molecular aspects of ATP-sensitive  $K^+$  channels in the cardiovascular system. *Jpn J Physiol* **47 Suppl 1**, S5-6.

- Jackson WF. (2005). Potassium channels in the peripheral microcirculation. *Microcirculation* **12**, 113-127.
- Jiang C & Haddad GG. (1997). Modulation of K<sup>+</sup> channels by intracellular ATP in human neocortical neurons. *J Neurophysiol* **77**, 93-102.
- Jiang C, Sigworth FJ & Haddad GG. (1994). Oxygen deprivation activates an ATP-inhibitable K<sup>+</sup> channel in substantia nigra neurons. *J Neurosci* **14**, 5590-5602.
- Johnson M. (2006). Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol* **117**, 18-24; quiz 25.
- Kang G, Chepurny OG, Malester B, Rindler MJ, Rehmann H, Bos JL, Schwede F, Coetzee WA & Holz GG. (2006). cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic beta cells and rat INS-1 cells. *J Physiol* **573**, 595-609.
- Kasemsri T & Armstead WM. (1997). Endothelin impairs ATP-sensitive K<sup>+</sup> channel function after brain injury. *Am J Physiol* **273**, H2639-2647.
- Kawabata H, Ryomoto T & Ishikawa K. (2001). Role of cardiac ATP-sensitive K<sup>+</sup> channels induced by angiotensin II type 1 receptor antagonist on metabolism, contraction and relaxation in ischemia-reperfused rabbit heart. *Jpn Circ J* **65**, 451-456.
- Kleppisch T & Nelson MT. (1995). Adenosine activates ATP-sensitive potassium channels in arterial myocytes via A2 receptors and cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **92**, 12441-12445.
- Knot HJ, Standen NB & Nelson MT. (1998). Ryanodine receptors regulate arterial diameter and wall [Ca<sup>2+</sup>] in cerebral arteries of rat via Ca<sup>2+</sup>-dependent K<sup>+</sup> channels. *J Physiol* **508** ( Pt 1), 211-221.
- Kondo C, Repunte VP, Satoh E, Yamada M, Horio Y, Matsuzawa Y, Pott L & Kurachi Y. (1998). Chimeras of Kir6.1 and Kir6.2 reveal structural elements involved in spontaneous opening and unitary conductance of the ATP-sensitive K<sup>+</sup> channels. *Receptors Channels* **6**, 129-140.
- Kubo M, Quayle JM & Standen NB. (1997). Angiotensin II inhibition of ATP-sensitive K<sup>+</sup> currents in rat arterial smooth muscle cells through protein kinase C. *J Physiol* **503** ( Pt 3), 489-496.

- Kuo A, Gulbis JM, Antcliff JF, Rahman T, Lowe ED, Zimmer J, Cuthbertson J, Ashcroft FM, Ezaki T & Doyle DA. (2003). Crystal structure of the potassium channel KirBac1.1 in the closed state. *Science* **300**, 1922-1926.
- Landry DW & Oliver JA. (1992). The ATP-sensitive K<sup>+</sup> channel mediates hypotension in endotoxemia and hypoxic lactic acidosis in dog. *J Clin Invest* **89**, 2071-2074.
- Li L, Shi Y, Wang X, Shi W & Jiang C. (2005). Single nucleotide polymorphisms in K<sub>ATP</sub> channels: muscular impact on type 2 diabetes. *Diabetes* **54**, 1592-1597.
- Li L, Wu J & Jiang C. (2003). Differential expression of Kir6.1 and SUR2B mRNAs in the vasculature of various tissues in rats. *J Membr Biol* **196**, 61-69.
- Light PE, Allen BG, Walsh MP & French RJ. (1995). Regulation of adenosine triphosphate-sensitive potassium channels from rabbit ventricular myocytes by protein kinase C and type 2A protein phosphatase. *Biochemistry* **34**, 7252-7257.
- Light PE, Bladen C, Winkfein RJ, Walsh MP & French RJ. (2000). Molecular basis of protein kinase C-induced activation of ATP-sensitive potassium channels. *Proc Natl Acad Sci U S A* **97**, 9058-9063.
- Light PE, Manning Fox JE, Riedel MJ & Wheeler MB. (2002). Glucagon-like peptide-1 inhibits pancreatic ATP-sensitive potassium channels via a protein kinase A- and ADP-dependent mechanism. *Mol Endocrinol* **16**, 2135-2144.
- Lin YF, Jan YN & Jan LY. (2000). Regulation of ATP-sensitive potassium channel function by protein kinase A-mediated phosphorylation in transfected HEK293 cells. *Embo J* **19**, 942-955.
- Locher KP, Lee AT & Rees DC. (2002). The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science* **296**, 1091-1098.
- Longmore J, Bray KM & Weston AH. (1991). The contribution of Rb-permeable potassium channels to the relaxant and membrane hyperpolarizing actions of cromakalim, RP49356 and diazoxide in bovine tracheal smooth muscle. *Br J Pharmacol* **102**, 979-985.
- Loutzenhiser RD & Parker MJ. (1994). Hypoxia inhibits myogenic reactivity of renal afferent arterioles by activating ATP-sensitive K<sup>+</sup> channels. *Circ Res* **74**, 861-869.
- Lu T, Hoshi T, Weintraub NL, Spector AA & Lee HC. (2001). Activation of ATP-sensitive K<sup>+</sup> channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol* **537**, 811-827.

- Ma D, Zerangue N, Raab-Graham K, Fried SR, Jan YN & Jan LY. (2002). Diverse trafficking patterns due to multiple traffic motifs in G protein-activated inwardly rectifying potassium channels from brain and heart. *Neuron* **33**, 715-729.
- Manning Fox JE, Nichols CG & Light PE. (2004). Activation of adenosine triphosphate-sensitive potassium channels by acyl coenzyme A esters involves multiple phosphatidylinositol 4,5-bisphosphate-interacting residues. *Mol Endocrinol* **18**, 679-686.
- Mao J, Wang X, Chen F, Wang R, Rojas A, Shi Y, Piao H & Jiang C. (2004). Molecular basis for the inhibition of G protein-coupled inward rectifier K<sup>+</sup> channels by protein kinase C. *Proc Natl Acad Sci U S A* **101**, 1087-1092.
- Marasciulo FL, Montagnani M & Potenza MA. (2006). Endothelin-1: the yin and yang on vascular function. *Curr Med Chem* **13**, 1655-1665.
- Masia R, Enkvetchakul D & Nichols CG. (2005). Differential nucleotide regulation of K<sub>ATP</sub> channels by SUR1 and SUR2A. *J Mol Cell Cardiol* **39**, 491-501.
- Matsuo M, Dabrowski M, Ueda K & Ashcroft FM. (2002). Mutations in the linker domain of NBD<sub>2</sub> of SUR inhibit transduction but not nucleotide binding. *Embo J* **21**, 4250-4258.
- Matsuo M, Kimura Y & Ueda K. (2005). K<sub>ATP</sub> channel interaction with adenine nucleotides. *J Mol Cell Cardiol* **38**, 907-916.
- Matsuo M, Kioka N, Amachi T & Ueda K. (1999). ATP binding properties of the nucleotide-binding folds of SUR1. *J Biol Chem* **274**, 37479-37482.
- Matsuo M, Tanabe K, Kioka N, Amachi T & Ueda K. (2000). Different binding properties and affinities for ATP and ADP among sulfonylurea receptor subtypes, SUR1, SUR2A, and SUR2B. *J Biol Chem* **275**, 28757-28763.
- Matsuoka T, Matsushita K, Katayama Y, Fujita A, Inageda K, Tanemoto M, Inanobe A, Yamashita S, Matsuzawa Y & Kurachi Y. (2000). C-terminal tails of sulfonylurea receptors control ADP-induced activation and diazoxide modulation of ATP-sensitive K<sup>+</sup> channels. *Circ Res* **87**, 873-880.
- Matsushita K, Kinoshita K, Matsuoka T, Fujita A, Fujikado T, Tano Y, Nakamura H & Kurachi Y. (2002). Intramolecular interaction of SUR2 subtypes for intracellular ADP-Induced differential control of K<sub>ATP</sub> channels. *Circ Res* **90**, 554-561.

- Michaelis UR & Fleming I. (2006). From endothelium-derived hyperpolarizing factor (EDHF) to angiogenesis: Epoxyeicosatrienoic acids (EETs) and cell signaling. *Pharmacol Ther* **111**, 584-595.
- Mikhailov MV, Campbell JD, de Wet H, Shimomura K, Zadek B, Collins RF, Sansom MSP, Ford RC & Ashcroft FM. (2005). 3-D structural and functional characterization of the purified K<sub>ATP</sub> channel complex Kir6.2-SUR1. *Embo J* **24**, 4166-4175.
- Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A, Gono T, Iwanaga T, Miyazaki J & Seino S. (1998). Defective insulin secretion and enhanced insulin action in K<sub>ATP</sub> channel-deficient mice. *Proc Natl Acad Sci U S A* **95**, 10402-10406.
- Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, Yamaguchi K, Koseki H, Iwanaga T, Nakaya H & Seino S. (2002). Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. *Nat Med* **8**, 466-472.
- Ming Z, Parent R & Lavallee M. (1997). Beta 2-adrenergic dilation of resistance coronary vessels involves K<sub>ATP</sub> channels and nitric oxide in conscious dogs. *Circulation* **95**, 1568-1576.
- Missiaen L, Sipma H, Parys JB, De Smet P, Callewaert G, Hill E, McCarthy TV & De Smedt H. (1999). IP<sub>3</sub>-induced Ca<sup>2+</sup> release in A7r5 vascular smooth-muscle cells represents a partial emptying of the stores and not an all-or-none Ca<sup>2+</sup> release of separate quanta. *Pflugers Arch* **437**, 691-694.
- Miura Y & Matsui H. (2003). Glucagon-like peptide-1 induces a cAMP-dependent increase of [Na<sup>+</sup>]<sub>i</sub> associated with insulin secretion in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* **285**, E1001-1009.
- Miyoshi H & Nakaya Y. (1995). Calcitonin gene-related peptide activates the K<sup>+</sup> channels of vascular smooth muscle cells via adenylate cyclase. *Basic Res Cardiol* **90**, 332-336.
- Miyoshi Y & Nakaya Y. (1991). Angiotensin II blocks ATP-sensitive K<sup>+</sup> channels in porcine coronary artery smooth muscle cells. *Biochem Biophys Res Commun* **181**, 700-706.
- Miyoshi Y, Wakatsuki T, Nomura M, Saito K, Nakaya Y & Inoue I. (1992). Endothelin blocks ATP-sensitive K<sup>+</sup> channels and depolarizes smooth muscle cells of porcine coronary artery. *Jpn J Pharmacol* **58 Suppl 2**, 338P.

- Moreau C, Prost A-L, Derand R & Vivaudou M. (2005). SUR, ABC proteins targeted by  $K_{ATP}$  channel openers. *J Mol Cell Cardiol* **38**, 951-963.
- Morrissey A, Rosner E, Lanning J, Parachuru L, Dhar Chowdhury P, Han S, Lopez G, Tong X, Yoshida H, Nakamura TY, Artman M, Giblin JP, Tinker A & Coetzee WA. (2005). Immunolocalization of  $K_{ATP}$  channel subunits in mouse and rat cardiac myocytes and the coronary vasculature. *BMC Physiol* **5**, 1.
- Murphy ME & Brayden JE. (1995). Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J Physiol* **486** ( Pt 1), 47-58.
- Nakhostine N & Lamontagne D. (1993). Adenosine contributes to hypoxia-induced vasodilation through ATP-sensitive  $K^+$  channel activation. *Am J Physiol* **265**, H1289-1293.
- Nelson MT, Huang Y, Brayden JE, Hescheler J & Standen NB. (1990a). Arterial dilations in response to calcitonin gene-related peptide involve activation of  $K^+$  channels. *Nature* **344**, 770-773.
- Nelson MT, Patlak JB, Worley JF & Standen NB. (1990b). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol* **259**, C3-18.
- Nelson MT & Quayle JM. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268**, C799-822.
- Neylon CB, Richards SM, Larsen MA, Agrotis A & Bobik A. (1995). Multiple types of ryanodine receptor/ $Ca^{2+}$  release channels are expressed in vascular smooth muscle. *Biochem Biophys Res Commun* **215**, 814-821.
- Nichols CG. (2006).  $K_{ATP}$  channels as molecular sensors of cellular metabolism. *Nature* **440**, 470-476.
- Nishida M & MacKinnon R. (2002). Structural basis of inward rectification: cytoplasmic pore of the G protein-gated inward rectifier GIRK1 at 1.8 Å resolution. *Cell* **111**, 957-965.
- Noma A. (1983). ATP-regulated  $K^+$  channels in cardiac muscle. *Nature* **305**, 147-148.
- Obata T. (2002). Adenosine production and its interaction with protection of ischemic and reperfusion injury of the myocardium. *Life Sci* **71**, 2083-2103.

- Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P & Mann M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635-648.
- Orito K, Satoh K & Taira N. (1993). Involvement of ATP-sensitive  $K^+$  channels in the sustained coronary vasodilator response to adenosine in dogs. *Eur J Pharmacol* **231**, 183-189.
- Park WS, Ko EA, Han J, Kim N & Earm YE. (2005). Endothelin-1 acts via protein kinase C to block  $K_{ATP}$  channels in rabbit coronary and pulmonary arterial smooth muscle cells. *J Cardiovasc Pharmacol* **45**, 99-108.
- Pearson WL, Skatchkov SN, Eaton MJ & Nichols CG. (2006). C-terminal determinants of Kir4.2 channel expression. *J Membr Biol* **213**, 187-193.
- Pegan S, Arrabit C, Zhou W, Kwiatkowski W, Collins A, Slesinger PA & Choe S. (2005). Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. *Nat Neurosci* **8**, 279-287.
- Piao H, Cui N, Xu H, Mao J, Rojas A, Wang R, Abdulkadir L, Li L, Wu J & Jiang C. (2001). Requirement of multiple protein domains and residues for gating  $K_{ATP}$  channels by intracellular pH. *J Biol Chem* **276**, 36673-36680.
- Pinkett HW, Lee AT, Lum P, Locher KP & Rees DC. (2007). An inward-facing conformation of a putative metal-chelate-type ABC transporter. *Science* **315**, 373-377.
- Ploug KB, Edvinsson L, Olesen J & Jansen-Olesen I. (2006). Pharmacological and molecular comparison of  $K_{ATP}$  channels in rat basilar and middle cerebral arteries. *Eur J Pharmacol* **553**, 254-262.
- Qu Z, Yang Z, Cui N, Zhu G, Liu C, Xu H, Chanchevalap S, Shen W, Wu J, Li Y & Jiang C. (2000). Gating of inward rectifier  $K^+$  channels by proton-mediated interactions of N- and C-terminal domains. *J Biol Chem* **275**, 31573-31580.
- Quayle JM, Bonev AD, Brayden JE & Nelson MT. (1994). Calcitonin gene-related peptide activated ATP-sensitive  $K^+$  currents in rabbit arterial smooth muscle via protein kinase A. *J Physiol* **475**, 9-13.
- Quayle JM, Nelson MT & Standen NB. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev* **77**, 1165-1232.



- Quinn KV, Cui Y, Giblin JP, Clapp LH & Tinker A. (2003). Do anionic phospholipids serve as cofactors or second messengers for the regulation of activity of cloned ATP-sensitive K<sup>+</sup> channels? *Circ Res* **93**, 646-655.
- Quinn KV, Giblin JP & Tinker A. (2004). Multisite phosphorylation mechanism for protein kinase A activation of the smooth muscle ATP-sensitive K<sup>+</sup> channel. *Circ Res* **94**, 1359-1366.
- Randall MD. (1995). The involvement of ATP-sensitive potassium channels and adenosine in the regulation of coronary flow in the isolated perfused rat heart. *Br J Pharmacol* **116**, 3068-3074.
- Randall MD & McCulloch AI. (1995). The involvement of ATP-sensitive potassium channels in beta-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **115**, 607-612.
- Reid JM, Paterson DJ, Ashcroft FM & Bergel DH. (1993). The effect of tolbutamide on cerebral blood flow during hypoxia and hypercapnia in the anaesthetized rat. *Pflugers Arch* **425**, 362-364.
- Reimann F, Ashcroft FM & Gribble FM. (2001). Structural basis for the interference between nicorandil and sulfonylurea action. *Diabetes* **50**, 2253-2259.
- Remillard CV & Yuan JXJ. (2006). TRP channels, CCE, and the pulmonary vascular smooth muscle. *Microcirculation* **13**, 671-692.
- Ren Y, Xu X & Wang X. (2003). Altered mRNA expression of ATP-sensitive and inward rectifier potassium channel subunits in streptozotocin-induced diabetic rat heart and aorta. *J Pharmacol Sci* **93**, 478-483.
- Rhodes HJ & Sutter MC. (1971). The action of diazoxide on isolated vascular smooth muscle electrophysiology and contraction. *Can J Physiol Pharmacol* **49**, 267-287.
- Rojas A, Wu J, Wang R & Jiang C. (2007). Gating of the ATP-sensitive K<sup>+</sup> channel by a pore-lining phenylalanine residue. *Biochim Biophys Acta* **1768**, 39-51.
- Rosenblum WI. (2003). ATP-sensitive potassium channels in the cerebral circulation. *Stroke* **34**, 1547-1552.
- Rudolphi KA, Schubert P, Parkinson FE & Fredholm BB. (1992). Adenosine and brain ischemia. *Cerebrovasc Brain Metab Rev* **4**, 346-369.
- Sakura H, Ammala C, Smith PA, Gribble FM & Ashcroft FM. (1995). Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium

- channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett* **377**, 338-344.
- Sakura H, Trapp S, Liss B & Ashcroft FM. (1999). Altered functional properties of K<sub>ATP</sub> channel conferred by a novel splice variant of SUR1. *J Physiol* **521 Pt 2**, 337-350.
- Sampson LJ, Davies LM, Barrett-Jolley R, Standen NB & Dart C. (2007). Angiotensin II-activated protein kinase C targets caveolae to inhibit aortic ATP-sensitive potassium channels. *Cardiovasc Res* **76**, 61-70.
- Sampson LJ, Hayabuchi Y, Standen NB & Dart C. (2004). Caveolae localize protein kinase A signaling to arterial ATP-sensitive potassium channels. *Circ Res* **95**, 1012-1018.
- Santa N, Kitazono T, Ago T, Ooboshi H, Kamouchi M, Wakisaka M, Ibayashi S & Iida M. (2003). ATP-sensitive potassium channels mediate dilatation of basilar artery in response to intracellular acidification in vivo. *Stroke; a journal of cerebral circulation* **34**, 1276-1280.
- Satoh E, Yamada M, Kondo C, Repunte VP, Horio Y, Iijima T & Kurachi Y. (1998). Intracellular nucleotide-mediated gating of SUR/Kir6.0 complex potassium channels expressed in a mammalian cell line and its modification by pinacidil. *J Physiol* **511 ( Pt 3)**, 663-674.
- Schulze D, Krauter T, Fritzenschaft H, Soom M & Baukrowitz T. (2003a). Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) modulation of ATP and pH sensitivity in Kir channels. A tale of an active and a silent PIP<sub>2</sub> site in the N terminus. *J Biol Chem* **278**, 10500-10505.
- Schulze D, Rapedius M, Krauter T & Baukrowitz T. (2003b). Long-chain acyl-CoA esters and phosphatidylinositol phosphates modulate ATP inhibition of K<sub>ATP</sub> channels by the same mechanism. *J Physiol* **552**, 357-367.
- Schwappach B, Zerangue N, Jan YN & Jan LY. (2000). Molecular basis for K<sub>ATP</sub> assembly: transmembrane interactions mediate association of a K<sup>+</sup> channel with an ABC transporter. *Neuron* **26**, 155-167.
- Seeholzer SH & Wand AJ. (1989). Structural characterization of the interactions between calmodulin and skeletal muscle myosin light chain kinase: effect of peptide (576-594)G binding on the Ca<sup>2+</sup>-binding domains. *Biochemistry* **28**, 4011-4020.
- Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L & Bryan J. (2000). Sur1 knockout mice. A model for K<sub>ATP</sub> channel-independent regulation of insulin secretion. *J Biol Chem* **275**, 9270-9277.

- Seino S. (1999). ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. *Annu Rev Physiol* **61**, 337-362.
- Seino S & Miki T. (2003). Physiological and pathophysiological roles of ATP-sensitive K<sup>+</sup> channels. *Prog Biophys Mol Biol* **81**, 133-176.
- Sheridan BC, McIntyre RC, Jr., Meldrum DR & Fullerton DA. (1997). K<sub>ATP</sub> channels contribute to beta- and adenosine receptor-mediated pulmonary vasorelaxation. *Am J Physiol* **273**, L950-956.
- Shi W, Cui N, Shi Y, Zhang X, Yang Y & Jiang C. (2007a). Arginine vasopressin inhibits Kir6.1/SUR2B channel and constricts the mesenteric artery via V1a receptor and protein kinase C. *Am J Physiol Regul Integr Comp Physiol* **293**, R191-199.
- Shi Y, Wu Z, Cui N, Shi W, Yang Y, Zhang X, Rojas A, Ha BT & Jiang C. (2007b). PKA phosphorylation of SUR2B subunit underscores vascular K<sub>ATP</sub> channel activation by beta-adrenergic receptors. *Am J Physiol Regul Integr Comp Physiol* **293**, R1205-1214.
- Shiota C, Larsson O, Shelton KD, Shiota M, Efanov AM, Hoy M, Lindner J, Kooptiwut S, Juntti-Berggren L, Gromada J, Berggren P-O & Magnuson MA. (2002). Sulfonylurea receptor type 1 knock-out mice have intact feeding-stimulated insulin secretion despite marked impairment in their response to glucose. *J Biol Chem* **277**, 37176-37183.
- Shyng S, Ferrigni T & Nichols CG. (1997). Regulation of K<sub>ATP</sub> channel activity by diazoxide and MgADP. Distinct functions of the two nucleotide binding folds of the sulfonylurea receptor. *J Gen Physiol* **110**, 643-654.
- Shyng S & Nichols CG. (1997). Octameric stoichiometry of the K<sub>ATP</sub> channel complex. *J Gen Physiol* **110**, 655-664.
- Shyng SL, Barbieri A, Gumusboga A, Cukras C, Pike L, Davis JN, Stahl PD & Nichols CG. (2000a). Modulation of nucleotide sensitivity of ATP-sensitive potassium channels by phosphatidylinositol-4-phosphate 5-kinase. *Proc Natl Acad Sci U S A* **97**, 937-941.
- Shyng SL, Cukras CA, Harwood J & Nichols CG. (2000b). Structural determinants of PIP(2) regulation of inward rectifier K<sub>ATP</sub> channels. *J Gen Physiol* **116**, 599-608.
- Shyng SL & Nichols CG. (1998). Membrane phospholipid control of nucleotide sensitivity of K<sub>ATP</sub> channels. *Science* **282**, 1138-1141.

- Smirnov SV & Aaronson PI. (1992).  $\text{Ca}^{2+}$  currents in single myocytes from human mesenteric arteries: evidence for a physiological role of L-type channels. *J Physiol* **457**, 455-475.
- Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ & Hunt JF. (2002). ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Mol Cell* **10**, 139-149.
- Snutch TP, Sutton KG & Zamponi GW. (2001). Voltage-dependent calcium channels--beyond dihydropyridine antagonists. *Curr Opin Pharmacol* **1**, 11-16.
- Spruce AE, Standen NB & Stanfield PR. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* **316**, 736-738.
- Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y & Nelson MT. (1989). Hyperpolarizing vasodilators activate ATP-sensitive  $\text{K}^{+}$  channels in arterial smooth muscle. *Science* **245**, 177-180.
- Stanley CA, Thornton PS, Ganguly A, MacMullen C, Underwood P, Bhatia P, Steinkrauss L, Wanner L, Kaye R, Ruchelli E, Suchi M & Adzick NS. (2004). Preoperative evaluation of infants with focal or diffuse congenital hyperinsulinism by intravenous acute insulin response tests and selective pancreatic arterial calcium stimulation. *J Clin Endocrinol Metab* **89**, 288-296.
- Stull JT, Kamm KE, Krueger JK, Lin P, Luby-Phelps K & Zhi G. (1997).  $\text{Ca}^{2+}$ /calmodulin-dependent myosin light-chain kinases. *Adv Second Messenger Phosphoprotein Res* **31**, 141-150.
- Tanabe K, Tucker SJ, Ashcroft FM, Proks P, Kioka N, Amachi T & Ueda K. (2000). Direct photoaffinity labeling of Kir6.2 by [ $\gamma$ -(32)P]ATP-[ $\gamma$ ]4-azidoanilide. *Biochem Biophys Res Commun* **272**, 316-319.
- Tanaka K, Kawano T, Nakamura A, Nazari H, Kawahito S, Oshita S, Takahashi A & Nakaya Y. (2007). Isoflurane activates sarcolemmal adenosine triphosphate-sensitive potassium channels in vascular smooth muscle cells: a role for protein kinase A. *Anesthesiology* **106**, 984-991.
- Thorin E, Nguyen TD & Bouthillier A. (1998). Control of vascular tone by endogenous endothelin-1 in human pial arteries. *Stroke* **29**, 175-180.
- Thorneloe KS, Maruyama Y, Malcolm AT, Light PE, Walsh MP & Cole WC. (2002). Protein kinase C modulation of recombinant ATP-sensitive  $\text{K}^{+}$  channels composed of Kir6.1 and/or Kir6.2 expressed with SUR2B. *J Physiol* **541**, 65-80.

- Thorneloe KS & Nelson MT. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. *Can J Physiol Pharmacol* **83**, 215-242.
- Thornton PS, MacMullen C, Ganguly A, Ruchelli E, Steinkrauss L, Crane A, Aguilar-Bryan L & Stanley CA. (2003). Clinical and molecular characterization of a dominant form of congenital hyperinsulinism caused by a mutation in the high-affinity sulfonylurea receptor. *Diabetes* **52**, 2403-2410.
- Tivesten A, Barlind A, Caidahl K, Klintland N, Cittadini A, Ohlsson C & Isgaard J. (2004). Growth hormone-induced blood pressure decrease is associated with increased mRNA levels of the vascular smooth muscle  $K_{ATP}$  channel. *J Endocrinol* **183**, 195-202.
- Toombs CF, McGee DS, Johnston WE & Vinten-Johansen J. (1993). Protection from ischaemic-reperfusion injury with adenosine pretreatment is reversed by inhibition of ATP sensitive potassium channels. *Cardiovasc Res* **27**, 623-629.
- Tucker SJ, Gribble FM, Proks P, Trapp S, Ryder TJ, Haug T, Reimann F & Ashcroft FM. (1998). Molecular determinants of  $K_{ATP}$  channel inhibition by ATP. *Embo J* **17**, 3290-3296.
- Tucker SJ, Gribble FM, Zhao C, Trapp S & Ashcroft FM. (1997). Truncation of Kir6.2 produces ATP-sensitive  $K^+$  channels in the absence of the sulphonylurea receptor. *Nature* **387**, 179-183.
- Verdon G, Albers SV, Dijkstra BW, Driessen AJ & Thunnissen AM. (2003). Crystal structures of the ATPase subunit of the glucose ABC transporter from *Sulfolobus solfataricus*: nucleotide-free and nucleotide-bound conformations. *J Mol Biol* **330**, 343-358.
- Wakatsuki T, Nakaya Y & Inoue I. (1992). Vasopressin modulates  $K^+$ -channel activities of cultured smooth muscle cells from porcine coronary artery. *Am J Physiol* **263**, H491-496.
- Wang R, Rojas A, Wu J, Piao H, Adams CY, Xu H, Shi Y, Wang Y & Jiang C. (2005). Determinant role of membrane helices in K ATP channel gating. *J Membr Biol* **204**, 1-10.
- Wang R, Su J, Zhang X, Shi Y, Cui N, Onyebuchi VA & Jiang C. (2006). Kir6.2 channel gating by intracellular protons: subunit stoichiometry for ligand binding and channel gating. *J Membr Biol* **213**, 155-164.

- Wang R, Zhang X, Cui N, Wu J, Piao H, Wang X, Su J & Jiang C. (2007). Subunit-stoichiometric evidence for kir6.2 channel gating, ATP binding, and binding-gating coupling. *Mol Pharmacol* **71**, 1646-1656.
- Wang X, Wu J, Li L, Chen F, Wang R & Jiang C. (2003). Hypercapnic acidosis activates K<sub>ATP</sub> channels in vascular smooth muscles. *Circ Res* **92**, 1225-1232.
- Wellman GC, Quayle JM & Standen NB. (1998). ATP-sensitive K<sup>+</sup> channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. *J Physiol* **507** ( Pt 1), 117-129.
- Wiener CM, Dunn A & Sylvester JT. (1991). ATP-dependent K<sup>+</sup> channels modulate vasoconstrictor responses to severe hypoxia in isolated ferret lungs. *J Clin Invest* **88**, 500-504.
- Williams DA & Fay FS. (1986). Calcium transients and resting levels in isolated smooth muscle cells as monitored with quin 2. *Am J Physiol* **250**, C779-791.
- Winkquist RJ, Heaney LA, Wallace AA, Baskin EP, Stein RB, Garcia ML & Kaczorowski GJ. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. *J Pharmacol Exp Ther* **248**, 149-156.
- Wu J, Cui N, Piao H, Wang Y, Xu H, Mao J & Jiang C. (2002a). Allosteric modulation of the mouse Kir6.2 channel by intracellular H<sup>+</sup> and ATP. *J Physiol* **543**, 495-504.
- Wu J, Xu H, Yang Z, Wang Y, Mao J & Jiang C. (2002b). Protons activate homomeric Kir6.2 channels by selective suppression of the long and intermediate closures. *J Membr Biol* **190**, 105-116.
- Xie LH, Horie M & Takano M. (1999). Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism. *Proc Natl Acad Sci U S A* **96**, 15292-15297.
- Xu H, Cui N, Yang Z, Wu J, Giwa LR, Abdulkadir L, Sharma P & Jiang C. (2001a). Direct activation of cloned K<sub>ATP</sub> channels by intracellular acidosis. *J Biol Chem* **276**, 12898-12902.
- Xu H, Wu J, Cui N, Abdulkadir L, Wang R, Mao J, Giwa LR, Chanchevalap S & Jiang C. (2001b). Distinct histidine residues control the acid-induced activation and inhibition of the cloned K<sub>ATP</sub> channel. *J Biol Chem* **276**, 38690-38696.

- Yamada M, Ishii M, Hibino H & Kurachi Y. (2004). Mutation in nucleotide-binding domains of sulfonylurea receptor 2 evokes Na-ATP-dependent activation of ATP-sensitive K<sup>+</sup> channels: implication for dimerization of nucleotide-binding domains to induce channel opening. *Mol Pharmacol* **66**, 807-816.
- Yamada M, Isomoto S, Matsumoto S, Kondo C, Shindo T, Horio Y & Kurachi Y. (1997). Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive K<sup>+</sup> channel. *J Physiol* **499** ( Pt 3), 715-720.
- Yamada M & Kurachi Y. (2004). The nucleotide-binding domains of sulfonylurea receptor 2A and 2B play different functional roles in nicorandil-induced activation of ATP-sensitive K<sup>+</sup> channels. *Mol Pharmacol* **65**, 1198-1207.
- Yang Y, Shi Y, Guo S, Zhang S, Cui N, Shi W, Zhu D & Jiang C. (2007). PKA-dependent activation of the vascular smooth muscle isoform of K<sub>ATP</sub> channels by vasoactive intestinal polypeptide and its effect on relaxation of the mesenteric resistance artery. *Biochim Biophys Acta*.
- Ye D, Zhou W & Lee HC. (2005). Activation of rat mesenteric arterial K<sub>ATP</sub> channels by 11,12-epoxyeicosatrienoic acid. *Am J Physiol Heart Circ Physiol* **288**, H358-364.
- Ye D, Zhou W, Lu T, Jagadeesh SG, Falck JR & Lee HC. (2006). Mechanism of rat mesenteric arterial K<sub>ATP</sub> channel activation by 14,15-epoxyeicosatrienoic acid. *Am J Physiol Heart Circ Physiol* **290**, H1326-1336.
- Yoo D, Flagg TP, Olsen O, Raghuram V, Foskett JK & Welling PA. (2004). Assembly and trafficking of a multiprotein ROMK (Kir 1.1) channel complex by PDZ interactions. *J Biol Chem* **279**, 6863-6873.
- Zerangue N, Schwappach B, Jan YN & Jan LY. (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K<sub>ATP</sub> channels. *Neuron* **22**, 537-548.
- Zhang H & Bolton TB. (1995). Activation by intracellular GDP, metabolic inhibition and pinacidil of a glibenclamide-sensitive K-channel in smooth muscle cells of rat mesenteric artery. *Br J Pharmacol* **114**, 662-672.
- Zhang HL & Bolton TB. (1996). Two types of ATP-sensitive potassium channels in rat portal vein smooth muscle cells. *Br J Pharmacol* **118**, 105-114.
- Zolnerciks JK, Wooding C & Linton KJ. (2007). Evidence for a Sav1866-like architecture for the human multidrug transporter P-glycoprotein. *FASEB J*.

## 15. Appendix: Publications

1. Shi Y, Chen X, Wu Z, Shi W, Jiang C, Harrison R. (2007) PKA Phosphorylation produces interdomain movements in SUR2B leading to activation of vascular K<sub>ATP</sub> channel. **Submitted**
2. Shi Y, Cui N, Shi W, Jiang C. (2007) A unique motif in Kir6.1 consisting of 4 phosphorylation repeats underlies vascular K<sub>ATP</sub> channel inhibition by protein kinase C. *J. Biol. Chem.* **in press**
3. Shi Y, Wu Z, Cui N, Shi W, Yang Y, Zhang X, Rojas A, Ha BT, Jiang C. (2007) PKA phosphorylation on SUR2B subunit underscores vascular K<sub>ATP</sub> channel activation by  $\beta$ -adrenergic receptors. *Am J Physiol.* **293**, R1205-14
4. Yang Y, Shi Y, Guo S, Zhang S, Cui N, Shi W, Zhu D, Jiang C. (2007) PKA-dependent activation of the vascular smooth muscle isoform of K<sub>ATP</sub> channels by vasoactive intestinal polypeptide and its effects on relaxation of the mesenteric resistance artery. *Biochimica Biophysica Acta.* **in press**
5. Shi W, Cui N, Shi Y, Zhang X, Yang Y, Jiang C. (2007) Arginine vasopressin inhibits Kir6.1/SUR2B channel and constricts the mesenteric artery via V1a receptor and protein kinase C. *Am J Physiol.* **293**, R191-9
6. Su J, Yang L, Zhang X, Rojas A, Shi Y, Jiang C. (2007) High CO<sub>2</sub> chemosensitivity versus wide sensing spectrum: a paradoxical problem and its solutions in cultured brainstem neurons. *J Physiol.* **578(Pt 3)**, 831-41.
7. Cui N, Li L, Wang X, Shi Y, Shi W, Jiang C. (2006) Elimination of allosteric modulation of myocardial K<sub>ATP</sub> channels by ATP and protons in two Kir6.2 polymorphisms found in sudden cardiac death. *Physiol Genomics.* **25**, 105-15



8. Li L, Shi Y, Wang X, Shi W, Jiang C.(2005) Single nucleotide polymorphisms in  $K_{ATP}$  channels: muscular impact on type 2 diabetes. *Diabetes*. **54**, 1592-7
9. Wang R, Rojas A, Wu J, Piao H, Adams CY, Xu H, Shi Y, Wang Y, Jiang C.(2005) Determinant role of membrane helices in  $K_{ATP}$  channel gating. *J Membr Biol*. **204**, 1-10.
10. Mao J, Wang X, Chen F, Wang R, Rojas A, Shi Y, Piao H and Jiang C (2004). Molecular basis for the inhibition of G-protein coupled inward rectifier  $K^+$  channels by protein kinase C, *Proc Natl Acad Sci U S A*. **101**, 1087-1092.
11. Wu J, Piao H, Rojas A, Wang R, Wang Y, Cui N, Shi Y, Chen F, Jiang C. (2004) Critical protein domains and amino acid residues for gating the Kir6.2 channel by intracellular ATP. *J Cell Physiol*. **198**, 73-81.