

**Functional analysis of regulatory phosphorylation
events in cardiac K_{ATP} channel subunits in ischaemia**

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Abstract

Protein kinase C (PKC)–mediated phosphorylation of ATP sensitive potassium channels (K_{ATP}) is believed to be involved in ischaemic preconditioning. Five consensus PKC phosphorylation sites in the Kir6.1 subunit, S354, S379, S385, S391 and S397, have been proposed as possible regulatory sites. The objective of this study was to investigate the role of these residues in regulating the functional properties of Kir6.1-containing K_{ATP} channels. HEK cells expressing Kir6.1/SUR2A or Kir6.1/SUR2B channels were used to assess electrical activity of the channels responding to PKC using the whole cell patch clamp technique. It was found that PKC activator, Phorbol 12-Myristate 13-Acetate (PMA), inhibited pinacidil-activated Kir6.1/SUR2A and Kir6.1/SUR2B currents. Mutation of serine 379 and serine 385 resulted in a significant reduction of the inhibitory effect of PKC in both Kir6.1/SUR2A and kir6.1/SUR2B channels. The inhibitory effect of PKC was also reduced in S397 mutant in Kir6.1/SUR2A channel.

An endosome targeting Kir6.1/SUR2A mutant was assessed for its functional response to PKC. The inhibitory effect of PKC was abolished in this mutant. Further investigation by immunofluorescence microscopy showed that incubating HEK cells expressing Kir6.1-HA tag/SUR2A with PMA for 30 minutes resulted in internalization of the Kir6.1 protein, but not in non-treated cells. In the presence of the dynamin GTPase inhibitor, dynasore, PMA-induced internalization was blocked indicating that internalisation was via a dynamin dependent mechanism.

To clarify which PKC isoform was responsible for K_{ATP} channel phosphorylation, Kir6.1 phosphorylation in rat ventricular myocytes was labelled with ^{32}P and stimulated with Adenosine A_1 receptor agonist, 2-chloro-N6-cyclopentyl-adenosine (CCPA). The phosphorylation was investigated in the absence and presence of permeable Tat-linked PKC inhibitor peptides. Kir6.1 phosphorylation was stimulated by CCPA and inhibited in the presence of Tat-coupled PKC ϵ inhibitor peptide but not Tat- PKC α , β , γ or δ inhibitor peptides.

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Abbreviations

5-HD	5-hydroxydecanoate
ABC	ATP-binding cassette transporter
ADP	Adenosine 5'-diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine 5'-diphosphate
bp	base-pair
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCPA	2-chloro-N6-cyclopentyl adenosine
cDNA	complementary DNA
DAG	diacylglycerol
DNA	deoxyribonucleic acid
EDTA	diaminoethanetetra acetic acid
Ek	Potassium equilibrium potential
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HA	Human influenza virus hemagglutinin polypeptide
HEK 293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	Immunoglobulin G
IPC	Ischemic preconditioning
K _{ATP}	ATP-sensitive Potassium channels
Kb	kilo base

KCO	Potassium channel opener
kDa	kilo dalton
Kir	Inward rectifying potassium channel
M1 and M2	Transmembrane segment 1 and 2
mitoK _{ATP}	mitochondrial ATP-sensitive potassium channels
NBD	Nucleotide binding domain
NBFs	nucleotide binding folds
pA	pico ampere
PCR	Polymerase chain reaction
pF	pico farad
PIP ₂	Phosphatidylinositol-4-5, biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
pS	pico siemens
RKR	endoplasmic reticulum retention sequence
ROS	reactive oxygen species
sarcK _{ATP}	Sarcolemmal ATP-sensitive potassium channels
SUR	sulphonylurea receptor
TAE	tris acetate EDTA
TMD	Transmembrane domain of SUR

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Chapter 1: Introduction

Ischaemic preconditioning (IPC) provides an effective mechanism of myocardial protection against subsequent lethal ischaemia. Brief periods of ischaemia prior to prolonged ischaemia can reduce myocardial infarct size, endothelial dysfunction, cardiac stunning, as well as cardiac arrhythmias (Cohen et al, 2000, Rezkalla and Kloner, 2007, London, 2007). This protection is thought to involve the protein kinase C (PKC)-mediated phosphorylation of ATP sensitive potassium channels (K_{ATP}) (Testai et al, 2007). One critical target of PKC phosphorylation is reported to be the pore-forming subunit, Kir6.1, which is found in mitochondria of cardiac myocytes and in vascular smooth muscle cells (Coung et al, 2005, Shi et al, 2008a, Singh et al, 2003). A study of vascular ATP-sensitive potassium channels (Kir6.1/SUR2B) showed that the PKC phosphorylation sites of Kir6.1 were in the distal C terminus, at Ser 379, Ser 385, Ser 391, and Ser 397 residues (Shi et al, 2008a). In the heart, among the five PKC phosphorylation sites in the C-terminal of the Kir6.1 subunit (Ser 354, Ser 379, Ser 385, Ser 391 and Ser 397), Ser 379 only has been shown to be a crucial site for the phosphorylation *in vitro* study (Singh, 2002, unpublished). Functional analysis of the effect of the PKC phosphorylation at this/these site(s) has not been studied in Kir6.1/SUR2A channel, a proposed mitochondrial K_{ATP} channel. Many studies of cardioprotection indicate the effects of PKC on K_{ATP} function (Hu et al, 2003, Jiao et al, 2008, Shi et al, 2008a). The explanation for the modulation of PKC on K_{ATP} ion channel activity is that it may result in an alteration of open probability or the trafficking of the channels (Light et al, 1996, Jiao et al, 2008). There is evidence indicating that PKC activation stimulates internalization of K_{ATP} channels in the Kir6.1/SUR2B subunit

combination channel. However, the mechanism by which PKC modulates the Kir6.1/SUR2A channel has not been studied. It is hypothesized that PKC phosphorylation of cardiac Kir6.1 affects internalization of the channel. The main objective of this study was to investigate the role of the five proposed phosphorylation sites of Kir6.1 in regulating cardiac Kir6.1 containing channel activity and to examine the effect of PKC on Kir6.1/SUR2A channel trafficking.

1.1 ATP- sensitive potassium channels

ATP sensitive potassium channels (K_{ATP} channels) were first described in the heart by Noma in 1983 (Noma, 1983). They act as molecular sensors of cellular metabolism by coupling cellular metabolic change to electrical activity of cell membranes. K_{ATP} channels play a vital role in many types of tissues such as pancreatic β -cells, skeletal muscle, smooth muscle, renal tubular cells, the central nervous system and, not least, in the cardiovascular system (Seino, 1999). Decreasing intracellular ATP results in opening of K_{ATP} channels, on the other hand, rising ATP brings about closing of these channels. These electrical activities of K_{ATP} channels are involved in cellular functions such as insulin secretion, smooth muscle relaxation, skeletal muscle excitability, neurotransmitter release and cardiac contraction (Isomoto and Kurachi, 1997). Although the regulation of these channels has been revealed over a number of years, many points are still challenging. Obviously, detailed understanding of their structure is also needed.

1.1.1 ATP sensitive potassium channels: Structure

K_{ATP} channels have a hetero-octameric stoichiometry, composed of two main subunits, pore-forming Kir6 and regulatory SUR polypeptides. The pore-forming subunit is a component part of inwardly rectifying potassium channels (Kir6), whereas the regulatory subunit, the sulfonylurea receptor (SUR), is an ATP-binding cassette (ABC) transporter protein (Bryan et al, 2004). K_{ATP} channels are formed by four Kir6 subunits accompanied by four SUR subunits (**Fig. 1.1**). Each Kir6 monomer has two transmembrane domains, M1 and M2, bridged by a pore-loop (P) of which some parts are extracellular (Aguilar et al, 1995, Bryan et al 2004). The M1 and M2 domains are also flanked by the cytoplasmic domains located at the N- and C- terminal. Together, these cytoplasmic domains are believed to be the sites for ATP action. It is agreed that the Kir subunits are responsible for K⁺ ion-selectivity and that the critical region for this mechanism is located in the H5 pore loop region. It is noteworthy that the H5 motif within the pore of the Kir6 family of glycine-tyrosine-glycine is different to the consensus sequence found in other Kir channel family members (Seino et al, 1999).

The SUR subunit is thought to be involved in trafficking of the assembled channel to the surface membrane and in controlling the gating of Kir6 pores (Chan et al, 2003). Unlike Kir6 subunits, the structure of the SUR subunit is more complicated, with 17 transmembrane spanning segments in 3 domains, TMD0, TMD1 and TMD2 (Seino et al, 1999). TMD1 and TMD2 both have six-helical transmembrane segments followed by a nucleotide-binding domain, resulting in one nucleotide binding fold domain (NBF1) between TMD1 and TMD2 and the second (NBF2) in the cytoplasmic C-terminal domain following the last transmembrane helix of TMD2. In addition, each NBF contains two Walker motifs, A and B, which are believed to play a role in

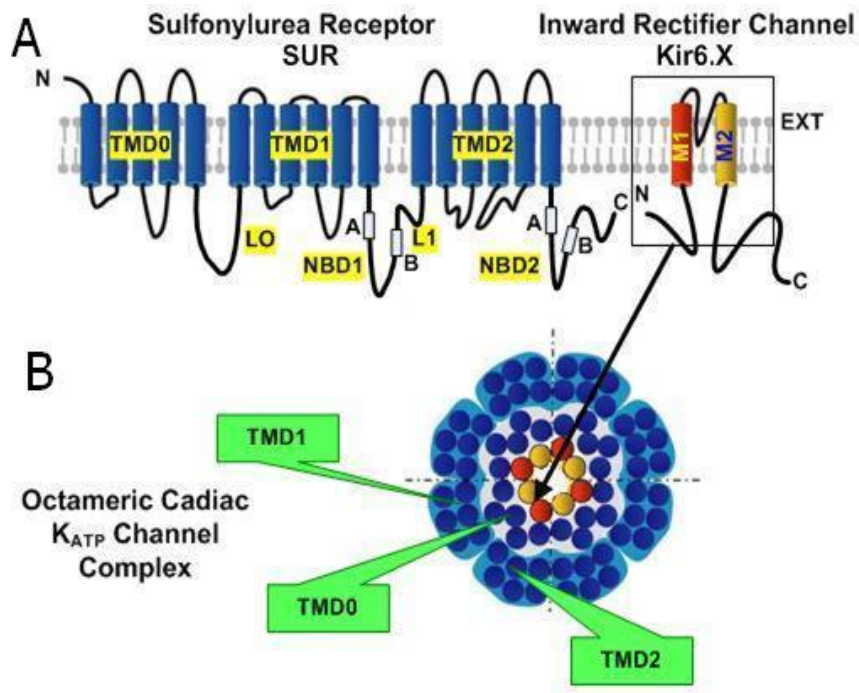


Figure 1.1 Topology of Kir6.2 and SUR subunits. (A) Transmembrane domain structure of Kir6 and SUR subunits. (B) K_{ATP} channels form a channel by assembly of four Kir monomers and four SUR monomers. (Adapted from Moreau, C., et al, 2005).

catalysing ATP hydrolysis (Bienengraeber, 2000). TMD0, the N-terminal transmembrane segment, has only five transmembrane helices, which are linked to TMD1 by cytoplasmic loop (L0) following the last transmembrane segment (Seino et al, 1999).

It is now clear that functional K_{ATP} channels must contain at least two subunit types, Kir and SUR subunits (Cui, 2001). Cloning and reconstitution studies of K_{ATP} channels showed that Kir or SUR subunits alone did not produce K^+ currents (Inagaki et al, 1995, Aguilar et al, 1995 in Babenko, 1998, Bryan et al, 2004). The genomic mapping of Kir6 and SUR subunits showed that these genes are encoded in pairs, ~4900 base pairs apart from each other on the same chromosome Kir6.2 and SUR1 on chromosome II and a larger distance between Kir6.2 and SUR2 on chromosome 12 (Babenko et al, 1998).

The physical association between the Kir6 and SUR subunits has been studied. By using truncated SUR1 constructs, Chan et.al (2003) demonstrated that full length SUR1 physically interacted strongly with Kir6.2 and that this was mediated by the presence of TMD0. In addition, using subunit chimaeras, Babenko et al (2003) found that SUR1 interacted with Kir6.2 through the TMD0 domain. In addition to association between Kir6.2/SUR1, physical association between Kir6.2 and SUR2A subunits of cardiac K_{ATP} has been studied by coimmunoprecipitation using a Kir6.2-specific antibody (Lorenz et al, 1999). The study showed that SUR2A could be immunoprecipitated by anti-Kir6.2 antibody only when it was in the complex of Kir6.2/SUR2A. Furthermore, coimmunoprecipitation of three maltose-binding protein

SUR2A polypeptide fusion proteins showed direct physical association between C-terminal fragments of SUR2A and the full length Kir6.2 (Rainbow et al, 2004). The trafficking of K_{ATP} channels needs a proper assembly of Kir and SUR subunit to mask RKR ER retention signal. Not only does SUR affect the assembly of functional channels, the specific SUR domains associated with Kir6 also play roles in determining gating and trafficking of K_{ATP} channels (Chan et al, 2003). Some studies showed that the TMD0 and L0 are particularly involved in gating or trafficking of K_{ATP} channels (Chan et al., 2003, Fang et. al, 2006).

1.1.2 Role of Kir6 and SUR subunits in gating mechanism

1.1.2.1 Kir subunit

The response to nucleotides of K_{ATP} channels is thought to be mediated via nucleotide binding sites of both the inward rectifying and regulatory subunits. It is now clear that there is at least one ATP binding site on the Kir6 subunit. By using electrophysiological study, Tucker et al (1997) found that even though the full length Kir6.2 subunit alone did not express functionally, truncated Kir6.2 (Kir6.2 Δ C26, Kir6.2 Δ C36) could produce currents. These currents responded to different intracellular ATP levels but were not activated by MgADP, as is the native channel. The enhancement of this inhibitory effect of ATP by the presence of the SUR1 subunit was also shown (Tucker et al, 1997). These studies made it clear that there is an ATP binding site on the Kir 6.2 subunit, which reacts to ATP in a Mg²⁺ independent manner. Further investigations using direct photoaffinity labelling of Kir6.2 by 8-Azido ATP and [γ -(32)P]ATP-AA confirmed the existence of ATP binding site on the Kir subunit (Tucker et al, 1999, Tanabe et al, 2000). These

studies showed that ATP can decrease photoaffinity labeling of Kir6.2 by [γ -(32)P]ATP-AA by about 50% and that Mg^{2+} did not alter the photoaffinity labelling, which correlated with former electrophysiological properties. It is thought that the ATP binding site is located at the C terminus of the M2 transmembrane segment. Vanoye et al (2002) examined the direct binding of ATP to the cytosolic domains of Kir1.1, and Kir6.1 and Kir6.2 and compared it to that of the ATP-insensitive channel, Kir2.1. They found that the COOH terminal domains of Kir1.1, Kir6.1 and Kir6.2 bound to a fluorescent ATP analogue but Kir2.1 did not. They also indicated an inability of the N termini of Kir6.1 and Kir1.1 to bind to an ATP analogue. These results suggested that the COOH terminal of K_{ATP} channel is essential for ATP binding, but not the NH_2 termini. However, the finding that Kir6.2 truncated at the N terminus decreased the sensitivity of the expressed channels to ATP, suggested that the ATP-binding site of the channel gating mechanism was more complicated (Koster et al 1999). One explanation for the role of the NH_2 terminus in channel regulation is that they probably interact with COOH terminus (Dong et al 2005). Dong et.al (2005) studied the nucleotide binding site of Kir6.2 and found that the N- terminus bound an ATP analogue but did not bind phosphatidylinositol 4,5-bisphosphate (PIP_2). Moreover, they found that directly linking the N and C termini of Kir6.2 enhanced the ATP binding, resulting in a decreased K_d and increased fluorescent enhancement factor (γ). From mutagenesis experiments, the basic residues in the N-and C- termini responsible for ATP binding are thought to be R50 in the N-terminus and K185 and R201 in the C terminus (Tucker et al 1998, Ribalet et al 2003, Trapp et al 2003). These positively charged residues affect the gating by the interaction with the phosphoryl groups of ATP. Dong et al (2005) showed that mutation of each of these residues brings about an

increased K_d for TNP-ATP binding. Unlike Kir6.2, Kir6.1 is proposed to possess two TNP-ATP binding sites, one is the same site as Kir6.2 on the C terminus and the other is on the distal C-terminus (Dong et al, 2005). Modelling of Kir6.2 based on X-ray crystallography of other inward rectifying channels has made models of the ATP binding site clearer. By using X-ray crystallography, researchers have succeeded in determining a structure of mouse Kir3.1, truncated bacterial channels KcsA and KirBac1.1 (Nishida et al, 2002, Doyle et al , 1998, Kuo A et al 2003) which then became the template for models of K_{ATP} channel structure (Haider et al, 2005).

1.1.2.2 SUR subunit

The sulfonylurea subunit (SURs) comprises two nucleotide binding domains, one is between TMD1 and TMD2 (NBF1) and the other is after TMD2 (NBF2) (Moreau et al, 2005). Walker A and B motifs in both domains complement the opposite motif in the other domain to form two nucleotide binding sites, which can bind and hydrolyze ATP. The functional role of the SUR subunit has been extensively studied. From reconstitution studies, it has been shown that the SUR subunit works together with the Kir subunit and regulates nucleotide sensitivity and pharmacological response (Babenko et al, 2003, Chan et al, 2003, 2006, Moreau et al, 2005). In homomeric C-35 truncated Kir6.2, Babenko et al (1999) found that SUR1 and SUR2 could restore a decreased open probability, decreased ATP sensitivity, as well as increased current density of the cell membranes. This suggested the importance of the regulatory subunit in the gating mechanism. Further studies showed that TMD0 and L0, a cytoplasmic loop between TMD0 and TMD1, of SUR are involved in the

interaction. By using coimmunoprecipitation, Chan et al (2003) showed the strong association between the SUR1 subunit and Kir6.2. They found that 6.2HA could be coimmunoprecipitated by a FLAG-tagged TMD0 of SUR1. To investigate whether TMD0 particularly regulates the gating mechanism of K_{ATP} channels, they compared single channel activity of Kir6.2 Δ C26, Kir6.2 Δ C26+TMD0, Kir6.2 Δ C26+SUR1 and found that the activity of Kir6.2 Δ C26+TMD0 and Kir6.2 Δ C26+SUR1 was similar and their burst duration was 25 times that of Kir6.2 Δ C26 alone. In the same way, Babenko et al (2003) found that TMD0, the increasing length of L0 and N-terminal segment of SUR1 all altered the Kir6.2 Δ C35 gating. Co-expression of Kir6.2 Δ C35 with TMD0 caused a 5-fold increased open probability (P_0 max) of the channels when compared to the baseline of Kir6.2 Δ C35. These findings suggested a crucial role of TMD0 and probably L0 as well. However, it is now unclear whether the other parts of SUR subunit are involved in the interaction (Bryan et al, 2004). It is proposed that L0 acts as the “tether” between SUR core domains and the TMD0/Kir assembly. Fang et al (2006) found that the burst kinetic of Kir 6.2 Δ C26 expressed with TMD0 of SUR1 and SUR2 was smaller than that of their corresponding SUR subunits with Kir6.2 Δ C26. However, the intraburst kinetics, which is represented by the time constant of the closed time component and the true mean open time, were similar, whereas the intrinsic gating pattern of these channels was different from that of Kir6.2 Δ C26 alone. These results suggest that the TMD0 confers the intrinsic gating characteristic to Kir6.2 Δ C26 but not all gating differences. To investigate the involvement of L0, Fang et al (2006) found that most single channel parameters of TMD0-L0 of SUR2A with Kir6.2 Δ C26 is somewhat between that of TMD0+Kir6.2 Δ C26 and SUR2A+Kir6.2 Δ C26. This underlined the involvement of

the L0 in defining the gating characteristics. It is proposed that L0 partially confers the burst duration properties of the channels.

1.1.3 ATP sensitive potassium channels: Modulation

1.1.3.1 Modulation by nucleotides

Elucidation of the gating mechanism of K_{ATP} channels is still challenging for researchers. It is widely accepted that K_{ATP} channels are regulated by intracellular nucleotides such as ATP and nucleotide diphosphates (NDPs), amongst which are ADP, UDP and GDP (Isomoto et al, 1997). Intracellular ATP (ATPi) in millimolar concentrations inhibits the channel activity, whereas NDPs effectively compete with ATP when the channels are in ATP-induced closed state leading to the opening of the channels (Seino et al, 1999). The regulation of K_{ATP} by nucleotides is complicated since their effectiveness seems to vary according to the concentration and the ratio of nucleotides and the absence or presence of magnesium (Babenko et al, 1998). It is thought that the Kir6 subunit is responsible for selectivity, inward rectification and unitary conductance, whereas the SUR subunit confers nucleotide sensitivity and pharmacological responses (Babenko et al, 1998). However, this issue is not clear cut because it has been shown that truncated Kir6.2 (Kir6.2 Δ C26) can constitute ATP-inhibited potassium channels without the presence of an SUR subunit (Tucker et al, 1997). Inhibition by ATP has been clearly shown to be mediated via a nucleotide triphosphate binding site constituted by the cytoplasmic N and C terminal domains of Kir6 subunits (Tucker et al, 1997).

ATPi regulates K_{ATP} channel activity by two actions, “ligand action” and “hydrolysis dependent” action (Isomoto et al, 1997). The first action refers to the binding of ATP to K_{ATP} channels directly, bringing about closure of the channels. The latter causes the restoration of K_{ATP} channel activity after run down in ATP-free solution (Isomoto et al, 1997). This restoration requires magnesium and hydrolysable ATP, suggesting that phosphorylation of the channel protein may be necessary in this action. The optimal concentration of ATPi required to inhibit the channels depends on K_{ATP} channel type, tissue and species. The binding sites for nucleotides are not characterized clearly. It is believed that there are binding sites for nucleotides both on Kir and SUR subunits (Seino et al, 1999). This is proposed to be the N terminal and C terminal region of Kir6. For the SUR subunit, the Walker A motif and Walker B motifs are thought to be involved since it was found that mutation of a lysine in the Walker A motif and aspartic acid in the Walker B motif resulted in weakened Mg^{2+} -independent high-affinity ATP binding. In SUR1, it is proposed that SUR confers both ATP and MgADP sensitivities. ATP is suggested to bind to the binding site in the NBF-1 region, whereas MgADP binds to NBF-2 and then antagonizes ATP binding to NBF-1 (Seino et al, 1999). A Study of Ammala et al (1996), which showed that SUR itself does not confer intrinsic channel activity, led to another model of regulation of K_{ATP} channels. This model proposed simultaneous action at both types of binding sites, that is, Kir6 subunit is bound by ATP leading to channel inhibition which is antagonized by binding of MgADP on SUR subunit (Seino, 1999).

1.1.3.2 Modulation by membrane phospholipids

Nucleotides are not the only molecules that regulate K_{ATP} channels. Membrane phospholipids (phosphatidylinositol polyphosphates, PIPs) also are able to activate channel activity. Some studies found that PIP binding to K_{ATP} channels brings about the open state of the channels (Fan et al, 1997, Huang et al, 1998). Shyng et al (1998) demonstrated that PIP_2 can restore run down of Kir6.2/SUR1 channels in the inside-out patch configuration. Moreover, it can decrease ATP sensitivity by over orders of magnitude. The mechanism of these responses is thought to act via the Kir6.2 subunit via two possible mechanisms. Firstly, PIP_2 binding may weaken the binding of ATP and, secondly, PIP_2 may change the gating of the channels, which then affects ATP sensitivity.

1.1.3.3 Modulation by potassium channel modulators

K_{ATP} channels can be modulated by a number of compounds including sulphonylureas, which have been used clinically as a diabetic treatment for a long time, and the potassium channel openers (KCOs), for which the mechanism of action is via K_{ATP} channels (Moreau et al, 2005). Both sulphonylureas and KCOs act on the SUR subunit. The sulphonylureas function by binding to transmembrane segments 14 and 15 in TMD2 of SUR, parts of the cytosolic loop (between segment 13 and 14) and parts of the linker between TMD0 and TMD1 (Moreau et al, 2005), whereas KCOs bind to the different sites. It has been demonstrated by D'haan et al (1999) that TMD2, segment 12-17, was a crucial region for the action of a cromakalim analog. Uhde et al (1999) found two regions, KCOI and KCO II, within TMD2 of rat SUR2B involved in binding the opener P1075, pinacidil and Levromakalim. In rat SUR2B, the KCO I region is probably at the connecting loop between helices 13 and

14, whereas the KCOII includes helices 16 and 17 and a short segment of NBD2. In SUR2- containing channels, TMD2 is thought to be the main region for the activation of the channels by cromakalim and pinacidil. The study of Moreau et al (2005) proposed two residues of TMD2, helix 17 (Leu 1249 and Thr1253 in SUR2A, Thr 1286 and Met 1290 in SUR1) might be a part of the binding site.

1.1.3.4 Modulation by phosphorylation

The effects of phosphorylation on K_{ATP} channels are varied and depend on the subtype of the channels. Moreover, K_{ATP} channels can be modulated by PKA or PKC-mediated phosphorylation which brings about the different effects. Extensive studies over the last decade have been performed to investigate K_{ATP} phosphorylation sites, as well as the regulatory effects of the protein kinases. PKA phosphorylation is involved in Angiotensin II mediated inhibition of rat arterial K_{ATP} channels. By using the whole cell patch clamp technique, Hayabuchi et al (2001) showed that Rp-cAMPS, a PKA inhibitor, reduced K_{ATP} currents of rat arterial smooth muscle cells containing 1mM ATP by $46.1 \pm 2.9\%$. The molecular mechanism of the phosphorylation was further investigated by Quinn et al (2004). By using recombinant K_{ATP} channels, they aimed to find out whether the phosphorylation occurred in recombinant channels, and whether the modulation was brought about by direct phosphorylation and which residues were responsible for this event. The result confirmed the involvement of PKA in regulating K_{ATP} channel activity. HEK 293 cells expressing Kir6.1/SUR2B channels were studied using the whole-cell configuration. It was found that application of a cAMP analogue stimulated Kir6.1/SUR2B channel. Further investigation was performed by using the

PKA inhibitor, Rp-cAMPS 200 $\mu\text{mol/L}$, in the intracellular solution (Quinn et al, 2004). It was found that, in the presence of Rp-cAMPS, Forskolin, the activator of adenylylase cyclase which can activate Kir6.1/SUR2B currents, could not stimulate Kir6.1/SUR2B channels. Moreover, this effect was believed to be the result of direct channel phosphorylation by PKA, rather than an indirect mechanism. By fusion a solubility enhancer, maltose-binding protein (MBP), to C terminal of Kir6.1 and nucleotide binding domains of SUR2B, it was found that MBP-Kir 6.1C, MBP-SUR2B-NBD1 and MBP-SUR2B-NBD2 can be substrates for PKA-mediated phosphorylation (Quinn et al, 2004).

A subsequent experiment was to test the effect of the catalytic subunit of PKA (PKAcat). The increased currents established by addition of PKAcat may indicate the direct phosphorylation by PKA. Quinn and colleagues (2004) also mapped the PKA phosphorylation sites of Kir6.1/SUR2B. By mutagenesis and biochemical and functional assay, they identified three phosphorylation sites in Kir6.1/SUR2B channels, S385 in Kir6.1 and T633 and S1465 in SUR2B subunit (Quinn et al, 2004). Shi et al (2007) found that the activation of Kir6.1/SUR2B by beta-adrenergic receptors occurs via the activation of PKA. The isoproterenol-activated Kir6.1/SUR2B current was eliminated when a PKA inhibitor (Rp-cAMP) or specific PKA inhibitory peptide (PK14-24) was applied to the pipette and bath solutions. However, mutation of the three previously identified PKA phosphorylation sites (Kir6.1 S385, SUR2B T633 and S1465) failed to inhibit the activation of Kir6.1/SUR2B current by PKA. Systematic mutational analysis showed that Serine 1351 and 1387 of NBD2 in SUR2B may be the functional PKA phosphorylation sites. *In vitro* phosphorylation and channel activity study showed that only the

S1387A mutation affected the phosphorylation and the current of the Kir6.1/SUR2B channel. In a further study, Shi, (2008b) showed that phosphorylation of S1387 caused a conformational change in NBD2 and strengthened the interaction of NBD2 and the intracellular linker between segment 9-10 of TMD1.

Protein kinase C modulation of K_{ATP} channels is involved in ischaemic preconditioning mechanisms in cardiac myocytes and also in the regulation of insulin secretion from beta cells (Brooks et al, 1996, Park et al, 2008). A functional study of PKC activation in cardiac myocytes showed that PKC affected the open probability of cardiac K_{ATP} channels (Light et al, 1996). A consistent result was found in recombinant Kir6.2/SUR2A and Kir6.2/SUR1 channels. It was found that PKC could increase the channel activity (NP_O) by $\sim 300\%$. In recombinant cardiac channels, it was thought that the phosphorylation site was located on the pore-forming subunit since the truncated Kir6.2 (Kir6.2 Δ C26) alone could be activated by a PKC activator and inhibited by chelerythrine, a PKC inhibitor. Further study showed that the phosphorylation occurred directly on a single residue in Kir6.2, T180. Point mutagenesis of this residue removed sensitivity of Kir6.2 Δ 26 channel currents to phorbol ester and reduced, although did not completely inhibit, direct PKC phosphorylation. Likewise, the open probability of the Kir6.1/SUR2B channel stimulated by pinacidil was decreased after addition of phorbol ester, a PKC activator, whereas pretreatment with chelerythrine caused the suppression of the inhibitory effect of PKC activator (Thorneloe et al, 2002). Together these results suggested the involvement of direct PKC phosphorylation in this type of channel. Interestingly, in K_{ATP} channels containing Kir6.2 subunits or combinations of Kir6.1 and Kir6.2 subunits, phorbol ester increased open probability, indicating a

differential regulation of K_{ATP} channel currents depending on the Kir6 subunits present.

In *in vitro* phosphorylation assays on cardiac myocytes, Kir6.1 was found to be phosphorylated by both PKC and PKA, whereas Kir6.2 was phosphorylated only by PKA (Singh, 2002). Since this PKC phosphorylation was completely inhibited by chelerythrine but not by the mitogen-activated protein kinase MAPK inhibitors, PKC seems to phosphorylate the channel directly. Singh (2002) demonstrated that PKC-mediated phosphorylation of Kir6.1 occurred on serine residues. A series of Kir6.1/Kir6.2 chimeras were used to map the PKC phosphorylation site to five potential serine residues within PKC consensus sites in the C-terminal domain. Point mutagenesis of these residues to alanine in full-length Kir6.1 revealed one mutant Kir6.1S379A which could not be phosphorylated, locating the site to this position. Similarly, Shi et al (2008a) also found that a phorbol ester inhibited Kir6.1/SUR2B channel activity, whereas it had no effect on Kir6.2/SUR2B channel. Using a similar approach to Singh (2002) Kir6.1/Kir6.2 chimeras were employed in functional assays to localize the PKC phosphorylation site. The result indicated that both N and C termini of Kir6.1 are essential for the PKC-dependent channel modulation. However, no PKC phosphorylation site was detected in the N terminus. Replacement of the distal C terminus of Kir6.1 by that of Kir6.2 resulted in a decrease in inhibition of channel activation by PMA. The possible five phosphorylation sites, S354, S379, S385, S391 and S397, were further tested after alanine mutagenesis both by patch clamp technique and by *in vitro* phosphorylation assay of subunit polypeptides. It was found that mutation of each of these residues decreased percentage of current inhibition by PMA. In addition, combined mutation of these serine residues led to a larger inhibition. Consistently, *in vitro*

phosphorylation assay indicated that these serine residues, but not S354, are critical for PKC phosphorylation (Shi et al, 2008a).

One explanation for the mechanism by which PKC affects K_{ATP} function is that PKC modulates the localization of K_{ATP} channels (Hu et al, 2003). Garg et al (2007) investigated whether PKC induces import of Kir6.2/SUR2A to mitochondria. By using Kir6.2/SUR2A transfected COS-7 cells, they found 100% increase in mitoplast localization of Kir6.2 in cells treated with PMA. Attenuation of this effect by PKC ϵ inhibitor (PKC ϵ V1-2) indicated that PKC ϵ is involved in this mechanism. Consistently, the Kir6.2 protein level in mitochondria increased 100% in response to PMA, whereas total cellular Kir6.2 protein remained unchanged. Jiao et al (2008) studied the effect of PKC on internalization of Kir6.1/SUR2B channels in transfected HEK 293 cells. They found that most of the Kir6.1/SUR2B channels are on the plasma membrane, which are then internalized to an intracellular location after treatment with PMA. This movement could be blocked by PKC ϵ inhibitor and methy- β cyclodextrin (M β CD), a drug which disrupts caveolae and lipid rafts. This indicated the involvement of PKC ϵ and caveolae in Kir6.1 internalization. The level of Kir6.1/SUR2B channel on the surface was also affected by PKC ϵ . The result correlated with functional study of the channel. By using whole cell patch clamp technique, they revealed the inhibitory effect of PMA on the whole cell current (Shi et al, 2008a, Jiao et al, 2008, Hu et al, 2009). However, the effect of PKC on other K_{ATP} channel subunit combination is still elusive. Moreover, the mechanism by which PKC affects the channel function required to be elucidation.

1.1.4 K_{ATP} channel function

The function and pharmacological properties of K_{ATP} channels varies according to the subunit combination, for example, reconstituted Kir6.2 and SUR2A, which forms cardiac type K_{ATP} channels, demonstrates a single channel conductance of 79 pS and cannot be activated by diazoxide, whereas Kir6.2/SUR1, the β cell type K_{ATP} channel, shows the same single conductance but responds to diazoxide. Properties of K_{ATP} channel subtype are described below.

1.1.4.1 Reconstituted Kir6.1/SUR2B channel

This combination channel is thought to resemble vascular smooth muscle K_{ATP} channels. Co-expression of these subunit results in small, 33 pS, conductance potassium channel. This channel is not inhibited by physiological ATP concentration, however, the channel is closed in the presence of high concentration ATP ($> 10^{-4}$ M) and open in response to pinacidil and nicorandil. It is also stimulated by UDP and GDP (Babenko et al, 1997).

1.1.4.2 Reconstituted Kir6.1/SUR 2A channel

There are few studies providing the electrophysiological and pharmacological properties of this recombinant channel. It was demonstrated that Kir6.1 is present in the inner membrane of mitochondria and also plasma membrane of rat cardiac myocytes (Counig et al 2005, Singh et al, 2003). Finding that 32 P Kir6.1 was co-immunoprecipitated with anti SUR2A suggests that Kir6.1/SUR2A may be a mito K_{ATP} channel (Singh, 2002). By using the inside-out configuration of the patch

clamp technique, it was shown that the Kir6.1/SUR2A channel was closed in the absence of millimolar of UDP (Kono et al, 2000). The activation of this channel was low, in 10% of patched cells only were Kir6.1/SUR2A channels detected which had a single channel conductance of ~ 33 pS in the presence of 10 mM UDP (Kono et al, 2000).

1.1.4.3 Reconstituted Kir6.1/SUR1 channel

Little is known about this subunit combination channel. This channel can be activated by diazoxide and metabolic inhibition. It is not clear about its unitary conductance because of a rapid rundown of the channel (Babenko et al, 1997).

1.1.4.4 Reconstituted Kir6.2/SUR1 channel

This recombinant channel resembles the classical β -cell type K_{ATP} channel. It can be activated by diazoxide and metabolic inhibition and can be blocked by sulphonylureas at low concentration ($IC_{50} < 10$ nM). This subtype is also expressed in brain and neuroendocrine cells (Babenko et al, 1997).

1.1.4.5 Reconstituted Kir6.2/SUR2A channel

This subunit combination channel is widely accepted as a $sarK_{ATP}$ channel in cardiomyocytes. It can be blocked by glibenclamide with IC_{50} of 1.2 μ M and activated by pinacidil and cromakalim but not diazoxide.

1.1.4.6 Reconstituted Kir6.2/SURB channel

This subtype of K_{ATP} channels was identified in colonic smooth muscle (Babenko, 1997, Flagg et al, 2010). Pharmacological properties of this combination channel are similar to K_{ATP} channel presented in vascular smooth muscle. It is activated by pinacidil and diazoxide and inhibited by ATP and glibenclamide.

1.1.4.7 Functional study of K_{ATP} channels

Patch clamp recording

Functional study of ion channels can be performed by using the patch clamp technique. The patch clamp technique is used to record the electrical properties of the cell membrane, the ion fluxes across a membrane, by using a micropipette attached to the cell membrane. The technique was established in 1976 by Erwin Neher and Bert Sakmann (Neher and Sakmann, 1978). According to Ohm's law, membrane potential of the cell depends on the current flow and the resistance (or conversely, the conductance) of the circuit. In the other words, the current flow across a membrane is the result of the driving force ($V_m - E_{rev}$) multiplied by the conductance where V_m is membrane potential and E_{rev} is the reversal potential. There are two types of the patch clamp technique called "Voltage clamp" and "Current clamp" technique. The popular one is voltage clamp in which the membrane potential is clamped at a desired level and the change in the currents is recorded at the same time. The conductance, which represents the ion channel activity, can be obtained by calculation. There are various types of patch clamp configurations that can be performed depending on the objective of the study, i.e.,

cell-attached patch, whole-cell, inside-out and outside-out excised patch, perforated patch configurations. The illustration is shown in **Figure 1.2**.

1.2 Ischaemic preconditioning

Ischemic preconditioning (IPC) was first described by Murry et al as repeated brief periods of sub-lethal ischemia providing protective effect against subsequent ischemic insult (Murry et al, 1986). The protective effect of IPC presents in two periods. One occurs immediately after the stimulus and terminates after 2-3 hours. This protection is called ‘classic’ or ‘early preconditioning’ (Zaugg et al, 2003, Testai et al, 2007). The other occurs 12-24 hours after the first stimulation; this is called ‘the second window of protection’ or ‘late/delayed preconditioning’ which is important during reperfusion (Zaugg et al, 2003, Yang et al et al, 2010). The protective effect of IPC is thought to be mediated, at least in part, by activation of ATP-sensitive potassium channels (K_{ATP}), which brings about the shortening of action potentials resulting in a reduction in Ca^{2+} influx and also muscle contraction (Noma et al, 1983, Reimer, 1996).

There are many substances involved in the mechanism of IPC (Downey et al, 2007). Adenosine, bradykinin and opioids, which are released during the ischaemic insult, are thought to be physiological triggers of IPC. Among these, adenosine provides the majority of the IPC trigger (Zaugg et al, 2003). Adenosine, bradykinin and opioids work together but through activation of different pathways. All triggers start with the binding of a Gi-protein coupled receptor. Opioids and bradykinin act through

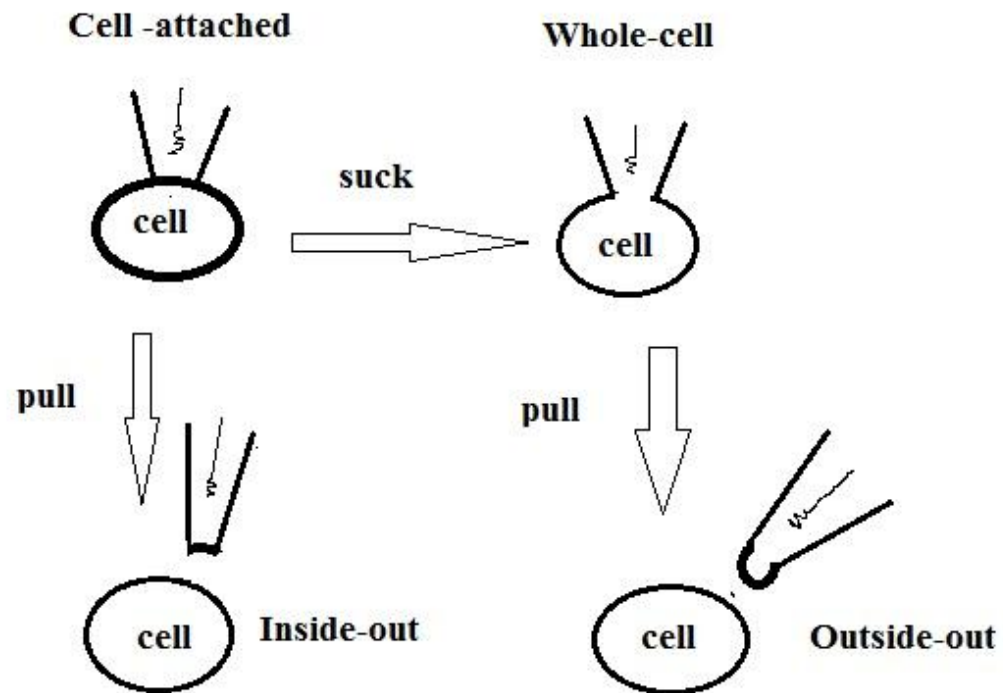


Figure 1.2 Patch clamp configurations. The attachment of micropipette to cell membrane can produce a gigaseal and the electrical activity can be recorded as cell-attached configuration. Application of slight suction on cell-attached configuration causes cell membrane rupture and allows a recording in whole cell configuration. Withdrawal the pipette while in whole cell configuration establishes outside-out configuration. Inside-out configuration was achieved by pulling the pipette away the cells while in cell-attached configuration.

activation of phosphatidylinositol-3 kinase (PI3-K) leading to phosphorylation of Akt (protein kinase B). Subsequently, Akt stimulates nitric oxide synthase resulting in guanylyl cyclase activation, which then stimulates protein kinase G. This action brings about the opening of mitochondrial ATP-sensitive potassium channels (mito K_{ATP}), leading to the production of reactive oxygen species (ROS), which in turn activate PKC. Unlike opioids and bradykinin, adenosine binds adenosine A1 and A3 receptors and then stimulates phospholipase C and phospholipase D to activate protein kinase C via raised intracellular Ca^{2+} . PKC is believed to be a common target of the IPC pathways, whereas mito K_{ATP} and sarcolemma ATP sensitive potassium channels (sarc K_{ATP}) are suggested to be the putative main end-effectors of IPC. Interestingly, PKC also plays a pivotal role in the memory mechanism of preconditioning in the second window of protection (Zaugg et al, 2003). The mechanism of IPC in both phases is depicted in **Figure 1.3**.

1.2.1 Ischemic preconditioning and adenosine receptor

It is believed that the release of adenosine, bradykinin and opioids triggers IPC mechanism (Cohe et al, 2007, Yang et al, 2010). Among these triggers, adenosine is likely to be the most powerful trigger (Zaugg et al, 2003). It is found that pre ischaemic administration of exogenous adenosine can attenuate post ischaemic myocardial dysfunction (Randhawa et al, 1995, Sekili et al, 1995). Lui et al (1996) also demonstrated that the cardioprotective effect of IPC could be abolished by adenosine receptor antagonists in rabbit heart. Further investigation revealed the involvement of adenosine receptors in IPC mechanism. Although 4 different

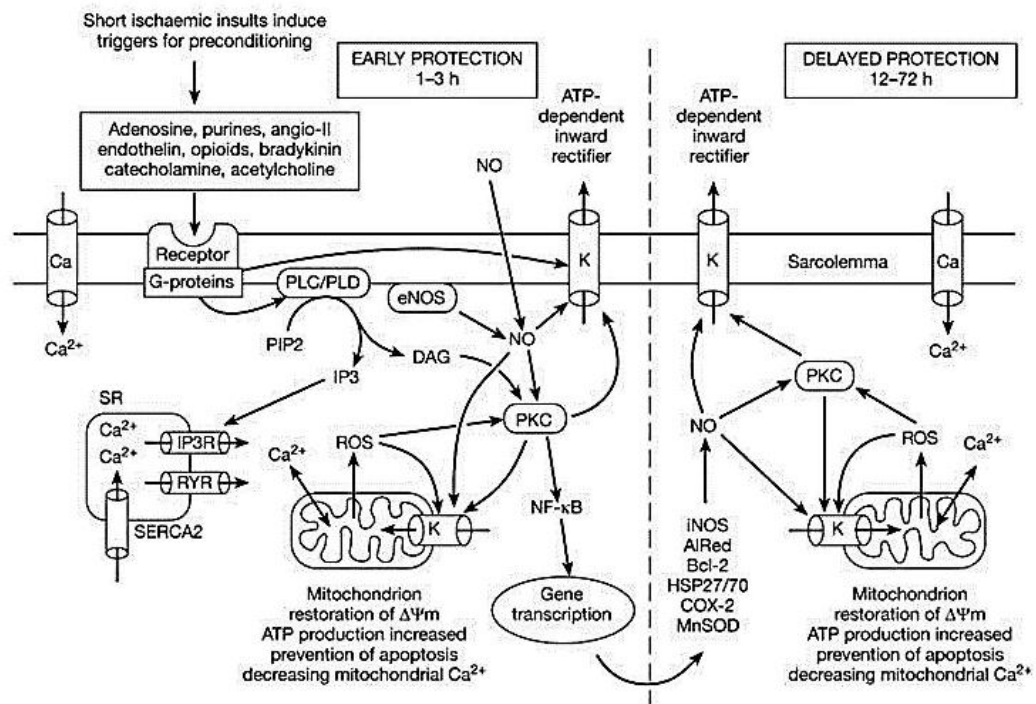


Figure 1.3 Schematic diagram showing a proposed IPC mechanism in early and delayed protection phases. The protective effect of IPC occurs in two periods which are early and delayed protection. PKC activation seems to be a centre of the mechanism in both periods, leading to activation of SarcK_{ATP} and mitoK_{ATP} channels. $\Delta\Psi_m$ =inner mitochondrial membrane potential; AlRed=aldose reductase; Bcl2=anti-apoptotic protein; Ca=sarcolemmal voltage-dependent Ca²⁺ channels; DAG=diacylglycerol; COX-2=cyclooxygenase type 2; eNOS=endothelial Nsynthase; G-proteins=heterotrimeric G-proteins; HSP27 and HSP70=heat shock proteins; iNOS=inducible NO synthase; IP3=inositol triphosphate; K=sarcolemmal and mitochondrial K_{ATP} channels; MnSOD=manganese superoxide dismutase; NF- κ B=nuclear factor- κ B; NO=nitric oxide; PKC=protein kinase PLC/PLD=phospholipases C and D; ROS=reactive oxygen species; RYR=ryanodine Ca²⁺-release channel; SERCA2=Ca²⁺ pump of the SR; SR=sarcoplasmic reticulum. (Taken from Zaugg et al, 2003).

receptor subtypes are thought to express in cardiac myocytes, only adenosine A_1 (A_1AR) and adenosine A_3 (A_3AR) receptors are believed to play a role in ischemic preconditioning (McIntosh and Lasley, 2011). Lui et al (1991) found that adenosine A_1 receptor agonists could reduce infarct size in rabbit heart. Moreover, it was also demonstrated that a selective A_1AR antagonist abolished the IPC cardioprotective effect in dog heart model (Auchampach, 1993). Lankford et al (2006) demonstrated that in A_1AR knockout mice, $A_1KO^{-/-}$ IPC did not decrease infarct size, whereas IPC could reduce the infarct size in WT, transgenic mice with increased cardiac A_1AR expression and in $A_1KO^{+/-}$ mice. They also found a strong correlation between A_1AR expression level and IPC effect. These studies suggest a role for A_1AR in IPC. However, Eckle et al (2007) did not find the absence cardioprotective effect of IPC in A_1AR knockout mice. They found a loss of IPC in $A_{2B}AR$ knockout mice instead. A role for A_3AR in IPC has been seen in rabbit. Lui et al (1994) demonstrated that IPC was abolished by A_3AR antagonist, BW-A1433. Moreover, A_3AR agonist, APNEA, could mimic IPC in rabbit heart. It was also found that highly selective A_3AR induced IPC in an experiment of Auchampach et al (1997).

The mechanism by which adenosine exerts cardioprotection has been revealed to some extent. One of hypothesis is that adenosine signalling may target ATP-sensitive potassium channels (McIntosh and Lasley, 2011). By using the patch clamp technique, it was found that application of adenosine could provoke K_{ATP} current both in whole cell and single channel configuration in rat ventricular myocytes (Kirch, 1990). Application of A_1AR agonist produced similar result to adenosine suggesting that the effect of adenosine was mediated via an A_1AR / G_i protein. However, the result of this latter experiment was inconsistent, since there were only 7 samples out

of 16 samples showing that characteristic (Kirch, 1990). Kim et al (1997) also showed an increasing K_{ATP} channel activity by A_1AR activation in rabbit ventricular myocytes. In contrast, it was also shown that A_1AR blocker could not alter the K_{ATP} opener-induced cardioprotection effect in ischemic rat and dog heart (Gross, 1997).

1.2.2 Ischemic preconditioning and PKC

The relation of PKC to IPC has been investigated over the last two decades. IPC is blocked by PKC inhibitors. For this reason, PKC is thought to be central to IPC mechanism. However, some points of the action of PKC are still elusive. The earliest studies hypothesized that the role of PKC relates to its translocation from cytoplasm to membrane (Cohen et al, 2000). It is thought that after activation of PKC by diacylglycerol and increased intracellular Ca^{2+} from phospholipase C activation or in response to NOS or ROS, isoform-specific and cytoskeleton-mediated translocation of cytosolic PKC to the membrane fraction occurs. It was found that PKC activity in the cytosol decreased whereas PKC in the particulate fraction increased after 10 minutes of ischaemic preconditioning (Zaugg et al, 2003). It was proposed that PKC translocation leads to phosphorylation and activation of $sarK_{ATP}$ and $mitoK_{ATP}$ (Zaugg et al, 2003). However, this hypothesis is still controversial. For example, one study found that there are no differences in subcellular distribution of total PKC in preconditioned and nonconditioned dog heart (Przyklenk, 1995). Armstrong et al (1996) found that the particulate fraction of PKC epsilon, alpha and zeta was not significantly different after preconditioning of rabbit cardiomyocytes with adenosine or 10 min ischaemia.

The possible mechanism by which PKC mediates its cardioprotective effect has been proposed by many groups. Recently, three explanations have been considered suggesting the role of PKC in IPC (Yang et al, 2010). Firstly, it is thought that PKCs in mitochondria, PKC ϵ 1 and 2, are responsible for the cells fate. It was shown that signalosome-induced PKC ϵ 1 activation leads to opening of mitoK_{ATP} channels, resulting in K⁺ influx, increased matrix pH and ROS production (Quinlan et al, 2008). At the same time, activation of PKC ϵ 2 can inhibit mitochondria permeability transition pore (mPTP) which prevents ATP depletion (Costa et al, 2008). However, Downey's group found that Chelerythrine, a non-selective PKC inhibitor, could not eliminate the A_{2B} adenosine receptor (A_{2B}AR)–mediated protective effect in rabbit heart (Kuno et al, 2007) which suggests that the mechanism may not mediated via PKC.

The second proposal indicated on the involvement of increasing adenosine level after IPC. Kitakaze et al (1995) demonstrated that IPC activated- PKC can increase ecto 5'nucleotidase activity. Therefore, IPC heart should have higher adenosine level than the naive heart. However, there are some arguments about this hypothesis, since the level of adenosine is quite difficult to measure.

The third hypothesis concerns about the adenosine receptor (AR) sensitivity. Kuno et al (2007) proposed that PKC activation increases A_{2B}AR affinity to adenosine, from low to high affinity. They found that a non-selective, potent A_{2B} adenosine agonist, NECA, can mimic the postconditioning effect and selective A_{2B}AR antagonists can abolish this effect.

1.2.2.1 PKC isoforms and ischemic preconditioning

There are at least 10 PKC isozymes which are classified into 3 subfamilies; the classical PKC (cPKC), the novel PKC (nPKC) and the atypical PKC (aPKCs). The cPKC, α , β I, β II and γ PKC, require the second messenger Ca^{2+} and diacylglycerol and the presence of phosphatidylserine for activation. The nPKC members, δ , ϵ and θ PKC, require diacylglycerol and phosphatidyl serine but not Ca^{2+} for activation. The third subtype comprises of λ/ι and ζ and requires a complex mixture of activators. (Mackay and Mochly-Rosen, 2001). It is believed that the localization and function of each PKC isozymes is unique. It is found that the existence of PKC isozymes in heart depends on species. Generally, PKC α , PKC δ and PKC ϵ are well documented for their expression in both neonatal and adult ventricular myocytes (Puceat et al, 1994, Rybin et al, 1997, Disatnick et al, 1994, Hudman et al, 2004) whereas nPKC is also found in neonatal and adult rat and rabbit heart (Erdbrugger 1997, Ping 1997). However, the existence of PKC β in the heart is still controversial (Rybin et al, 1994, Hudman et al, 2004).

It is still unclear which PKC isoforms are responsible for IPC. Hund et.al (2007) found critical effects on electrical coupling induced by ischaemic preconditioning by PKC epsilon but not PKC delta. Ostadal et.al (1998) found that PKC isoforms α , δ , η and ζ were expressed in the ventricular myocardium of neonatal rat heart after ischaemic preconditioning. Mitchell et.al (1995) also found the translocation of PKC α and δ isozymes in ischaemic preconditioned rat heart and translocation of PKC δ and ζ in rat hearts preconditioned with phenylalanine. Hudman et al (2004) found that PMA reduced reperfusion injury, improved functional recovery. Pretreatment

ventricular myocytes with PMA resulted in prolonged onset of contraction failure of cells in the presence of metabolic inhibition. Translocation of PKC- α , β , γ , δ , ι , λ / ζ to plasma membrane in response to PMA was observed (Hudman et al, 2004). Other studies reported the translocation of PKC α , δ and ϵ after IPC in rat hearts (Gray et al, 1997, Yoshida et al, 1997). The different findings may be due to differences in species studied and pathological stimuli. In human, Hassouna et.al (2004) demonstrated the role of PKC ϵ and PKC α in the signal transduction pathway of IPC. They proposed that the activation of PLC and PLD leads to PKC ϵ phosphorylation, which then causes opening of mito K_{ATP} channel. After that, ROS are released, which in turn activate PKC ϵ leading to activation of p38MAPK and the end effectors of IPC. However, the use of atrial myocardium and the limited selectivity of the PKC isoform blockers used were limitations of this study. A clear understanding of the PKC isoforms which are responsible for IPC and their role is still needs to be elucidated.

A role for isoform specific PKC regulation of mitochondrial function has been demonstrated. Pravdic et al (2009) found the involvement of PKC ϵ in delaying of mPTP opening in isoflurane induced IPC in rat heart. By using confocal microscopy, mPTP opening time was observed in volatile anesthetic-induced preconditioning (APC) in rat ventricular myocytes in the presence or absence of specific PKC inhibitor. It was found that in the presence of PKC ϵ inhibitor, prolong mPTP time induced by APC was abolished. Furthermore, it was also found that, in rat retinal model, PKC ϵ and PKC δ inhibitor could attenuate IPC effect mimicked by the opening of K_{ATP} channel by injection of diazoxide (Dreixler et al, 2008). A study

showed that in rat hippocampal mitochondria, where both Kir6.1 and Kir 6.2 subunits were found, PKC ϵ agonist ($\Psi\epsilon$ RACK) could mediate neuroprotection and mitoK_{ATP} inhibitor diminished that effect (Raval et al, 2007). This suggests the involvement of PKC ϵ and mitoK_{ATP} channel in IPC

1.2.3 Ischaemic preconditioning and K_{ATP} channels

In addition to PKC activity, K_{ATP} channels also play a key role in the mechanism of IPC. It is suggested that sarcolemmal and mitochondrial K_{ATP} channels (sarcK_{ATP} and mitoK_{ATP}) are putative end effectors of IPC (Zaugg et al, 2003). Activation of sarcK_{ATP} is suggested to result in shortening of the action potential, whereas activation of mitoK_{ATP} may bring about depolarization of the inner membrane leading to ROS production. The links between IPC and K_{ATP} have been investigated. Singh H (2002) found that the phosphorylation of cardiac K_{ATP} channels induced by adenosine A1 receptor agonist occurred on the Kir6.1 subunit and could be blocked by PKC inhibitors. He also found that PKC mediated phosphorylation of Kir 6.1 was mapped *in vitro* to serine residue 379. However, whether there is phosphorylation of SUR subunits is still unknown.

It is now clear that in the heart, K_{ATP} channels are heteromultimeric complexes, which comprise of four inwardly rectifying potassium channel subunits such as Kir6.1 or Kir6.2, and four sulfonylurea subunits such as SUR1, SUR2A or SUR2B (Aguilar-Bryan, 2000). It is most likely that Kir6.2 expresses dominantly in sarcolemma,

whereas Kir6.1 expresses predominantly in the mitochondria (Singh et al, 2003, Coung, 2005). Both sarcK_{ATP} and mitoK_{ATP} have been shown to play a role in IPC.

1.2.3.1 Role of sarcK_{ATP} channel in ischemic preconditioning

It is widely accepted that sarcK_{ATP} channel is a heteromultimer of Kir6.2 and SUR2A (Yokoshiki et al, 1998). However, both Kir6.1 and Kir6.2 have been found in the heart and also SUR1 and SUR2A (Singh et al, 2003, Bever et al, 2004, Isidoro, 2007). The role of sarcK_{ATP} channels in IPC is under debate. The finding that sarcK_{ATP} channel blocker HMR 1883 reduced diazoxide-induced recovered contractile function of rat ventricular myocytes suggests a role of sarcK_{ATP} channels in cardioprotection (Rodrigo et al, 2004). Suzuki et al (2002) showed that, in Kir6.2 knockout mice, IPC did not reduce the infarct size. Furthermore, the contractile function of WT heart was significantly impaired when treated with HMR 1098, a sarcK_{ATP} channel blocker comparable to that of KO mice. They also found that the cardioprotective effect of diazoxide, a specific mitoK_{ATP} channel blocker, was abolished in Kir6.2 knockout mice (Suzuki et al, 2003). It was suggested the cardioprotective effect of K_{ATP} channel openers was involved in shortening of action potential duration (APD) (Grover et al, 2000). Moreover, administration of glibenclamide, a K_{ATP} channel blocker, worsened postischemic dysfunction of barbitol-anesthetized dog heart with inhibition of the reduction of APD (Yao et al, 1993). These studies suggest a role for sarcK_{ATP} channel in IPC. However, there has been controversy surrounding the role of sarcK_{ATP} channels in IPC. Some studies demonstrated that shortening of APD was not necessary for IPC (Yao et al, 1994, Hamada et al, 1998). Hamada et al (1998), demonstrated that pinacidil and IPC improved regional contraction of anesthetized

dog heart and that did not shorten APD. The finding that diazoxide, a specific mitoK_{ATP} opener, reduced cellular injury as well as the finding that 5-hydroxydecanoic acid (5-HD), a specific mitoK_{ATP} could abolish IPC effect suggests a role of mitoK_{ATP} in IPC rather than sarcK_{ATP} (Liu et al, 1998, Sato et al, 2000).

1.1.3.2 Role of mitoK_{ATP} channel in ischemic preconditioning

The role of mitoK_{ATP} in IPC has been studied extensively. The finding that IPC was mimicked by diazoxide and blocked by 5HD supported the role for mitoK_{ATP} in IPC (Sato et al, 2000, Lui et al, 1998). Garlid et al (1997) also found that diazoxide and cromakalim, a K_{ATP} channel opener, improved postischaemic functional recovery of isolated rat heart with similar potency. However, diazoxide activated sarcK_{ATP} current less than that of cromakalim and also the shortening of APD. Both diazoxide and cromakalim could activate mitochondrial K_{ATP} activity. The cardioprotection of these agents was blocked by 5HD and glibenclamide. These findings suggest that IPC mechanism may be via mitoK_{ATP} channel.

1.3 Trafficking of membrane proteins: Overview

There are many processes involved in membrane protein trafficking as depicted in **Figure1.4**. Membrane proteins are synthesized, folded and checked at endoplasmic reticulum and then targeted to membranes via the trans-Golgi network (TGN). This is called the secretory pathway. However, membrane proteins are recycled or lysed by the endocytic pathway. The surface density of membrane protein on cell membrane is a result of both secretory and endocytic pathway.

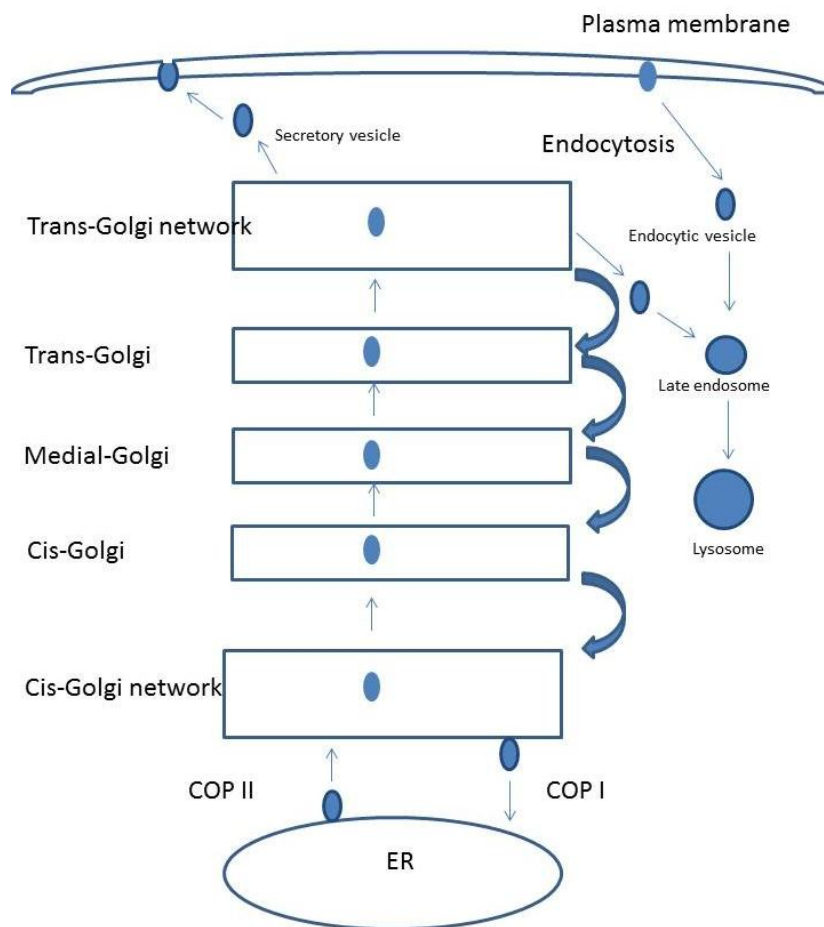


Figure 1.4 Schematic diagram showing membrane protein trafficking. The secretory pathway starts in the ER. The membrane proteins finally reach the plasma membrane via the trans-Golgi network. Coat protein II (COP II) is necessary for forming vesicle whereas the unqualified proteins are captured back from the Golgi complex to ER by coat protein I (COP I) vesicles. Membrane proteins can be internalized and traffic to their destinations by endocytosis.

1.3.1 Secretory pathway

The first crucial step of secretory pathway takes place in endoplasmic reticulum where proteins are synthesized, folded and quality controlled. After being checked, membrane proteins exit the ER via COP II-mediated budding and then traffic to Golgi apparatus-cis Golgi and Golgi trans network (GTN), then fuse to membranes. Although the mechanism of the trafficking is not fully understood, some processes are revealed. ER exiting of membranes protein requires coat proteins (COP) II which helps membrane proteins to form vesicles. The recognition of membrane proteins by COP II involves ER export motif of the proteins. The mechanism by which COP II recognize ER export motif is believed to be via a temperature-sensitive secretion(sec) 24 , component of COP II (Homan, 2009). To form vesicles, GTPase binding protein, Rab protein, together with V SNARE proteins are necessary for budding, targeting, docking and fusion process. When the cargo proteins are transported to target membrane, coat protein are uncoated and shedded causing the expose of V SNARE proteins to attach to target membrane. The transport from Golgi apparatus to plasma membrane is not well understood (Lodish, 2003).

1.3.2 Endocytic pathway

Endocytosis is a process that moves proteins from plasma membranes to the cytoplasm to be degraded or recycled. The endocytosis mechanism compose of a non-coat dependent and a coat independent pathway called clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). The mechanism of CME is well studied, whereas that of clathrin-independent endocytosis still needs more works to

clarify. However, both types bring the cargo to a common pathway via endosome to be sorted and then lysed or recycled.

Clathrin-mediated endocytosis

Clathrin mediated endocytosis requires the protein named clathrin to develop coated vesicles. The first step of this mechanism is forming clathrin coated pits (CCPs) and budding. This stage needs adaptor and accessory proteins, for example, Epsin, amphiphysin, to aid clathrin aggregation and assembly. Next, clathrin polymerization and Bin/Amphiphysin/Rvs (BAR) proteins work together for full formation of CCPs and invagination. Then, the membrane scission is made by the aids of dynamin, a large GTPase, which releases the vesicle (CCVs) into intracellular side. The coated vesicle then becomes uncoated by auxilin and hsc70. The naked vesicle further traffics to the appropriate destination (reviewed in Doherty et al, 2009). The mechanism of CME is depicted in **Figure 1.5**

The mechanism by which clathrin binds to lipid membrane has been revealed clearly. The membrane is not attached to clathrin directly. Adaptor proteins are required in this step. Clathrin interacts with adaptor proteins via a clathrin binding motif. In the other way, adaptor proteins bind to phosphatidylinositol(4,5) biphosphate (PtdIns4,5P₂) located in plasma membranes acting like the bridge between clathrin and cell membrane. The transmembrane protein cargo is also recruited by adaptor proteins. The selection of protein cargo is made by the recognition of internalization signals of the cargo by adaptor proteins. (Owen et al, 2011).

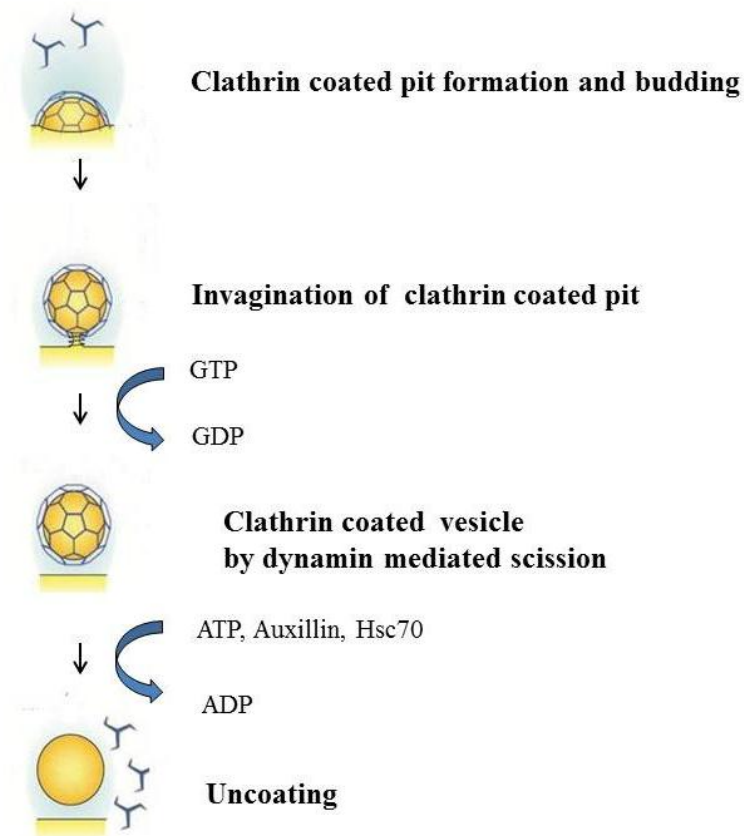


Figure 1.5 Schematic diagram showing the formation of clathrin coated vesicle (CCV). After Clathrin coated pit (CCP) has been formed, CCV formation is established and then released from the membrane. CCV requires uncoating proteins to produce naked CCV which will then be delivered to appropriate compartment. (Adapted from Doherty GJ, McMahon T, 2009)

Clathrin-independent endocytosis

The mechanism of clathrin-independent endocytosis (CIE) is not so clear. These pathways are caveolae-/caveolin1-dependent, clathrin-independent carrier/GPI-anchored protein-enriched early endosomal compartment (CLIC/GEEC), IL2R β pathway, Arf6-dependent, Flotillin dependent, macropinocytosis, circular dorsal ruffles and entosis. It is now known that these pathways are cholesterol dependent. More studies on these CIE are required to elucidate the role and responsibility of these pathways in protein trafficking.

1.3.3 Trafficking of K_{ATP} channels

The transport of ATP- sensitive potassium channels begins with the proper assembly of Kir6 and SUR subunits. To check the property of the assembly, Arginine-based endoplasmic reticulum localization signals (RKR motifs) presenting in both Kir6.2 and SUR subunits is needed (**Figure1.6A**). This checkpoint will not allow unassembled or inappropriately assembled channels to traffic to cell membranes. These ER localization motifs are detected by coatamer protein I (COPI) resulting in channel retrieval to endoplasmic reticulum. There is some evidence showing that these motifs are also recognized by 14-3-3 protein which in turn masks this motif from COP I binding. By using 14-3-3 scavenger, Heusser et al (2006) showed that in the presence of pGpLI-R18, a 14-3-3 scavenger, cell-surface expression of Kir6.2/SUR1 recombinant channel is reduced by more than 50% suggesting a role for 14-3-3 protein in channel trafficking. The involvement of arginine based motifs in the decrease in surface expression was also demonstrated. Physical association

between 14-3-3 proteins and K_{ATP} channels in INS-1-b cell was also demonstrated. Schwappach et al (2005) proposed the trafficking of K_{ATP} channels as **Figure 1.6B**.

Little is known about the endocytosis of K_{ATP} channels. It is still unclear whether the pathway is clathrin or caveolin-dependent. However, some studies indicated the role of caveolin in K_{ATP} internalization (Jiao et al 2008, 2009, Sampson et al, 2007, 2010, Cole et al, 2010). Sampson et al (2007) confirmed the existence of vascular K_{ATP} channels in caveolae. They found that angiotensin II modulated K_{ATP} channels by promoting translocation PKC ϵ to caveolae in aortic smooth muscle cells. Similarly, Jiao et al (2008) investigated the effect of caveolae disruption using methyl- β -cyclodextrin(M β CD), a cholesterol depleting drug, on the PKC-mediated inhibitory effect on vascular K_{ATP} channels. They found that M β CD treated cells showed less inhibition of PMA-induced K_{ATP} current inhibition implicating a role for caveolae. Moreover, overexpression of caveolin-1 in Kir6.2/SUR2B transfected HEK cells resulted in increased inhibitory effect of PMA on K_{ATP} current. This suggests a role for caveolin on channel function. Jiao et al (2008) also found the internalization of K_{ATP} channels by PKC to be dynamin dependent. In 2009, Hu et al demonstrated the localization of cardiac K_{ATP} channels (Kir6.2) in caveolin-enriched microdomains and colocalization of K_{ATP} channels with caveolin-3. In adult rat cardiac myocytes, the disruption of caveolae with M β CD could reduce adenosine-stimulated K_{ATP} channel current by >70% (Hu et al, 2009). Two possibilities have been proposed for the mechanism by which caveolin affects K_{ATP} channel activity. Firstly, caveolin plays a role in internalization of the channels. Secondly, caveolin directly interacts with K_{ATP} channels and then reduces channel open probability (Jiao et al, 2008, Davies et al, 2010, Cole et al, 2010).

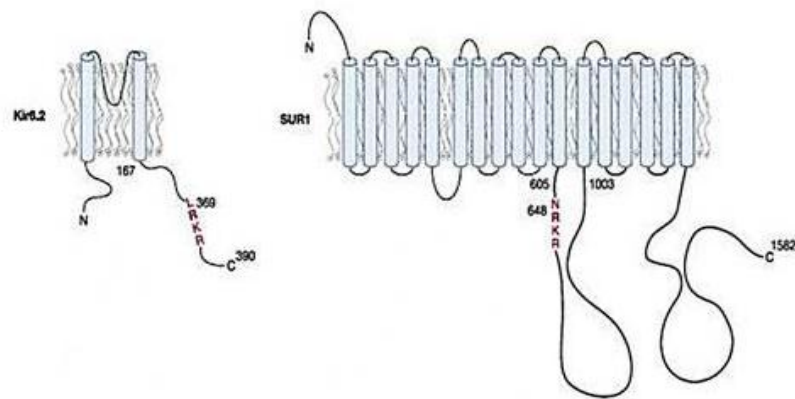
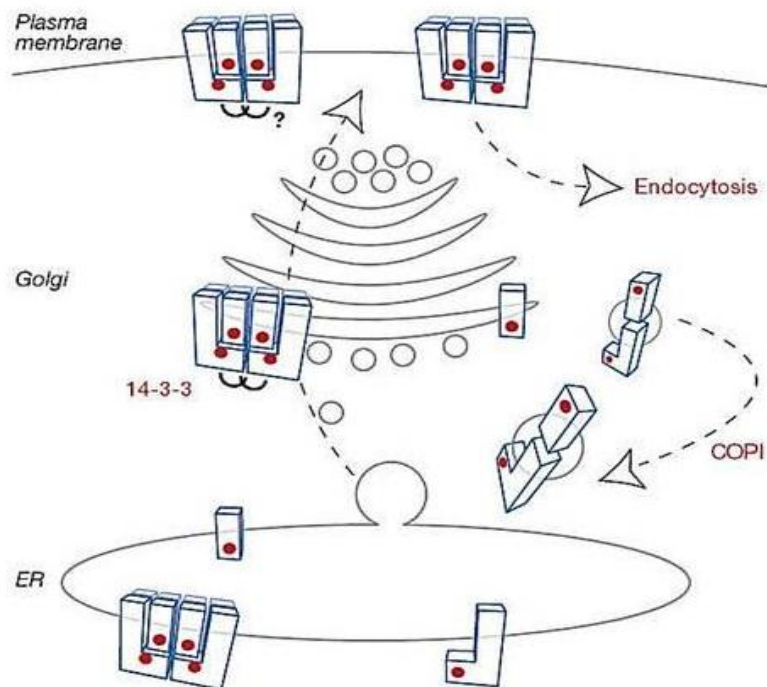
A**B**

Figure 1.6 Showing the arginine based motif and trafficking of KATP. A) The RKR motif in K_{ATP} channel subunits which presents on C terminus of both Kir and SUR subunits B) Trafficking of K_{ATP} channels. The assembly of Kir subunit (large rectangular shape) and SUR subunit (small rectangular shape) with 14-3-3 proteins can mask arginine based motifs (red dot) from recognition of COP I. The picture was taken from Husser and Schwappach, 2005.

1.4 Aims

A number of studies have indicated the involvement of PKC-mediated phosphorylation of K_{ATP} channels in ischaemic preconditioning. The pore-forming subunit which expresses in cardiac myocyte mitochondria, Kir 6.1, has been found to be a substrate for PKC-mediated phosphorylation (Singh, 2002, Shi, 2008a). Structural studies suggested that a serine residue in the cytoplasmic C-terminal of the subunit, S379, is a crucial site. To confirm its potential, the functional effect of PKC-mediated phosphorylation of this site is needed. Moreover, the mechanism by which such phosphorylation affects the localization and number of K_{ATP} is necessary to be investigated.

The main objectives of this project are:

1. To investigate the functional effect of PKC and PKC-mediated phosphorylation at proposed phosphorylation sites of relevant recombinant Kir6.1/SURx channels using the whole cell patch clamp technique. The baseline channel activity and PKC activation of wild type and mutant will be tested.
2. To investigate the effect of PKC on Kir6.1/SU2A trafficking using immunofluorescence and patch clamping.
3. To investigate which PKC isoform(s) is (are) responsible for A_1AR -mediated Kir6.1 phosphorylation by using radioactive labelling.

Chapter 2: Materials and Methods

2.1 Cell culture and transfection

Human embryonic kidney (HEK 293) cells were resuscitated from liquid nitrogen and then cultured in full growth medium, alpha-Minimal Eagle Medium (α MEM) containing 10% fetal calf serum and 10 mM L-glutamine (Gibco), and incubated at 37°C, 5% CO₂. Cells at passages 4-30 were used. The medium was changed every 3-4 days. The cells were split every 3-4 days and prepared for the next transfection. Cells were dissociated by mechanical vibration and suspended. Five millilitres of the cell suspension was aspirated and re-seeded into a 75 ml flask with 10 ml full growth medium. Sterilized coverslips were prepared by coating with poly-L lysine (1:10 dilution of 0.1%w/v Poly-L-Lysine). Then cells were plated in a 6 well plate containing coverslips at a density of 5×10^5 cells/well and incubated at 37 °C with 5% CO₂. When the confluence of the cells was 60-70%, which usually took 24-48 hours, the cells were transfected. Fugene® HD was used as transfection reagent. According to the manufacturer's guideline, the transfection was optimized and the best protocol determined. Two micrograms each of the EGFP-tagged DNAs, pIRES2-EGFP-F cKir6.1 or pIRES2-EGFP-F Kir 6.2 and pIRES-EGFP-F SUR2A or SUR2B DNA was added into 100 ml of serum free medium, (UltraMEM, Gibco). After that, 16 μ l of Fugene® HD was added into the DNA mixture, mixed well and incubated at room temperature for 15 minutes. DNA-reagent complex was added on to cells after changing the cell medium, mixed well and incubated at 37°C with 5% CO₂. The cells were used between 48-72 hours after transfection.

2.2 Molecular biology methods

2.2.1 Purification of plasmid DNA

Plasmids were purified using the QIA filter plasmid midi kit (Qiagen). Bacterial cells were harvested by centrifugation at 3000 x g for 30 minutes and the supernatant removed. The cells were resuspended with 6 ml buffer P1 (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and lysed by 6 ml buffer P2 (200 mM NaOH, 1% SDS (w/v)). The lysate was precipitated by adding 6 ml chilled P3 buffer (3.0 M potassium acetate, pH 5.5). To bind the DNA, the lysate was then passed through the anion exchange resin and washed with 20 ml buffer QC (1.0 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol(v/v)). Next, the DNA was eluted by using QF buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol (v/v) and then precipitated with 3.5 ml isopropanol. After that, the precipitated DNA was filtered using a midi filter and eluted by buffer 1 ml TE (10mM Tris-Cl, pH 8.0, 1 mM EDTA). Forty microlitres of sodium acetate (pH 5.2) was added, topped up with absolute ethanol (~ 1 ml) and kept at -20°C overnight. The next day, the mixture was centrifuged 13,000 rpm and the ethanol solution removed. The pellet was air dried and the plasmid DNA dissolved with 50 µl NANO water or 10mM Tris pH 8.0. Finally, the concentration of the DNA was measured using spectrophotometric analysis (Qubit, Invitrogen).

2.2.2 Construction of mutants

2.2.2.1 Construction of Kir6.1S379A, Kir6.1S385A, Kir6.1S397A,

The clones, S379A, S385A, and S397A mutant provided by Dr. D. Lodwick (Department of Cardiovascular Sciences, University of Leicester) were digested

with restriction endonuclease enzymes, *BspE1/BamH1* with the following ratio; 5 µg of DNA: 4 µl NEB 3 buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM Dithiothreitol): 3 µl *BspE1*: 1 µl of *BamH1* and NANO water in total volume of 40 µl, and incubated at 37°C overnight. After that, the digested DNAs were purified using MinElute reaction clean up kit (Qiagen). The digested DNA was then run on 0.8% agarose gel. The band of 1210bp was purified using a MinElute gel extraction kit (Qiagen) and kept as insert. In the meantime, pcDNA3.1/Kir6.1 was digested with *BspE1/BamH* (2.5 µg DNA: 4 µl NEB 3 buffer: 3 µl *BspE1*: 1 µl *BamH* and nanoH₂O in total volume of 40 µl), incubated at 37°C overnight. Subsequently, the digested DNA was treated with Antarctic phosphatase buffer (50 mM Bis-Tris-Propane-HCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, pH 6.0) at 37°C for 30 minutes to decrease the vector background and then heat shocked at 65°C for 10 minutes. The digested DNA was purified using MinElute reaction clean up kit, then run on 0.8% an agarose gel, the fragment of 5770 bp was purified using MinElute gel extraction kit kept as a vector. All DNAs were quantified by running 2 µl of each DNA on a 0.8% agarose gel and then visualized under UV cabinet linking to AlphaImager computer system to quantify DNA amount. Next, insert was ligated into vector using 90 fmol of insert and 30 fmol of vector with 4 µl of 5X ligase buffer and 1 µl of T4 DNA ligase in total volume of 20 µl, incubated at room temperature for 30-60 minutes. The control ligation which contained vector only was also set up at this stage. Competent cells, *E. coli* (DH5α, Invitrogen) were transformed by adding 2 µl of each ligation to 50 µl of DH5α, the mixture then incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds and kept on ice for 2 minutes. SOC medium (0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose) 950 µl was added and incubated on shaker at 37°C for 1

hour. The mixture was then spun hard for a short time, supernatant removed. The remaining transformation mixture was spread onto LB (1.0% Tryptone, 0.5% Yeast Extract, 1.0% Sodium, Chloride (NaCl), pH 7.0) plate containing ampicillin (1:1000) and incubated at 37°C for 16-18 hours. The selected colonies were then inoculated into 5 ml aliquote of LB medium containing Ampicillin and grown at 37°C with shaking overnight. The DNA was isolated using a miniprep kit (Qiagen). Some of constructs were checked by restriction digestion (approved by Dr.D. Lodwick). However, all constructs were sent to The Protein Nucleic Acid Chemistry Laboratory (PNACL, University of Leicester) for DNA sequencing with 1:100 cc6.1A primer.

2.2.2.2 Construction of Kir 6.1 S354A

Polymerase chain reaction (PCR) was set up with 61U/S354AR and S354AF/61D as primers and pcDNA3.1myc/hisA/kir6.1 as template. The mixture contained 10 µl of 5X Phusion HF reaction buffer (BioLabs), 1 µl of deoxynucleotidetriphosphates (dNTP), 0.5 µl of DNA polymerase (Pfu), 2.5 µl of each primer, 1 µl of template with Nano water in total volume of 50 µl. PCR reaction was allowed overnight (Hybaid PCR Sprint). The products was checked by running on a 0.8% Agarose gel and then purified with PCR purification kit (Qiagen). Products of 473 and 225bp, respectively, were kept as templates. Next, an overlap PCR using 61U/61D as primers and the templates (products of 473 and 225 bp) was performed. The mixture for PCR reaction was 10 µl of 5X Phusion HF reaction buffer, 1 µl of dNTP, 0.5 µl of DNA polymerase (Pfu), 2.5 µl of each primer, 1 µl of each template with Nano water in total volume of 50 µl. The mixture was allowed for PCR reaction for 2 hours. The obtained DNA was

purified by PCR purification kit and then run on a 0.8% agarose gel giving the band of 672 bp which was further purified by using gel extraction kit (Qiagen). Concentration of the DNA was calculated. The obtained DNA was digested with BstEII/BspE1. Briefly, 10 µl of DNA was incubated with 2 µl 10X buffer NEB3 (New England BioLabs), 1 µl of BspE1, 0.2 µl BSA, 5.8 µl Nano water, incubated at 37°C overnight. Then, 1 µl BstEII was added and incubated at 60°C for 4 hours. The DNA was further run on a 0.8% agarose gel and a band of 557bp was isolated and kept for ligation as insert. pcDNA3.1myc/hisA/kir6.1 was also digested. BstEII 1 µl mixing with 2 µl 10X buffer NEB3, 0.2 µl BSA and 5.8 µl Nano water was incubated with 10 µl of DNA for 4 hours at 60°C. After that, 1 µl BspE1 was added and incubated at 37°C overnight. The digested DNA was treated with Shrimp alkaline phosphatase (SAP) and run on 0.8% agarose gel. A band of 6423bp was isolated and kept for ligation (vector). The two products obtained from the digestion were ligated with T4 DNA ligase enzyme (30 ng insert, 57 ng vector, 4 µl 5X ligase buffer, 1 µl T4 DNA polymerase and 12.6 Nano water). DH5 α was transformed (see section 2.2.2.1) and selected recombinants was sent to PNACL for sequencing with BGHR sequencing primer (Invitrogen).

2.2.2.3 Construction of Kir 6.1 S391A

Generally, the process for the construction of Kir6.1S391A construction was the same as for Kir6.1S354A. PCRs were set up with 61U/S391AR and S391AF/61D as primers and pcDNA3.1myc/hisA/kir6.1 as template. Products were 583 and 115 bp respectively. Overlap PCR was set up with 61U/61D as primers and the products from previous PCRs (583 and 115 bp products) as template. The reaction

was loaded on a 0.8% agarose gel and a product of 67 bp was purified. The purified DNA then was digested with BstEII/BspE1 and a band of 557 bp isolated from an agarose gel. Digestion of pcDNA3.1myc/hisA/kir6.1 with BstEII/BspE1 and SAP treatment were carried out and a band of 6423bp was purified. DNAs of 557bp and 6423bp obtained from the previous step were ligated with T4DNA ligase and then transformed into *E. coli*, DH5 α competent cells. Selected recombinants were sent to PNACL for DNA sequencing with BGHR as primer.

2.2.3 *Reaction cleanup*

To purify double-strand DNA fragments from enzymatic reaction, MinElute reaction cleanup kit was used. Buffer ERC 300 μ l was added to the enzymatic reaction and mixed thoroughly. The sample was applied to the MinElute column placing in 2 ml collection tube and then centrifuged for 1 minute at 10,000 x g, the flow through discarded. The MinElute column was washed by adding 750 μ l buffer PE and the column was then centrifuged for 1 minute, the flow-through discarded. Additional centrifugation for 1 minute at maximum speed was applied. After that, buffer EB (10 mM Tris-Cl, pH 8.5) was added to the column to elute DNA.

2.2.4 *Gel extraction of DNA fragments*

To extract and purify DNA from a agarose gel, MinElute gel extraction kit (Qiagen) was used. DNA fragment was excised from the agarose gel and then weighed. Buffer QC (composition unknown) was added to the gel in volume ratio of 3:1 (100 mg of gel is $\sim$ 100 μ l), incubated at 50°C for 10 minutes. After the gel

has dissolved, 1 gel volume of isopropanol was added and mixed thoroughly. The sample was applied to the MinElute column which was placed in a 2 ml tube to bind DNA and then centrifuged for 1 minute. The flow-through was discarded. The MinElute column was further spun at 10,000 x g for 1 minute and placed in a clean 1.5 ml tube. PDNA was eluted by adding 10 µl of buffer EB. The purified DNA was obtained by leaving the column stand for 1 minute and further centrifugation for 1 minute.

2.2.5 PCR purification

PCR purification was attempt to purify double-strand DNA fragments from PCR reaction. MinElute reaction cleanup kit was used. Buffer PB (composition unknown) was added to the PCR reaction in the ratio of 5:1 and mixed thoroughly. The sample was put into the MinElute column placing in 2 ml collection tube and then centrifuged for 1 minute at 17,900 x g. The flow through was discarded. The MinElute column was washed by adding 750 µl buffer PE and the column was then centrifuged for 1 minute and the flow-through discarded. Additional centrifugation of the column for 1 minute at maximum speed was applied. Then, buffer EB (10 mM Tris-Cl, pH 8.5) was added to the column to elute DNA.

2.2.6 Agarose gel electrophoresis

To make agarose gels, 0.8% Agarose was mixed with TAE solution (2 M Tris-Acetate containing 50 mM EDTA and 1.74 M glacial acetic acid, pH 8.5) with 5% ethidium bromide. The solution was then poured into horizontal gel cast and

allowed to set.. DNA was loaded into precast wells in the gel. The gel was run in TAE solution with 5% ethidium bromide at 95 V for 1 hour. DNA bands were then visualized under UV light by using MultiImage Light cabinet and Alpha imager software (AlphaInnotech).

2.3 Electrophysiological recordings

2.3.1 Whole-cell patch clamp technique

Patch clamp recording was performed at 25-28°C on the stage of a fluorescent inverted microscope (Nikon). Transfected HEK cells were bathed and continuously perfused in the chamber containing bath solution (135 mM NaCl, 6 mM KCl, 0.33 mM NaH₂PO₄, 5 mM Na pyruvate, 10 mM glucose, 10 mM HEPES, 1 mM MgCl₂, 2 mM CaCl₂, pH 7.4). Cells expressing EGFP-F, which were green, were selected. Junction potentials were adjusted to zero when the pipette entered the bath solution. The selected cell was attached to a fire-polished micropipette having a resistance of 3-6 MΩ, filled with pipette solution (140 mM KCl, 2 mM MgCl₂, 2.5 mM EGTA, 10 HEPES, 1 mM CaCl₂, 3 mM K₂ATP, 100 μM NaADP, 100 μM GTP, pH 7.2) using a micromanipulator. The free calcium concentration in pipette solution was ~ 90 nM (using online standard software, <http://maxchelator.stanford.edu/CaEGTA-NIST.htm>). After a giga-seal was established, gentle suction was applied to go into the whole cell configuration. The membrane capacitance and series resistance were compensated. The protocols used to obtain currents in current recording experiments were constant voltage clamp or step pulse protocols. The constant voltage clamp protocol clamped the membrane at 0 mV constantly and recorded the current continuously. The step pulse protocol held the membrane potential at -40 mV and then stepped to various

desired voltages. In this experiment, the pulse train stepped from -100 mV to +80 mV in 20 mV increments lasting for 150-200 ms. The application of activators and/or inhibitors to the bath solution was made via a perfusion system. Recordings were obtained using an Axopatch 200B amplifier (Axon Instruments) and a Digidata A to D convertor (Digidata 1200). Whole cell currents were recorded and displayed using p-clamp 10 software. The data were expressed as current (pA) or current density when normalized with cell capacity (pA/pF).

2.4 Phosphorylation experiment

2.4.1 Isolation of rat cardiac myocytes

Adult male Wistar rats were used to prepare rat cardiomyocytes. Rats weighing around 250-300 grams were sacrificed by cervical dislocation. The heart was then removed quickly and placed into cold Ca^{2+} -free Tyrode solution (135 NaCl, 5 KCl, 0.33 NaH_2PO_4 , 10 mM Glucose, 5 Na Pyruvate, 10 MgCl_2 , 10 HEPES, 2 mM EGTA, pH 7.3), subsequently mounted on a Lagendorff reverse perfusion system. The ischaemic time in this step was normally less than 5 minutes. The heart then was retrogradely perfused with warm (37°C) Ca^{2+} -free Tyrode solution for 6 minutes. The solution used to perfuse the heart was bubbled with 100% O_2 throughout the experiment. To isolate cardiac myocytes, the heart was digested by perfusing warm Ca^{2+} free Tyrode solution with Type I collagenase from *Clostridium histolyticum* and protease type XIV from *Streptomyces griseus* and bovine serum albumin (30 ml of Ca^{2+} free solution, 50 mg BSA, 20 mg Protease, 30 mg Collagenase type I) for 8-10 minutes. Next, the heart was flushed with warm normal Tyrode solution (135 NaCl, 5 KCl, 0.33 NaH_2PO_4 , 10 mM Glucose, 5 Na Pyruvate, 10 MgCl_2 , 10 HEPES, 2 mM CaCl_2 , pH 7.3) for 3 minutes and

then the atria were removed before releasing the ventricle into a flask. Isolation of ventricular myocytes was completed by shaking the ventricles in 37°C water bath for about five minutes. Then the Tyrode solution containing cardiac myocytes was kept and the remaining ventricles were further repeatedly shaken as before for 6-8 times yielding 6-8 fractions of cardiac myocytes. All fractions then were sieved and placed in test tubes. Cell viability was evaluated by using light microscopy. The cells in the fraction were allowed to settle and then washed with normal Tyrode solution. The ventricular myocytes were transferred to test tubes and kept on ice and transferred to the phosphorylation laboratory immediately (within 15 minutes).

2.4.2 Phosphorylation of Kir6.1 in response to CCPA stimulation in isolated rat ventricular myocytes

Rat ventricular myocytes were pooled in a test tube and allowed to settle for 10 minutes. After that, the cells were washed twice with phosphate free solution (135 mM NaCl, 5 mM KCl, 10 mM Glucose, 5 mM Na-pyruvate, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, pH 7.3). The cells then were examined for viability and counted. A number of cells, at least, 1×10^6 , with more than 60% viability was required in this experiment. The cells were then incubated with 37 MBq ³²P-orthophosphate (radioactive specific activity) 37°C for 2 hours. The cells were divided into 10 fractions and 100 nM of PKC inhibitors and activators were added as detailed in chapter 7 for 10 minutes at 37°C. After incubation, cells were centrifuged at 10,000 rpm in a cooled benchtop centrifuge and ³²P removed by aspiration. and the cells were washed twice with 1 ml phosphate free solution containing phosphatase substrates and inhibitors (phosphate free Tyrode buffer

containing 1 mM EDTA, 1mM EGTA, 1 mM Na orthovanadate, 10 mM Na glycerophosphate, 50 mM NaF, 5 mM Na pyrophosphate, pH 7) by centrifugation of samples at 10,000 rpm for a short time. The cells were then lysed with 0.2 ml cold lysis buffer containing protease inhibitor (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na orthovanadate (added just before use), 10 mM Na glycerophosphate, 50 mM NaF, 5 mM Na pyrophosphate, 0.1 % protease inhibitor cocktail), centrifuged at 13,000 rpm and the supernatant was removed for immunoprecipitation. Kir6.1 was immunoprecipitated with 4 µl anti-Kir6.1 antiserum and 5 mg 1:1 slurry of protein A-Sepharose in 0.15 ml of supernatant. The samples were incubated at 4°C on roller overnight. After that, the beads were spun down and supernatant removed. The beads were washed 4 times with solution containing 0.1% Nonidet-P40, 500 mM KCl, 20 mM Tris pH 7.4 and incubated with denature buffer for 30 minutes at room temperature. The samples were spun for 1 minute at 13,000 rpm, supernatant removed for electrophoresis. The elution was loaded on 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gels were fixed and dried and exposed to Kodak autoradiography film. The optical intensity of the band corresponding phosphorylated Kir6.1 was measured by densitometry and normalized to control phosphorylation by CCPA alone.

2.4.3 Polyacrylamide Gel electrophoresis

Running gels were poured between minigel plates from a mixture of 6.5 ml distilled H₂O, 5.32 ml 30% acrylamide solution (Bio-Rad), 4 ml 1.5M Tris HCl pH 8.8, 160 µl 10% sodium dodecyl sulfate (SDS), 160 µl 10% ammonium peroxidisulphate (AMPS), 40 µl Tetramethylethylenediamine (TEMED). Poured

gels were gently layered with industrial methylated spirit (IMS) and allowed to polymerise for 1 hour at room temperature. Stacking gels, composed of 6.1 ml distilled H₂O, 1.3 ml 30% acrylamide solution, 2.5 ml 1M Tris HCl pH 6.8, 100 µl 10% SDS, 100 µl 10% AMPS and 40 µl TEMED, were poured put on top of the running gel where IMS removed and a comb inserted to form loading wells. . After well setting, the gel was put in to electrophoresis tank containing running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS). After loading of samples, electrophoresis was performed at 150 V for 45 minute with molecular weight standard.

2.5 Immunofluorescence assay

2.5.1 Cell preparation

HEK 293 cells were plated in 6 well plates containing 13 mm round poly –L- Lysine coated coverslips at 1×10^5 cells/well. The cells were allowed to grow up in α MEM with growth supplement medium (see section 2.1) overnight or until a confluency of 70% had been reached. The cells then were transfected with 2 ug of Kir6.1 HA tag Ds- Red and 2 µg of pIRES-2 SUR2A Ds-red using Fugene HD transfection reagent (see section 2.1 for the transfection protocol). To locate cell membranes, pcDNA3.1 myc hisA mCherry-F was co-transfected with the above DNAs. The cells were left to grow for 72 hours then were processed for immunodetection of HA label.

2.5.2 Staining of Kir6.1-containing channels

Protocol I: Labelling of cell-surface Kir6.1-containing channels

To detect surface Kir6.1 containing channels, transfected HEK 293 cells were washed once with normal Tyrode solution at 4°C and then treated with 100 nM phorbol-12 myristate 13 acetate (PMA) or PMA plus 60 µM dynasore, a dynamin inhibitor, for 5 or 30 minutes. After 3 washes, the cells were fixed with 4% paraformaldehyde for 25 minutes and 100 mM glycine for 25 minutes. To block non-specific proteins, 5% BSA in Tyrode was put onto the cells for 1 hour. The cells then were incubated with 1:250 primary antibody (Rat anti-HA, Roche) overnight. After washing, cells were incubated with 1:250 secondary antibody (anti-rabbit IgG FITC conjugate, Roche) for 2 hours in the dark. All steps were conducted at 4°C. Immunolabelled cells were then washed three times with chilled normal Tyrode solution. The coverslips of labelled cells were mounted to slide using mounting medium (VECTASHIELD®) and subjected to confocal microscopic study.

Protocol II: Labelling of Kir6.1-containing channels for detection of channel

internalization

Generally, the protocol was similar to protocol I except for the sequence and the temperature of each step. HEK 293 cell expressing Kir6.1-HA channels were blocked with 5% BSA at 4°C for 30 minutes. After that, primary antibody was incubated at 4°C and left overnight. After washing, secondary antibody was incubated at 4°C for 2 hours in the dark. Cells then were washed thoroughly for 3 times and treated with PMA or dynasore at 37°C for 5 or 30 minutes. Finally, after extreme washing the cells were incubated with paraformaldehyde and glycine.

The solution, concentration of antibodies and chemicals used in this protocol were the same as protocol I.

2.5.3 Confocal microscopy

Immunolabelled cells were visualized using a laser scanning confocal microscope (Nikon C1si) under oil-immersed 60x objective lens. The images of cells expressing Kir6.1 which was labeled with FITC (495 nm peak excitation, 519 nm peak emission) were obtained using 488 nm filters of an argon laser whereas the plasma membrane stained with mCherry-F (587 nm excitation, 610 nm emission) was visualized with a 561 nm filters. The images were analyzed by EZ-C1 acquisition and analysis software.

Quantification of green fluorescence intensity was performed using EZ-C1 software. The mean pixel intensity of single cells was measured at a region of interest (intracellular side) and then normalized by the mean pixel intensity of the background measured proximal to the cell.

2.6 Materials

Chemicals were obtained from Sigma-Aldrich unless stated. P1075 was purchased from Torcis, Vectashield mounting medium from Vector laboratories, ³²P-orthophosphate from PerkinElmer. Rat anti-HA High affinity was obtained from Roche. Borosilicate capillary glass and all glassware used in patch clamp

experiment were from Harvard Apparatus. Coverslips were from Warner Instruments.

2.7 Data analysis and statistics

All data are shown as mean $\pm$ S.E.M. The analysis of the data was done using Graphad Prism5 software. The relative current was calculated from the current over the entire time divided by the maximal current. The current density was calculated from the current divided by membrane capacitance. The reversal potential was calculated from the Nernst equation. In phosphorylation experiment, the data are normalized by that of control. Statistical comparison between two groups was done using two tailed, unpaired t-Test or Wilcoxon signed rank test depending on characteristics of the data. To evaluate the differences between three or more groups One way ANOVA following by Dunnett post-test was used. A p value of <0.05 was considered as significant.

Chapter3: Optimization of electrophysiological recording conditions for a novel Kir6.1/SUR2A subunit combination

The finding that Kir6.1 is present in mitochondria (Raval, 2007, Coung, 2005, Singh, 2003) led to Kir6.1 being proposed as a pore-forming subunit of mito K_{ATP} channels. By using specific subunit antisera to localize K_{ATP} channels in rat ventricular myocytes, Singh et al found Kir6.1, Kir6.2 and SUR2A in mitochondria (Singh et al, 2003). In a study to identify whether K_{ATP} subunits in ventricular myocytes were substrate for phosphorylation, Kir6.1 was shown to be the major substrate for both protein kinase A and protein kinase C. A surprising observation was that phosphorylated Kir6.1 was immunoprecipitated by anti-SUR2A antibody, as well as Kir6.2. No evidence was found for co-immunoprecipitation of Kir6.1 with Kir6.2. This suggests that Kir6.1/SUR2A may be the subunit combination susceptible to regulation during IPC and that it may be the subunit combination responsible for the formation of mitoK_{ATP} channels, for which a definite identification remains elusive. The study on this subunit combination channel was very rare (Kono, 2000).

The involvement of PKC in Kir6.1 phosphorylation has been studied to some extent. It was shown that Kir6.1 phosphorylation was mediated by PKC and the phosphorylation site was serine 379 *in vitro* (Singh, 2000). Shi et al (2008a) demonstrated that PKC phosphorylation sites of Kir 6.1 were S379, S385, S391 and S397. However, further investigation showed that the potential residues for functional response of vascular type K_{ATP} (Kir6.1/SUR2B) channel to PKC were S354, S379, S385, S391 and S397. Little is known about Kir6.1/SUR2A channel activity and also

its response to PKC. The potential of proposed PKC phosphorylation sites on channel activity remains unknown. To explore this discrepancy, the effect of PKC phosphorylation on the function of wild type and mutant Kir6.1/SUR2A was further investigated. Optimization of transfection and basic electrophysiological characterization of the novel subunit combination was required. Recombinant Kir6.2/SUR2A was selected as a positive control since it has been well studied and its properties well characterized. The objective of this part of study was to enhance the transfection conditions for functional Kir6.1/SUR2A channel. HEK 293 cells were transfected with Kir6.2/SUR2A to determine the optimal condition and then these conditions were applied to Kir6.1/SUR2A channel. The expression and the function of recombinant channels were assessed by fluorescence microscopy and the patch clamp technique, respectively.

3.1 Functional expression of Kir6.2/SUR2A

HEK 239 cells were used because of their low endogenous current (Ammala et al, 1996). Transfection of DNA was achieved using FugeneHD following the manufacture's guideline. It was found that using the proportions of Kir: SUR: transfection reagent in 1:1: 3.5-4 resulted in the best transfection efficiency, with minimum toxicity. Transfection with 1 µg of each DNA yielded around 70 % transfection efficiency. Green fluorescence was observed in transfected cells from the first day after transfection but they were not bright. Cells on the second or third day after transfection were used routinely in electrophysiological study. The whole-cell configuration was performed by clamping the membrane potential at 0 mV constantly. After breaking into the cells, membrane capacitance and series resistance were

compensated and then the baseline currents were recorded. Subsequently, a K_{ATP} channel opener, 100 μ M P1075, was perfused to the cell and current was recorded again. To test the response of the channels to sulphonylureas, 10 μ M glibenclamide, a specific K_{ATP} channel blocker, was applied after the peak current was seen. The recordings showed that there was no baseline channel activity at 0 mV. In this condition, P1075 could activate the channel with tiny currents and glibenclamide abolished the P1075– stimulated current effectively. The averaged peak current was 66.5 ± 21.4 pA (n=4), whereas the averaged current density was 13.1 ± 1.2 pA/pF (n=3). A representative current is shown in **Figure 3.1A**

To obtain more functional channels, the transfection protocol was modified. The amount of each DNA was increased to 2 μ g each with 14 μ l of transfection reagent. The transfection efficiency and the brightness of the cells remained the same as that of previous protocol. The same electrophysiological protocol was applied. As before, no baseline current was observed at 0 mV but P1075 activated a much greater K_{ATP} channel current, resulting in mean current of 2159 ± 300.9 pA. When cells were transfected with 2 μ g of each DNA, the current density of was 281.9 ± 40.9 pA/pF (n=8). The P1075-activated current was then eliminated completely with glibenclamide (10 μ M). Comparison of the currents and current density obtained from the cells transfected with different protocols is shown in **Figures 3.1 and 3.2**.

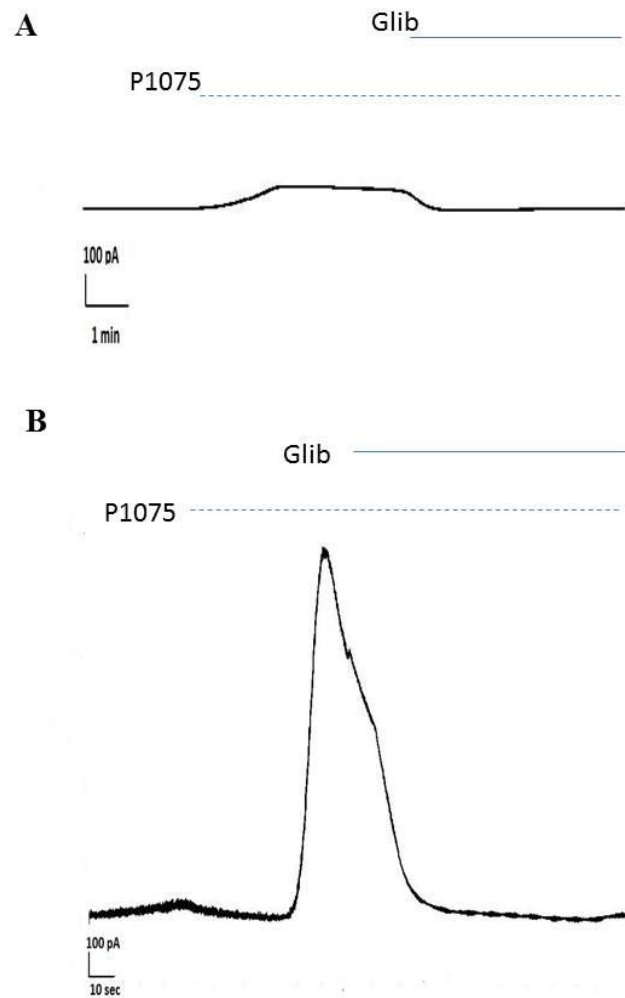


Figure 3.1 Representative currents obtained from HEK 293 cells transfected with different amounts of DNA encoding Kir6.2 and SUR2A subunits. A)

Representative current from the channels resulting from expression with 1 μ g of each DNA (B) Representative current obtained from the cell transfected with 2 μ g of each DNA. Currents were activated by addition of 100 μ M P1075 and blocked by addition of 10 μ M glibenclamide.

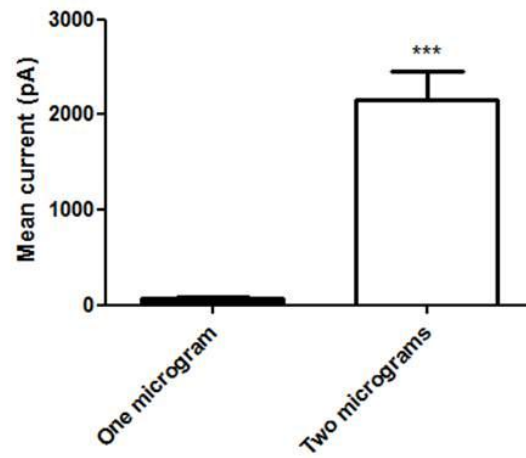
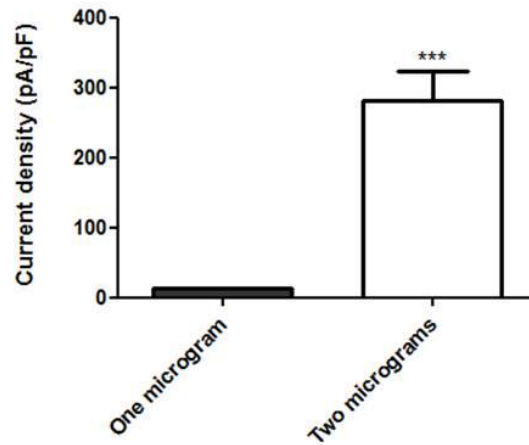
A**B**

Figure 3.2 Comparison of mean current and current density of HEK 293 cells transfected with different amounts of DNA encoding Kir6.2 and SUR 2A subunits A) Comparison of mean current obtained from HEK cells transfected with 1 and 2 μg of each DNA ($n=4, 8$, respectively). B) Currents obtained on expression of Kir6.2 with SUR2A were corrected for cell capacitance and the current density given as pA/pF ($n=3$ for $1\mu\text{g}$ and 8 for $2\mu\text{g}$).

3.2 Functional expression of recombinant Kir6.1/SUR2A channel

To study the function of Kir6.1/SUR2A channel, HEK 293 cells were transfected with 2 μ g of Kir6.1 and 2 μ g of SUR2A. Since there have been few studies of the Kir6.1/SUR2A subunit combination (Kono et al, 2000), currents obtained from Kir6.1/SUR2A channel were further characterized by determination of the current voltage relationship and investigation of the response to K_{ATP} modulators. It has been shown previously that intracellular uridine diphosphate (UDP) is necessary for Kir6.1/SUR2A channels to be activated (Kono et al, 2000). For this reason, the modulation of the channel by UDP was also determined using the whole-cell patch clamp technique, both by constant or stepped membrane potential clamping. In constant clamping model, the membrane potential was held at 0 mV constantly throughout the recording. To obtain the current-voltage relationship, the membrane potential was stepped to various levels. Briefly, the membrane potential was held at -40 mV and then the pulse test was started from -100 mV with 20 mV increments until membrane potential reached +40 mV. Current pulses lasted for 150-200 ms. To test the effect of intracellular UDP, currents in the absence or presence of 10 mM UDP in the pipette solution were recorded.

The transfection efficiency given by the proportion of green fluorescent cells of recombinant Kir6.1/SUR2A channels did not differ from that of Kir6.2/SUR2A (70%), however this subunit combination was not active when the membrane potential was clamped at 0 mV. Application of P1075 in the absence of UDP resulted in a small outward current. The averaged peak current was 189.4 ± 71.4 pA and the mean current density was 23.6 ± 7.9 pA/pF ($n=3$). When 10 mM UDP was added into

pipette solution, there was still no current at 0 mV, but on activation by 10 μ M P1075 the currents varied greatly, from 202.8 pA to 1173.4 pA. The averaged current was 715.1 ± 416.6 pA, whereas the mean current density was 61.6 ± 41.5 pA/pF (n=10). These currents were abolished completely by 10 μ M glibenclamide. However, it was noted that only 50% of successfully Kir6.1/SUR2A transfected cells could be activated by pinacidil (72 out of 144 cells) even in the presence of UDP whereas a 72 % of that of Kir6.2/SUR2A channel was observed (14 out of 18 cells). An example of Kir6.1/SUR2A currents is shown in **Figure 3.3**. The size of the peak current in the presence of UDP was significantly different from current recorded in its absence (715.1 ± 416.6 versus 189.4 ± 71.4 pA, $p = 0.02$, n=3, 5). The current density of the two groups was also significantly different (61.6 ± 41.5 versus 23.6 ± 7.9 pA/pF, $p = 0.03$, n=3, 5). The comparison of the maximal current and the current density in the absence or presence of UDP was shown in **Figures 3.4**.

3.3 Current-voltage relationship of Kir6.1/SUR2A current

To characterize Kir6.1/SUR2A currents further, the current-voltage (I-V) relationship was obtained by using an episodic recording protocol. The channel was stimulated by 100 μ M P1075 and inhibited by glibenclamide. Without P1075 activation, the Kir6.1/SUR2A channel responded moderately to various steps of applied voltage. The maximum current was usually obtained at the most positive membrane potential applied at +80 mV. The averaged maximum current was 283.4 ± 71.5 pA. Although some of the currents showed weak inward rectification, the averaged I-V relationship did not represent that characteristic (n=5). The averaged reversal potential was ~ -40 mV. Perfusion of P1075 to bath solution could increase the currents in some extent.

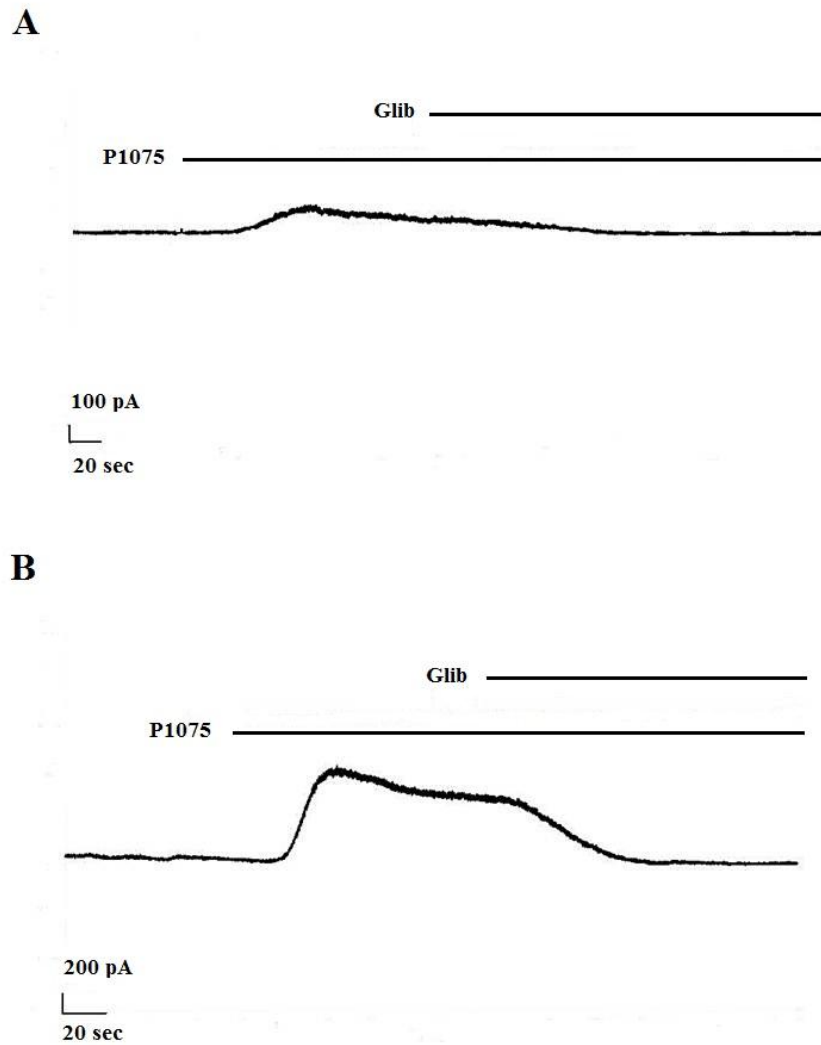


Figure 3.3 Representative currents recorded from the Kir6.1/SUR2A channels in the absence and presence of intracellular UDP. *A) Representative current recorded in the absence of intracellular UDP. B) Representative current recorded in the presence of 10 mM intracellular UDP. Currents were activated by the addition of 100 μ M P1075 and inhibited by 10 μ M glibenclamide.*

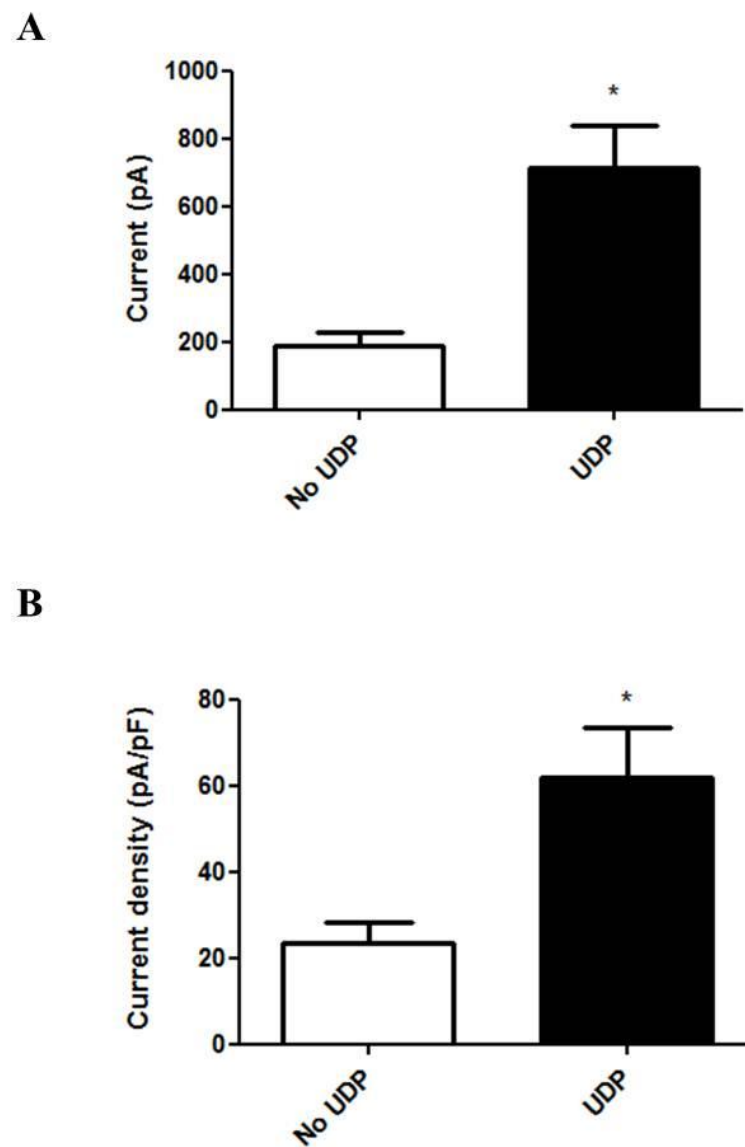


Figure 3.4 Comparison of Kir6.1/SUR2A channel activity activated by P1075 in the absence and presence of intracellular UDP. A) The difference in maximal current B) The comparison of the current density in the absence and presence of UDP (n=3 for No UDP and 5 for UDP group).

The averaged P1075-activated maximum current was 496.35 ± 114.82 pA (n=4). The representative of the currents and I-V relationship was shown in **Figure 3.5**.

3.4 The rundown of Kir6.1/SUR2A current activated by P1075

It was noticed that the currents activated by 1075 had some degree of running down. To characterize the Kir6.1/SUR2A channel rundown further, the currents activated by P1075 were recorded over 10 minutes at the holding potential of 0 mV (n=3) and the rundown observed currents were normalized to the peak current in each trace and the relative current determined for each minute in the traces. The averaged relative current declined to 0.37 ± 0.1 at the 5 th minute and 0.22 ± 0.01 at the 10 th minute after peak current was achieved (n=3 and 2 respectively). A representative current showing the rundown and the relative current over time are shown in **Figure 3.6**

To investigate whether rundown of Kir6.1/SUR2A channels depended on channel activation by 1075, the stability of current in the presence of a second K_{ATP} channel opener, pinacidil, was investigated. Pinacidil (100 μ M) was added to the bath solution after going whole cell and then the current was recorded according to the gap free mode protocol. It was shown that pinacidil could stimulate the channel and that while present, rundown of current was more limited. The averaged pinacidil-activated maximum current was 263.8 ± 50.6 pA, whereas the current density was 155.2 ± 53.6 pA/pF. The remaining current was 85 ± 1 % and 68 ± 4 % of maximum current at 5 and 10 minutes respectively (n=4). An example of this current is shown in **Figure 3.7**

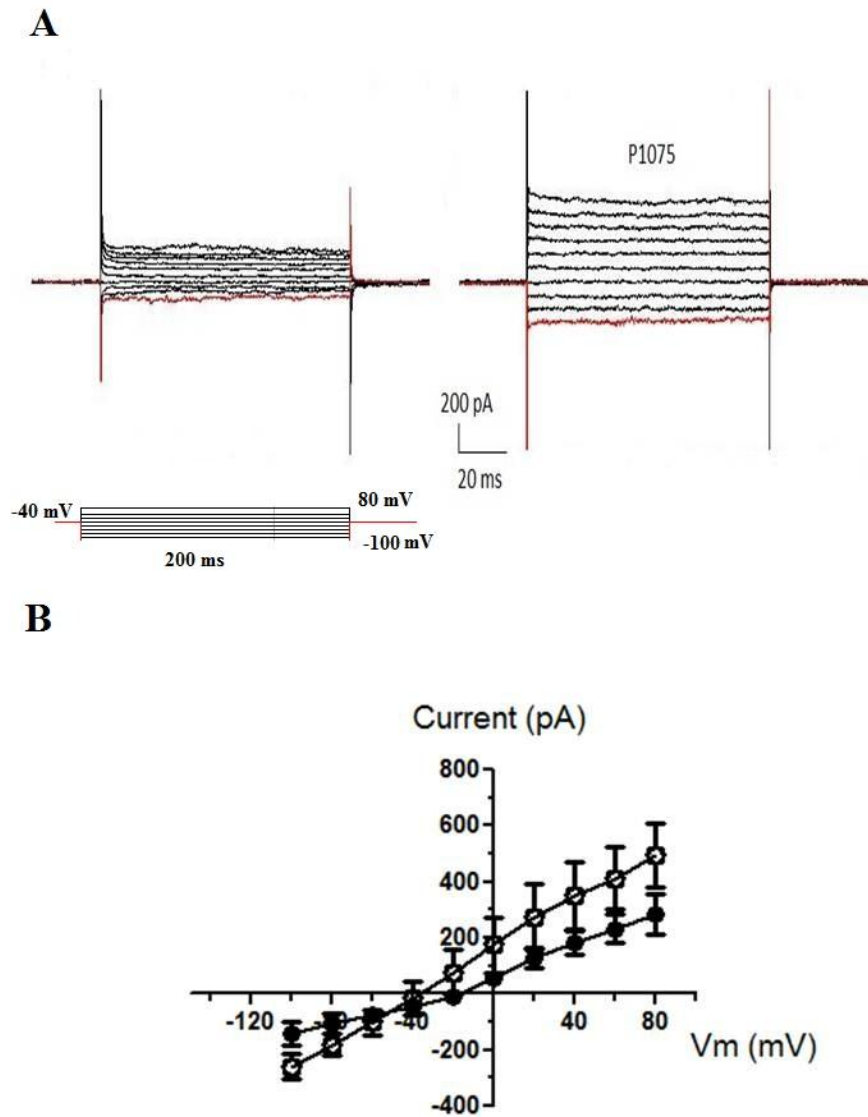


Figure 3.5 The response of Kir6.1/SUR2A to various membrane potentials in the absence and presence of P1075 A) The currents obtained from a cell using the episodic protocol B) The averaged I-V relationship of Kir6.1/SUR2A currents obtained in the absence and presence of P1075 activation (n=6, black circle=control, white circle= with P1075).

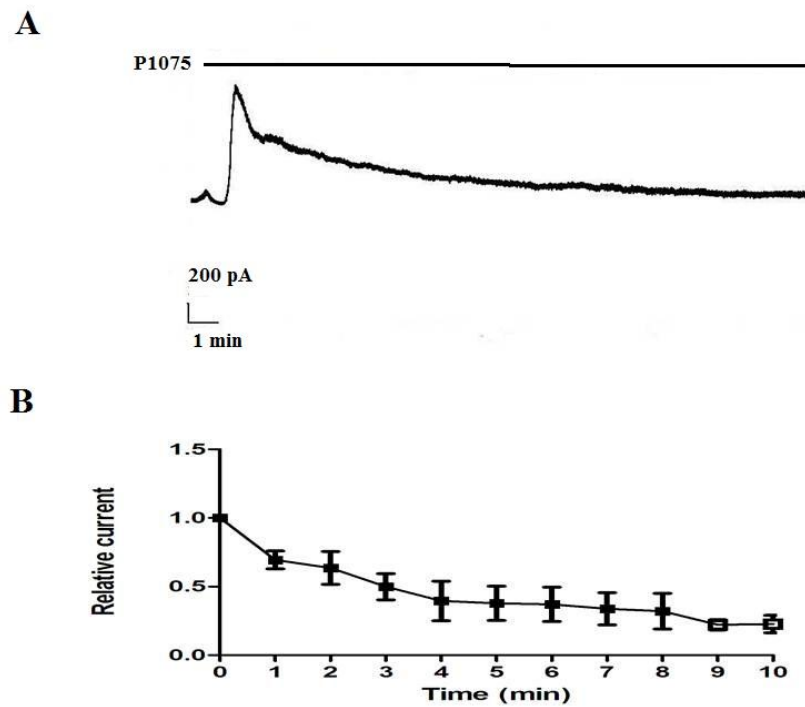


Figure 3.6 Rundown of Kir6.1/SUR2A current activated by P1075. A) A representative P1075-stimulated current trace over 10 minutes showing run down current. B) Averaged relative current (relative to the peak current at 0 min) at one different time interval up to 10 minutes (black rectangle = $n=3$, white rectangle = $n=2$).

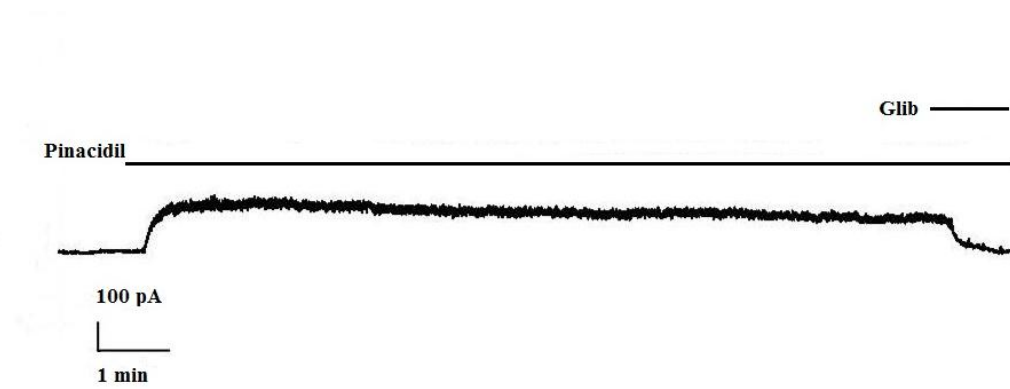


Figure 3.7 Representative Kir6.1/SUR2A channel current activated by pinacidil. *Pinacidil 100 μ M stimulated Kir6.1/SUR2A current with slow rundown. The current was abolished in the presence of glibenclamide.*

Summary

Optimization of transfection for the functional study of recombinant Kir6.1/SUR2A channels was done initially by using recombinant Kir6.2/SUR2A as a model. In the case of Kir6.1/SUR2A channels, it was found that currents could be activated by both P1075 and pinacidil and that these currents could be blocked completely by glibenclamide. In addition, it was found that greater currents could be obtained in the presence of 10 mM intracellular UDP in the pipette. The current density obtained from recombinant Kir6.1/SUR2A channels was very variable. Although some run-down of currents was seen for both P1075 and pinacidil-stimulated currents, this was much greater for the currents activated by P1075.

Chapter 4: Electrophysiological recording of functional responses to protein kinase C phosphorylation of wild type and mutant Kir6.1 subunits expressed in combination with SUR2A subunits

There are 7 consensus phosphorylation sites in intracellular C-terminal of Kir6.1, which are, serine 234 (S234), threonine 345 (T345), serine 354 (S354), serine 379 (S379), serine 385 (S385), serine 391 (S391) and serine 397 (S397) (Singh, 2002). By using chimeras of Kir6.1 and Kir6.2, Singh et al (2002) found that phosphorylation by PKC occurred on the C terminal half of Kir6.1, between residues I²⁸⁰-S⁴²⁴. This result excluded S234 from being a possible PKC phosphorylation site. Further investigation demonstrated that serine but not threonine residues were phosphorylated, permitting the conclusion that T354 could not be phosphorylated and suggesting that serine(s) at positions 354, 379, 385, 391 and 397 are potential sites for PKC phosphorylation. However, when these residues were mutated, phosphorylation by PKC was prevented only in the Kir6.1S379A mutant. Seemingly, the S379 site was the most crucial position for PKC-mediated Kir6.1 phosphorylation. An *In vitro* phosphorylation study of Shi et al (2008a) confirmed the importance of S379, but indicated the additional involvement of S385, S391 and S397, but not S354, in PKC phosphorylation. The functional consequences of PKC phosphorylation of these sites was also studied by Shi (2008a) by using the Kir6.1 and SUR2B subunit combination. It was found that individual mutation of these sites (including S354) led to a reduction in channel inhibition by PKC to the same limited extent for each serine residue. This evidence suggested that the nature of PKC-mediated Kir6.1 phosphorylation and regulation remains unresolved according to *in vitro* and *in vivo* studies to date.

The Kir6.1/SUR2A subunit combination has not been studied for its response to PKC phosphorylation to date. Given the implication of K_{ATP} channels as a common target in IPC signalling pathways, the demonstration that Kir6.1 is the predominant substrate for PKC phosphorylation in isolated ventricular myocytes and the demonstrated association of Kir6.1 with SUR2A by co-immunoprecipitation from these cells (Singh, 2002), it was hypothesised that PKC phosphorylation of the Kir6.1/SUR2A would lead to an increase in Kir6.1/SUR2A channel activity. This could contribute to IPC signalling by both reducing sarcolemmal excitability and/or conferring protective ion fluxes to mitochondria (Yao, 1994, Yamada, 1998). The whole cell patch clamp technique was used to measure the function of the recombinant Kir6.1/SUR2A channel expressed in HEK 293 cells. To clarify the role of five proposed PKC phosphorylation sites, serines at positions 354, 379, 385, 391 and 397 was replaced individually by alanine, a non-phosphorylatable amino acid. The functional response of these point mutants to PKC stimulation was then assessed.

4.1 Inhibitory effect of PKC on Kir6.1/SUR2A current

Preliminary studies herein showed that P1075 and pinacidil can both activate Kir6.1/SUR2A channel activity, however, P1075-stimulated current showed considerably more rundown (see section 3.4). Pinacidil was chosen, therefore, for further studies. In the control group, after forming the whole cell configuration, the membrane potentials of HEK 293 cells expressing Kir6.1/SUR2A were clamped at 0 mV in normal physiologic solution and dialysis allowed with intracellular fluid for 1 min. At this stage, the expressed channels showed no activity. Pinacidil 100 μ M was then perfused through the perfusion system to the bath solution. It was shown that pinacidil can open the channel instantly with a mean current of 116.8 ± 42.0 pA

and current density of 92.0 ± 32.0 pA/pF (n=6). The cell capacitance of this group varied from 2 to 10 pF. Pinacidil-stimulated Kir6.1/SUR2A channels also showed only slow rundown of the current of $17.1 \pm 6.7\%$ at the ninth minute after activation (n= 6). A representative recording is shown in **Figure 4.1 A**.

To investigate the effect of PKC phosphorylation on Kir6.1/SUR2A function, 100 nM Phorbol 12-myristate 13-acetate (PMA), a PKC activator, was applied to the bath solution after the current reached steady state, normally 4 minutes after pinacidil application. The effect of PKC was measured 5 minutes later (at ninth minute after activation). The current then was blocked by glibenclamide to confirm that the current passed through K_{ATP} channels and to confirm the electrical seal remained intact. In this group, the mean current density was 111.7 ± 55.7 pA/pF and cell capacitance was between 2-27 pF. The effect of PMA was quantified as percentage of current inhibition (see chapter 2). It was found that PMA inhibited Kir6.1/SUR2A current by $65.1 \pm 5.5 \%$ five minute after application. This reduction was significantly greater than the rundown of the control group ($p < 0.005$). The comparison between the rundown in control currents and inhibitory effect of PKC on Kir6.1/SUR2A current is shown in **Figure 4.1**. The currents studied were blocked totally by glibenclamide.

To confirm the involvement of PKC in the inhibitory action of PMA, a PKC inhibitor, 100 nM PKCi 20-28, was added to the pipette solution. It was clear that, in this case, PMA had no effect on the current. The percentage of inhibition was 14.8 ± 9.8 (n=4) which was significantly different from that of PMA without PKCi ($p < 0.005$) indistinguishable from control current rundown ($p = 0.83$).

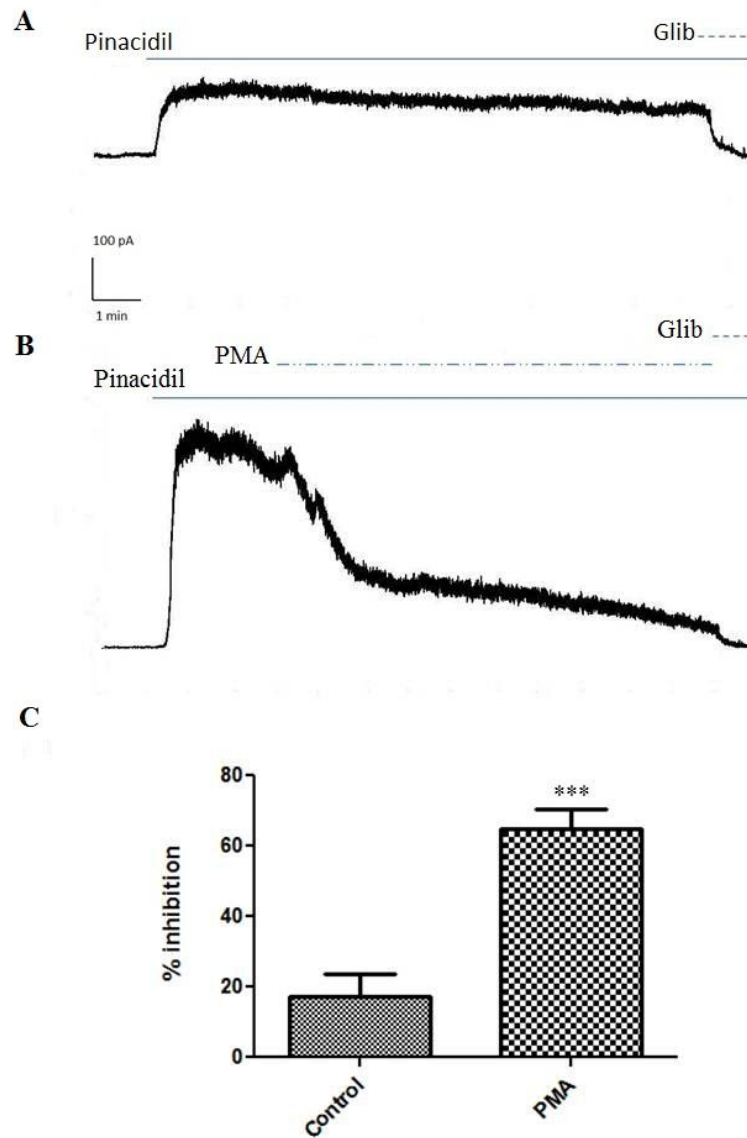


Figure 4.1 Effect of PKC stimulation on Kir6.1/SUR2A channel function. A) An example of current activated with $100\ \mu\text{M}$ pinacidil with slow rundown. B) Effect of $100\ \text{nM}$ PMA on a representative Kir6.1/SUR2A current. C) Comparison between the percentage of rundown and percentage of inhibition by PMA on WT Kir6.1/SUR2A channel current ($n = 6, 8$ for control and PMA group respectively, $p < 0.005$).

Representative data and the comparison of the percentage inhibition between PMA alone and PMA+PKCi groups is shown in **Figure 4.2**.

To ensure PMA action was stimulating PKC and not eliciting effects on channel activity non-specifically, 4 α -phorbol 12,13-didecanoate (4 α -PDD), an inactive analogue of PMA, was used as a negative control. It was found that incubation of cells with 100 nM 4 α -PDD did not inhibit pinacidil-activated Kir6.1/SUR2A current. The percentage of inhibition of 4 α -PDD on the current was 3.5 ± 3.6 (n=6). The inhibition of kir6.1/SUR2A current on PMA-stimulation was significantly different from that after addition of 4 α -PDD to the bath as shown in **Figure 4.3**.

The results so far demonstrated that PKC activator could inhibit Kir6.1/SUR2A channel currents, however, it was not clear whether PKC was acting through the Kir subunit, SUR subunit or both. To explore this question, it was hypothesised that PKC regulates K_{ATP} channel function through phosphorylation of the Kir subunit. Since Kir6.1 but not Kir6.2 had been shown to be a direct substrate for PKC it was hypothesised that Kir6.2/SUR2A channels would be insensitive to PKC stimulation. When Kir6.2/SUR2A subunits were expressed in HEK 293 cells expressed Kir6.2/SUR2A and stimulated with 100 nM PMA, it was found that recombinant kir6.2/SUR2A current was not inhibited (**Figure 4.4**).

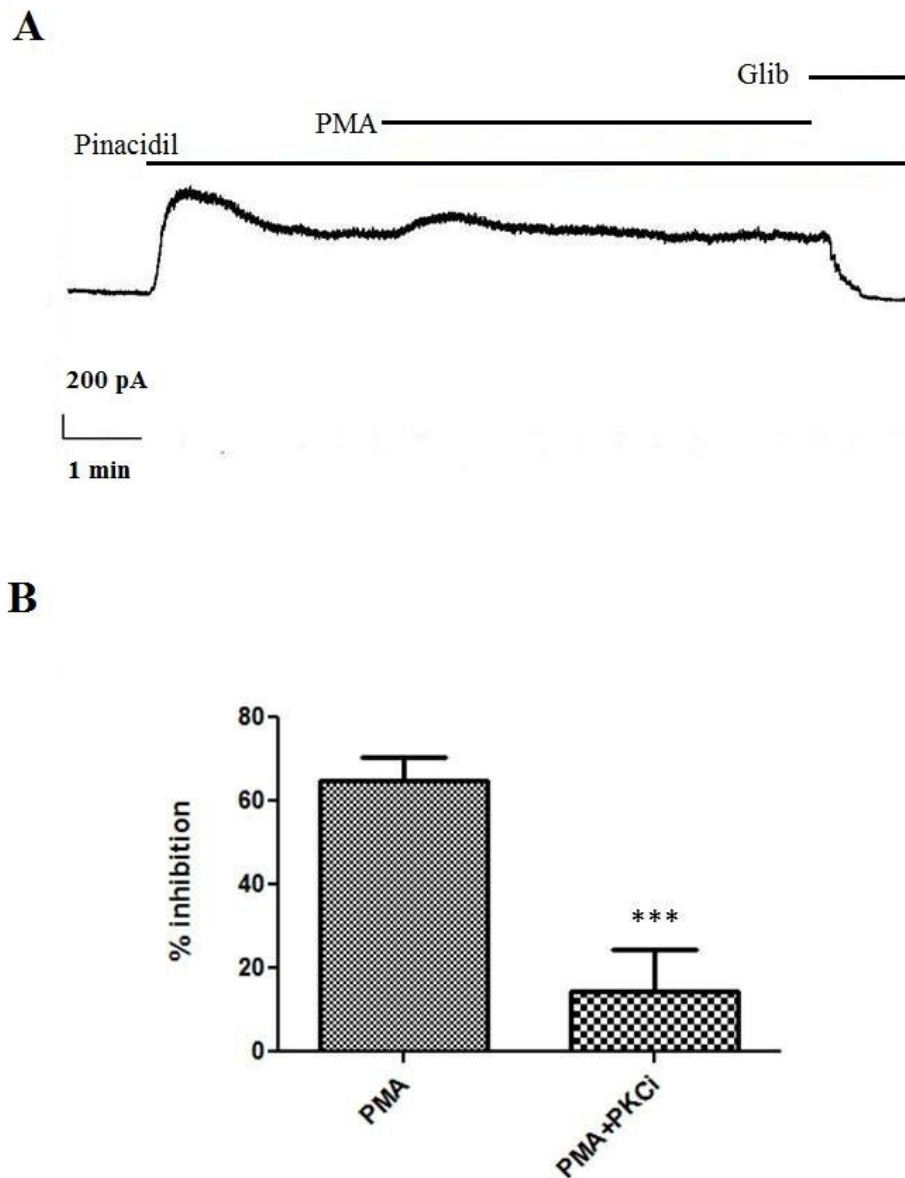


Figure 4.2 Effect of PKC inhibitor on the inhibitory effect of PMA on Kir6.1/SUR2A channel currents. A) A representative kir6.1/SUR2A current recording showing the absence of inhibitory effect of PMA in the presence of PKCi peptide in the internal solution. B) Comparison of the percent inhibition produced by PMA stimulation alone and in the presence of PKCi after 5 minute application. (n= 8 in PMA group and 4 in PKCi group, $p < 0.005$.)

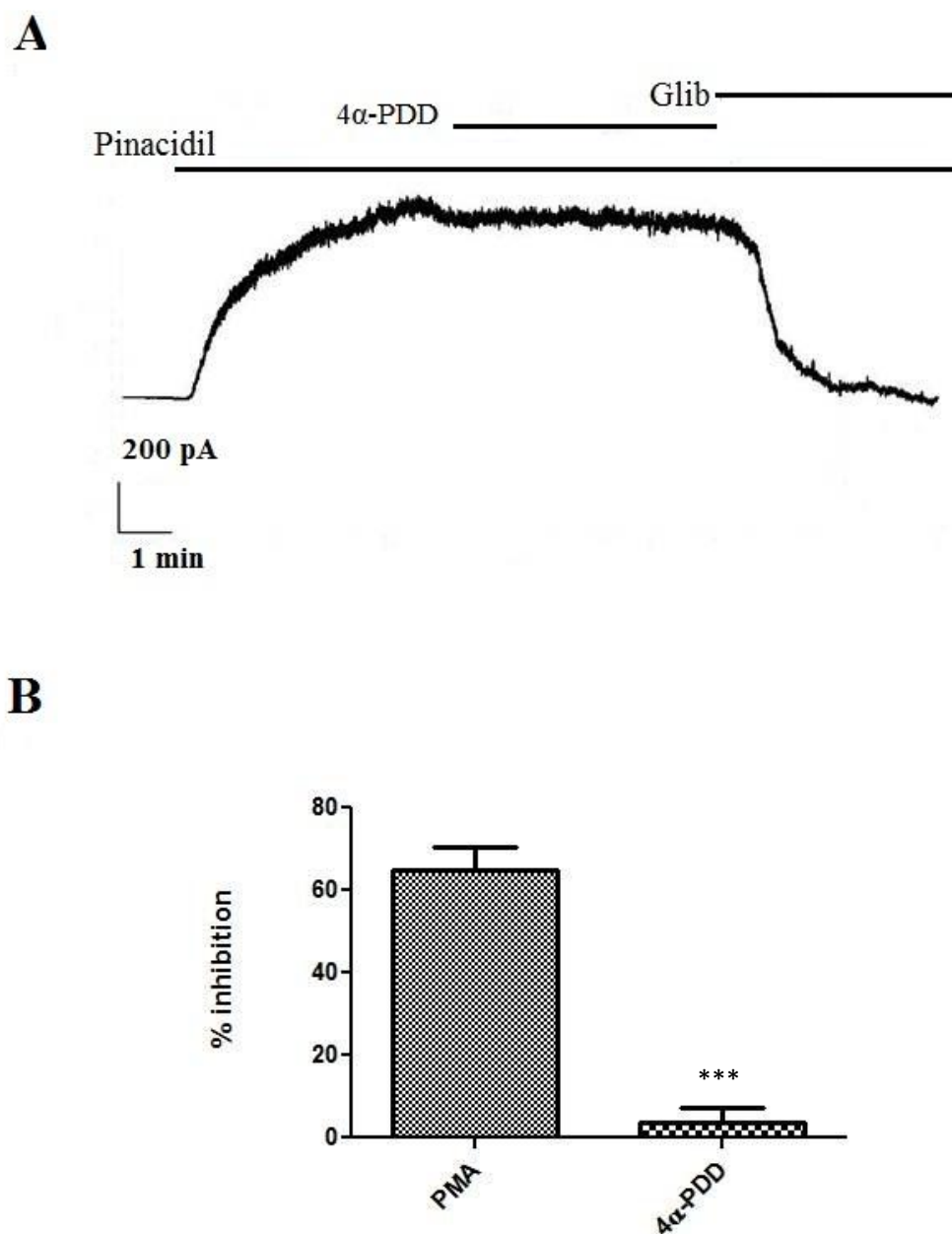


Figure 4.3 Effect of inactive analogue of PMA on Kir6.1/SUR2A channel currents. *A) A representative recording showing the absence of effect of 4 α -PDD on Kir6.1/SUR2A current. B) Bar graph comparing the inhibition of Kir6.1/SUR2A current by PMA and 4 α -PDD after 5 minute incubation. Inhibitory effect of PMA was significantly greater than that of 4 α -PDD ($n=6$, $p<0.001$).*

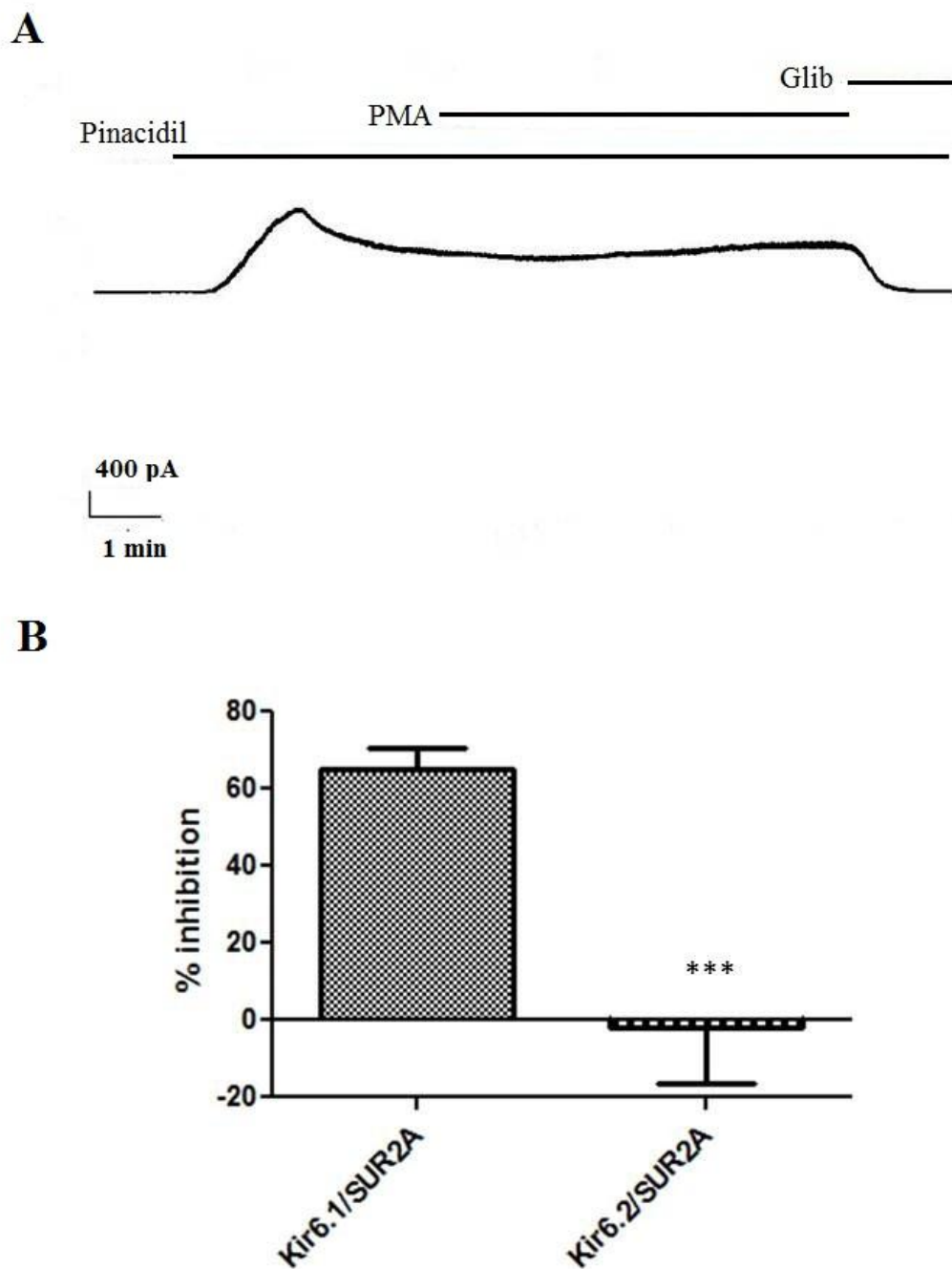


Figure 4.4 Effect of PMA on recombinant Kir6.2/SUR2A channel. A) A tracing obtained from Kir6.2/SUR2A expressed cell showing a slightly increasing current after PMA application. B) A bar graph comparing inhibitory effect of PMA on Kir6.1/SUR2A and Kir6.2/SUR2A current

4.2 Functional response to PKC of mutated Kir6.1/SUR2A channels

To confirm the role of the five PKC consensus sites on PKC-mediated inhibition of Kir6.1/SUR2A function individually, serine at positions 354, 379, 385, 391 and 397 sites were replaced with alanine by site directed mutagenesis (see section 2.9.2). The channel activity of these mutants was then examined. Generally, the transfection efficiency evaluated by the proportion of green fluorescent cells was not different between the different Kir6.1 mutant/SUR2A channels (data not shown). The current density of WT and Kir6.1mutant containing channel stimulated by pinacidil did not differ significantly. It was noted that the percentage of cells which yielded >100 pA current when activated by pinacidil was lower in WT and Kir6.1S391A than that of other mutants. The details are shown in **Table 4.1**.

4.2.1 Effect of PKC stimulation on Kir6.1S354A/SUR2A channel currents

S354 is thought to be one of the phosphorylation sites located in C-terminal of Kir6.1. Shi et al (2008a) has demonstrated an ambiguous effect of PKC on a mutant in this position. They found that the inhibitory effect of PKC on the Kir6.1S354A/SUR2B channel was significantly lower than that of WT, suggesting a role for this residue, whereas their *in vitro* phosphorylation study suggested that mutation of this site did not alter the level of Kir6.1 phosphorylation by PKC. Absence of the involvement of this residue in Kir6.1 phosphorylation was also suggested by Singh (Singh, 2002). To clarify the role of this residue in the functional response of Kir6.1 channel to PKC

Table 4.1 Current density and proportion of cells expressing current > 100 pA

Recombinant channel	Current density (pA/pF) (n)	% of cells yielding currents (n)*
Kir6.1/SUR2A (WT)	111.70 $\pm$ 55.7 (8)	50 (144)
Kir6.1S354A/SUR2A	99.17 $\pm$ 39.3 (8)	68 (22)
Kir6.1S379A/SUR2A	116.7 $\pm$ 31.1(6)	75 (28)
Kir6.1S385A/SUR2A	87.39 $\pm$ 15.75 (7)	88 (18)
Kir6.1S391A/SUR2A	95.02 $\pm$ 28.4 (7)	28.5 (28)
Kir6.1S397A/SUR2A	107.4 $\pm$ 16.2 (6)	68.4 (19)

*% of cells yielding current = (number of cells which can be activated by pinacidil and produce a maximum current >100 pA/number of cell which are successfully patched) x 100.

phosphorylation in a novel channel, electrophysiology of Kir6.1S354A/SUR2A channels expressed in HEK 293 cells was investigated.

In this mutant channel, application of PMA inhibited Kir6.1S354A/SUR2A current by 68.5 ± 7.4 % (n=8) which was not significantly different to that of WT. **Figure 4.5** illustrates a representative recording of current in the presence of PMA and comparison of inhibitory effect of PKC on WT and Kir6.1S354A/SUR2A channels.

The absence of inhibitory effect of PKC on mutated Kir6.1S354 suggests that the residue plays no role in PKC-mediated inhibition of Kir6.1/SUR2A channel currents.

4.2.2 Kir6.1S379A reduces Kir6.1/SUR2A channel sensitivity to PKC

Amongst the five serine residues within PKC consensus phosphorylation sites, S379 seems to be the most important site for PKC-induced Kir6.1 phosphorylation. Shi et al (2008a) found that mutation of S379 resulted in some reduction in the inhibitory effect of PKC on Kir6.1/SUR2B current. In their ^{32}P incorporation phosphorylation study, it was shown that addition of the S379 mutation to a Kir6.1 construct containing S385, S391, S397 almost completely inhibited the phosphorylation. Singh (2002) also found that Kir6.1S379A mutant could not be phosphorylated at all. It was hypothesized that Kir6.1S379 is involved in PKC regulation of Kir6.1/SUR2A channel and that mutation of S379 would reduce inhibitory effect of PKC.

Mutation of this site (S379A) resulted in a reduction in the inhibitory effect of PKC compared to that of WT channel. The percentage of inhibition was 31.3 ± 3.3 (n=7). An example of recording and the pooled data for effect of this mutant on the sensitivity to PMA stimulated PKC activity is shown in **Figure 4.6**.

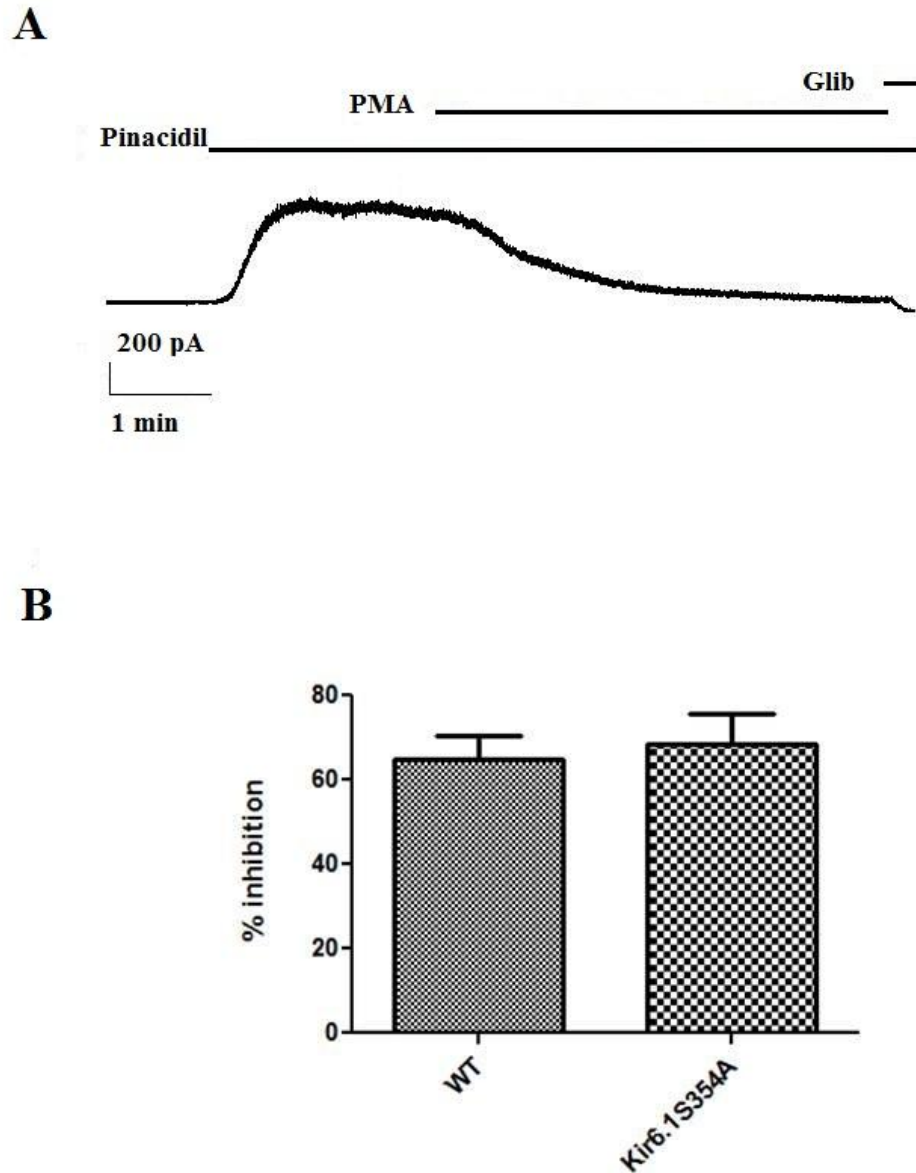


Figure 4.5 Effect of PKC activator PMA on Kir6.1S354A/SUR2A mutant channel currents. A) Representative recording obtained from the Kir6.1S354A/SUR2A mutant showing strong current inhibition on stimulation with PMA. B) Bar graph comparing the percentage of current inhibition by PMA of Kir6.1S354A/SUR2A mutant and WT channels ($n=8$). Inhibitory effect of PKC on WT and Kir6.1S354 was not significantly different.

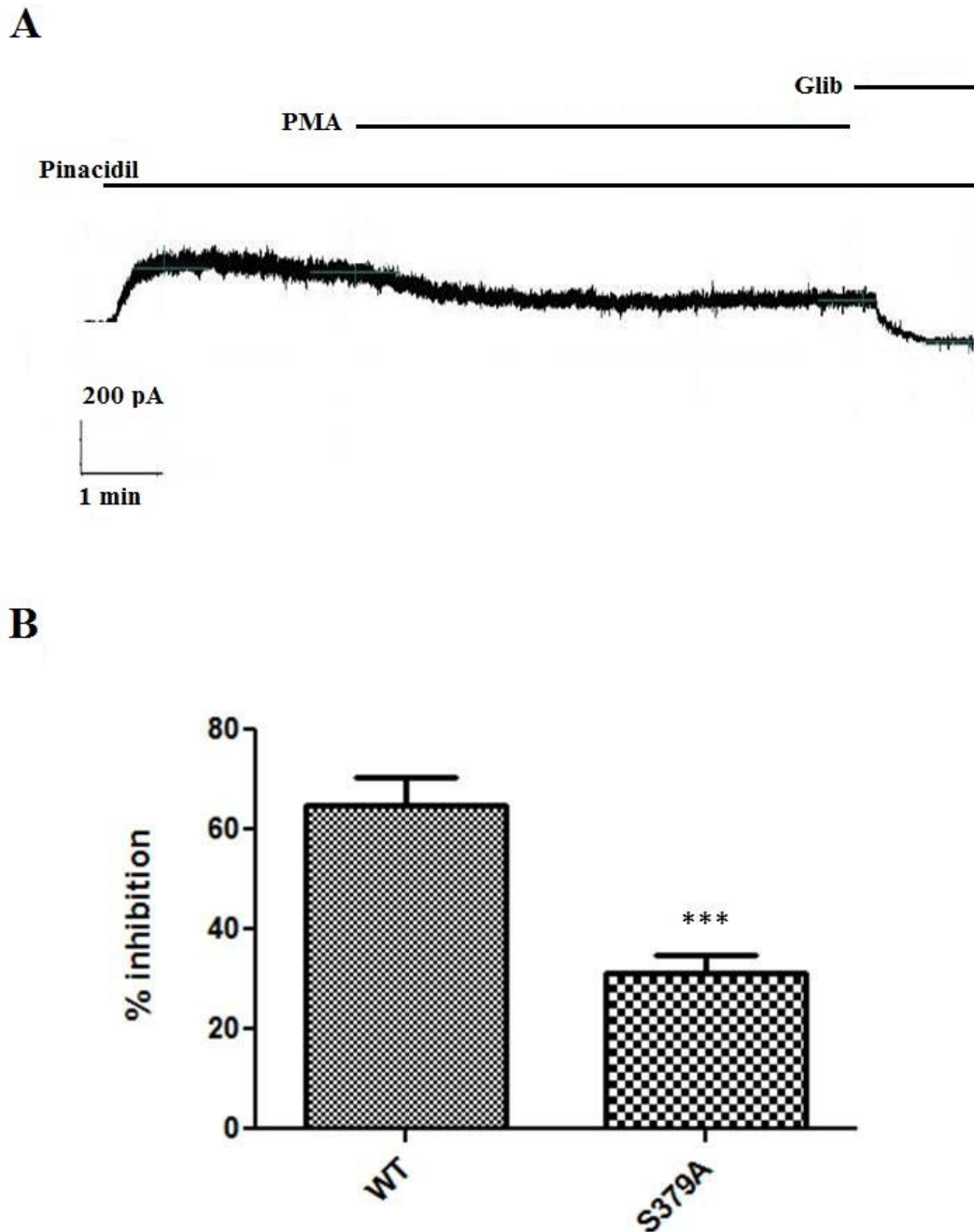


Figure 4.6 Inhibitory effect of PMA on Kir6.1S379A/SUR2A channels. A) Representative recording showing the effect of PMA on Kir6.1S379A/SUR2A whole cell current. B) Comparison of the inhibitory effect of PKC on WT and Kir6.1S379A/SUR2A channel currents. The inhibition of the mutant was significantly lower than that of WT channels ($p < 0.05$).

The reduction in Kir6.1S379/SUR2A channel activity in response to PKC activation suggests a role for S379 in PKC-mediated Kir6.1 phosphorylation.

4.2.3 Mutation of Kir6.1S385 reduces inhibitory effect of PMA on Kir6.1/SUR2A channel currents.

One of the interesting phosphorylation sites in Kir6.1 is S385 because this site is a potential substrate for both PKA and PKC phosphorylation (Quinn, 2004, Shi, 2008a, Singh, 2002). Shi et al (2008a) demonstrated that Kir6.1S385A/SUR2B current was slightly inhibited by PMA which correlated with their measurement of phosphorylation at this site which showed that S385 site is a substrate for PKC-mediated Kir6.1 phosphorylation. Singh et al (2002,unpublished) also proposed S385 as a potential residue for PKC phosphorylation, however, mutation of this site still yielded in Kir6.1 phosphorylation to levels the same as in WT (Singh, 2002). To confirm the role of S385 in functional response to PKC in the Kir6.1/SUR2A channel, serine S385 was mutated to alanine and then the inhibitory effect of PMA channel was observed.

Whole cell patch clamp recording revealed that Kir6.1S385A/SUR2A channel activity was not affected by PMA which was significantly different from WT channel. The channel inhibition in the mutant was $26 \pm 5.7\%$ (n=6), which was not significantly different from the rundown, but was statistically different from that of WT ($65.1 \pm 5.5\%$, $p < 0.05$). A representative recording and a bar graph comparing the effect of PMA on WT and the Kir6.1S385A mutant is shown in **Figure 4.7**. The reduction in inhibition of Kir6.1S385/SUR2A channel activity by PKC activation implies that S385 site is a potential substrate for PKC-mediated Kir6.1 phosphorylation.

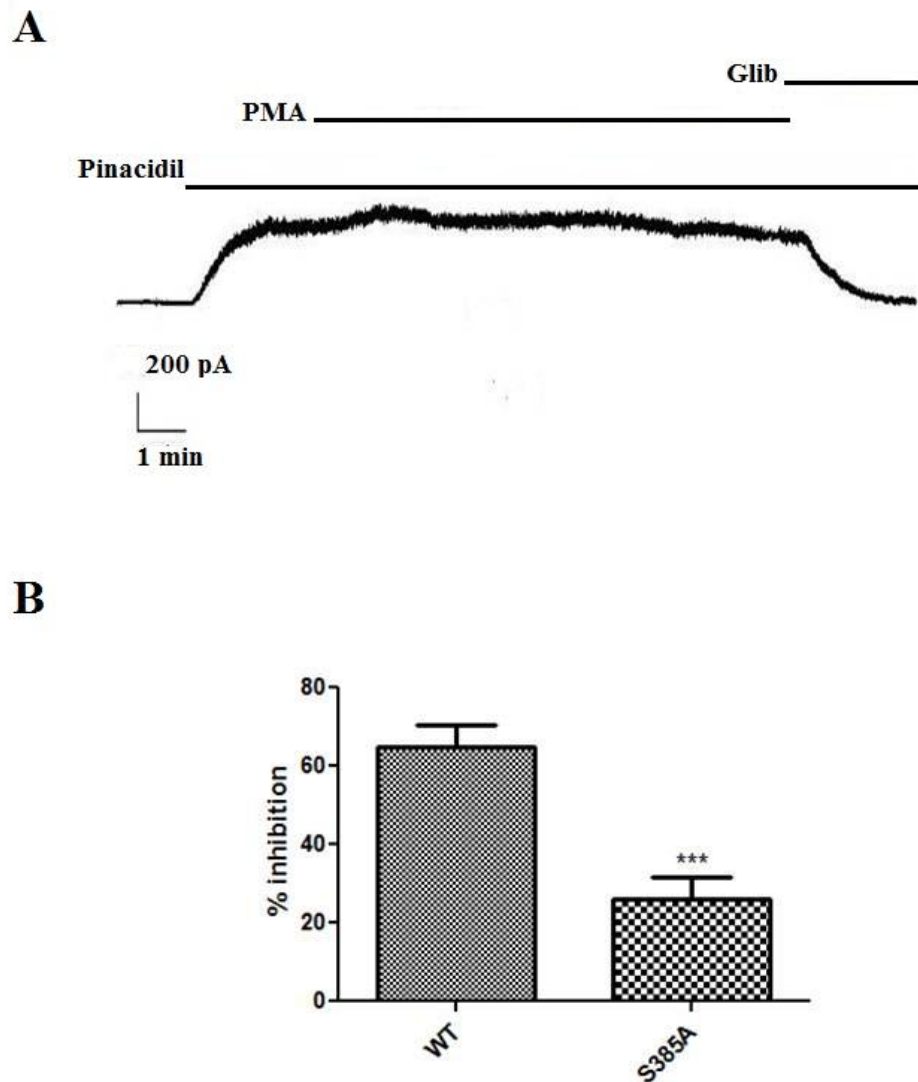


Figure 4.7 Showing the effect of PMA on Kir6.1S385A/SUR2A channel activity.

A) Representative recording showing the effect of PMA on Kir6.1S385A/SUR2A current. B) Comparison of channel inhibition by PMA of WT and Kir6.1S385 mutant. The inhibitory effect of PMA on Kir6.1S385 mutant channel was significantly decreased.

4.2.4 Mutation of Kir6.1S391 does not affect PKC-mediated inhibition of Kir6.1/SUR2A channels

The responsibility of S391 site in Kir6.1 for PKC phosphorylation was investigated by Shi et al (2008a). They found that the inhibitory effect of PKC on Kir6.1S391A/SUR2B current was smaller than that of WT. An assay of direct phosphorylation also suggested an involvement of S391 in PKC-mediated Kir6.1 phosphorylation. On the contrary, Singh et al (2002) did not demonstrate any involvement of S391 in Kir6.1 phosphorylation. In Singh's case, mutation of Kir6.1S391 to alanine had no effect on PKC-mediated phosphorylation. Due to this discrepancy, the elucidation whether S391 is involved in the regulation of channel activity in response to PKC was required.

In this study, Kir6.1S391A/SUR2A current was inhibited by PMA to the same extent as WT. The percentage of channel activity inhibition was $65.6 \pm 3.8 \%$, $n=6$, as shown in **Figure 4.8**. The inhibition of Kir6.1S391 mutant channels by PKC suggests that S391 is not involved in PKC mediated Kir6.1 phosphorylation.

4.2.5 Mutation of Kir6.1S397 inhibits consequences of PKC activation on Kir6.1/SUR2A channels

Kir6.1 serine 397 was believed to be one of the phosphorylation sites in Kir6.1 sensitive to PKC. Shi et al (2008a) found that mutation of this site resulted in reduction of channel inhibition by PMA by about 20% in Kir6.1/SUR2B channel. In Kir6.1/SUR2A combination, it was found that inhibitory effect of PKC on Kir6.1S397A/SUR2A current was only slightly reduced. Pinacidil-activated current

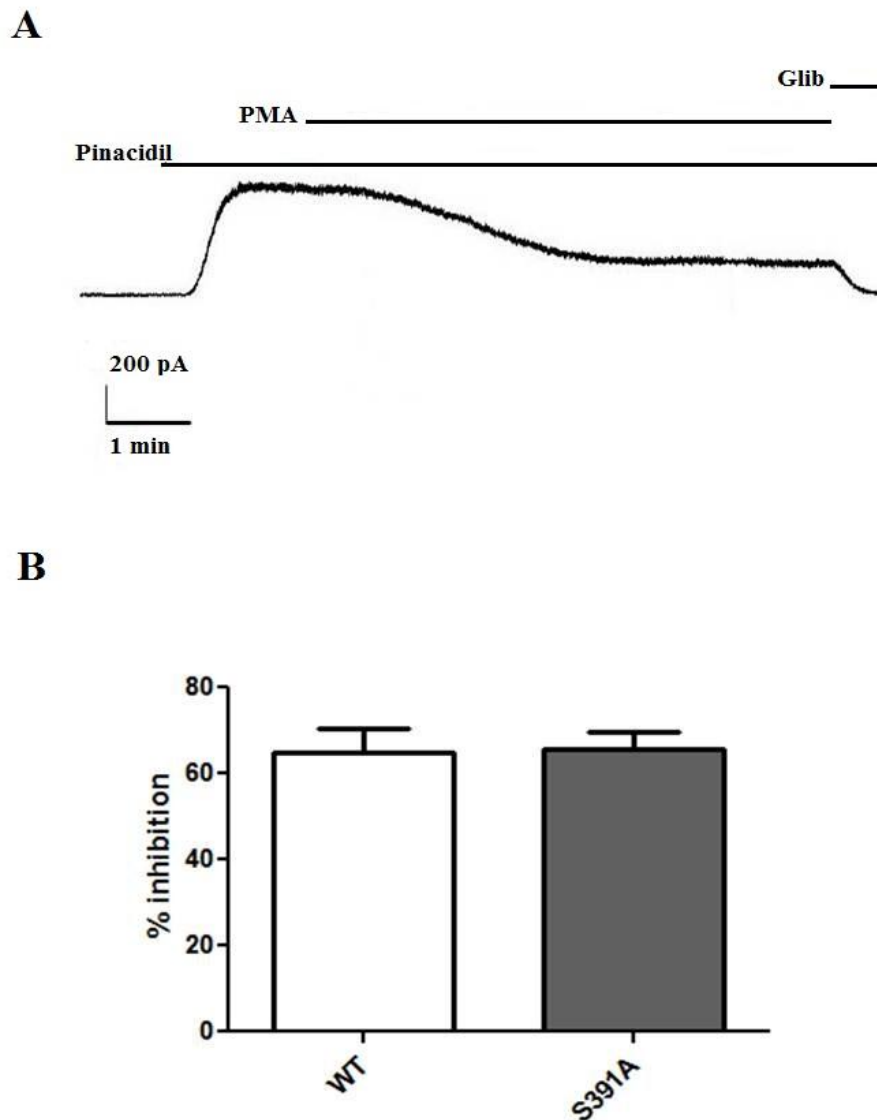


Figure 4.8 Effect of PMA on Kir6.1S391A/SUR2A channel. *A) Representative recording of Kir6.1S391A/SUR2A channel current in the presence of 100 nM of PMA. B) Bar graph comparing the effect of PMA on WT and Kir6.1S391A/SUR2A channel current. The inhibition of Kir6.1S391A/SUR2A channel by PKC was not significantly different from that of WT ($n=6$, $p=0.94$).*

was inhibited by PMA by 48.1 ± 2.7 % which was statistically different ($p=0.02$). The effect of PMA on this recombinant channel is shown in **Figure 4.9**. The decrease in the sensitivity of Kir6.1/SUR2A channel to PKC by mutation of S379 in the Kir6.1 subunit suggests that S397 also plays a role in PKC mediated Kir6.1 phosphorylation.

4.2.6 Mutation of endosome targeting Kir6.1 reduces inhibitory effect of PKC on Kir6.1/SUR2A channels

To understand how PKC activation inhibits Kir6.1/SUR2A, the current of endosome targeting Kir6.1 mutant, Kir6.1364IL365AA/SUR2A, was observed during exposure to PKC activator. It was found that the inhibitory effect of PKC was reduced in this mutant indicating the involvement of Kir6.1 trafficking in response to PKC. In the presence of 100 nM PMA, endosome targeting mutant channel current was not inhibited. The percentage of inhibition was 27.3 ± 7 which was significantly lower than that of WT (65.0 ± 5.5) as shown in **Figure 4.10**. The loss of inhibition of PKC activation on endosome mutant implied that the effect of PKC is involved in channel trafficking.

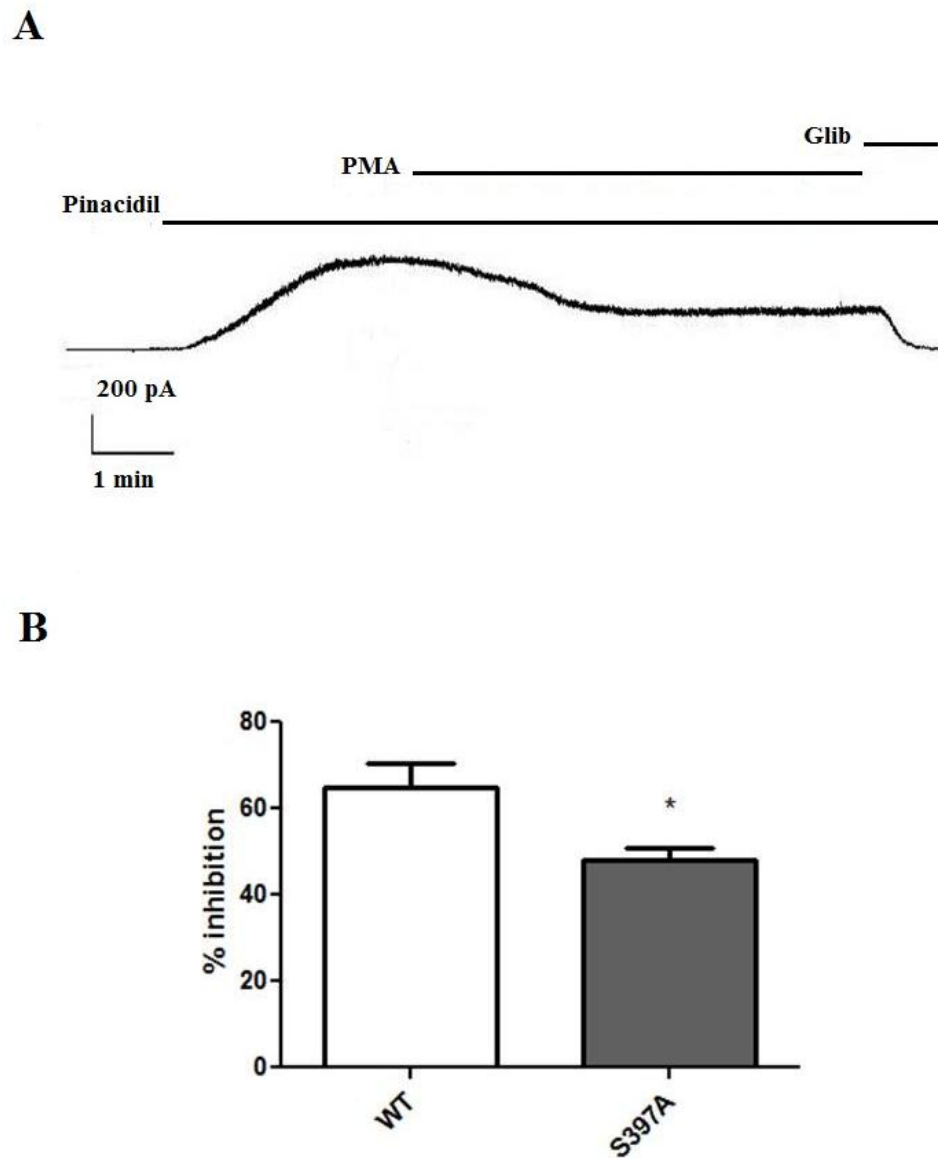


Figure 4.9 Inhibitory effect of PKC on Kir6.1S397A/SUR2A current. A) Representative recording showing the effect of PMA on Kir6.1S397A/SUR2A channel activity. Application of PMA inhibited the currents. B) Bar graph comparing the percentage of channel inhibition between WT and Kir6.1S397A/SUR2A channels. The channel inhibition by was significantly lower in the mutant ($p=0.02$, $n=6$).

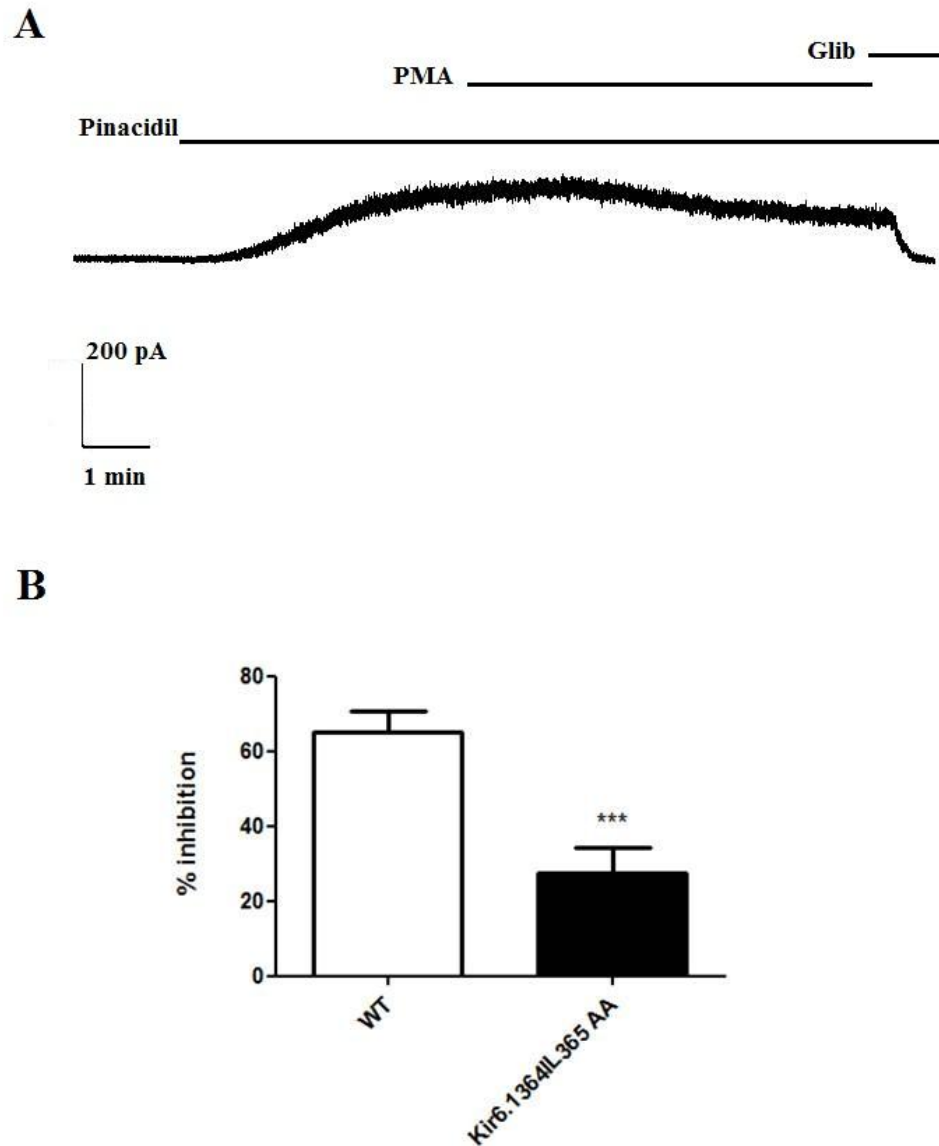


Figure 4.10 Showing the inhibitory effect of PMA on Kir6.1364IL365AA/SUR2A channel currents. A) Representative recording showing slight reduction of Kir6.1364IL365AA currents in the presence of 100 nM PMA. B) Comparison of percentage of WT and Kir6.1364IL365AA mutant current inhibition by PMA. Inhibitory effect of PMA was statistically lower in the mutant channel ($p < 0.005$, $n=9$).

Summary

Recombinant Kir6.1/SUR2A channels expressed in HEK 293 cells could be activated by pinacidil with only slow rundown. The PKC activator, PMA, inhibited recombinant Kir6.1/SUR2A current by about 65%. The inactive form of PMA failed to show an inhibitory effect on this channel. The effect of PMA could be abolished by the PKC inhibitor, PKCi (20-28). On the contrary, PMA did not inhibit Kir6.2/SUR2A current. Mutation of S379, S385 and S397 in Kir6.1 subunit led to a significantly decreased inhibitory response to PMA stimulation, whereas PMA-induced inhibition of mutated Kir6.1S354 and Kir6.1S391/SUR2A current was similar to the WT. Together, these experiments confirmed three crucial phosphorylation sites responsible for Kir6.1/SUR2A channel.

Chapter 5: Electrophysiological recording of functional responses to protein kinase C phosphorylation of wild type and mutant Kir6.1 subunits expressed in combination with SUR2B subunits

Recombinant Kir6.1/SUR2B channels show characteristics mimicking vascular smooth muscle type K_{ATP} channels (Yamada, 1997). It is believed that the regulation of this channel is mediated in part by channel phosphorylation by cyclic AMP dependent protein kinase (PKA) and protein kinase C (PKC). PKA phosphorylation leads to activation of the channel, whereas PKC inhibits the channel current (Quinn, 2004, Shi et al, 2008b). It was found that PKA phosphorylation sites are located at serine 385 on the kir6.1 subunit, and threonine 633 and serine 1465 on the SUR2B subunit (Quinn, 2004, Shi et al, 2008b). PKC phosphorylation sites were located on the Kir6.1 subunit only (Shi et al, 2008a). The identity of the PKC phosphorylation sites on Kir6.1 subunit is still debated. The direct phosphorylation experiment of Singh et al suggested that S379 is a potential site for PKC phosphorylation (Singh, 2002). On mutation of consensus site serine residues to non-phosphorylated alanine, Shi et al (2008a) demonstrated a potential functional significance of S354, S379, S385, S391 and S397 in PKC-mediated inhibition of Kir6.1/SUR2B channels, since inhibition was reduced in each case. On direct *in vitro* phosphorylation, however, no evidence was found in support of S354 in Kir6.1 phosphorylation (Shi et al, 2008a). Recently, functional study of Kir6.1/SUR2A channel in chapter 4 also showed reduction in inhibitory action of PKC when S379, S385, S 397, but not S354 nor S391 were mutated (see chapter 4). While the evidence for the involvement of Kir6.1 S379, S 385 and S397 in PKC-mediated inhibition was consistent in both SUR2A (this study) and SUR2B (Shi et al, 2008a) containing channels, results for

Kir6.1 S391 were inconsistent. Moreover, although Shi et al (2008a) reported functional evidence in favour of an involvement of Kir6.1 S354, this study did not (chapter 4). In addition, Shi et al (2008a) were not able to demonstrate evidence of direct phosphorylation of this residue. Together these results raised doubt over the involvement of Kir6.1 S354 in the channel regulation by PKC. The purpose of this study was to confirm which phosphorylation sites are responsible for the inhibition of Kir6.1/SUR2B channel activity in response to PKC phosphorylation, to elucidate whether regulation of Kir6.1 containing channels is common for all SUR isoform combinations with Kir6.1 or whether the nature of the SUR isoform co-expressed imposes a further level of complexity to the regulation of Kir6.1-containing channels by PKC.

To do this, Kir6.1/SUR2B channels expressed in HEK 293 cells were electrophysiologically studied using the whole cell patch clamp technique (see section 2.9). The effect of PKC stimulation on the channel activity of wild type and the Kir6.1 mutants in combination with SUR2B, (Kir6.1S354A/SUR2B, Kir6.1S379A/SUR2B, Kir6.1S385A/SUR2B, kir6.1S391A/SUR2B and Kir6.1S397A/SUR2B) was examined. The experimental design was as described in section 2.3. Briefly, after going whole cell, 100 μ M pinacidil was perfused into the bath solution to activate the channel. When the activated current was stable, normally at the 4th minute of recording, 100 nM PMA was applied for 5-8 minutes. The effect of PMA was quantified at five minutes after application. After that, a K_{ATP} channel blocker, 100 μ M glibenclamide, was added at the end of recording to confirm that currents studied were carried by K_{ATP} channels.

5.1 Expression of Kir6.1/SUR2B recombinant channel in HEK cells

To study the functional effect of PKC activation on Kir6.1/SUR2B channels, it was necessary to optimize the transfection. HEK 293 cells were transfected with Kir6.1/SUR2B in a proportion of 2 μ g:2 μ g following the protocol used in Kir6.1/SUR2A experiment (chapter 4). The cells were patched after 48 hours of transfection. The expression of Kir6.1/SUR2B channels in HEK cells examined by green fluorescent cells was about 60%, which was not different from that of Kir6.1/SUR2A channel. The Kir6.1/SUR2B channel was not active when the membrane potential was clamped at 0 mV. In contrast to Kir6.1/SUR2A channels and although channel expression was suggested by the green fluorescence appearing in cells, transfection with 2 μ g of each DNA did not yield pinacidil-activated currents (n=25). Therefore, the transfection was optimized further by increasing each DNA to 3 μ g. With this amount of DNA, some but not all green fluorescent cells responded to pinacidil. The percentage of cells responding to pinacidil with a current >100 pA was 50%. The mean current density was low compared to Kir6.1/SUR2A channel (chapter 4) at 46.6 ± 10.7 pA/pF. The current was blocked completely by 100 μ M glibenclamide confirming that it was carried by K_{ATP} channels. The Kir6.1/SUR2B current had a slow rate of rundown decreasing in amplitude by 17.1 ± 5.7 % at the ninth minute of recording. The degree of rundown within a representative of the current is shown in **Figure 5.1**

5.2 PKC inhibits Kir6.1/SUR2B current

There is evidence showing that PKC inhibits Kir6.1 containing channels (Thorneloe, 2002). Shi et al (2008a) demonstrated a 70% inhibition of Kir6.1/SUR2B current by

PKC, whereas a 60% reduction was found in Kir6.1/SUR2A (see section 4.1). To confirm this effect, 100 nM PMA was applied as above. In this study, it was shown that PMA reduced pinacidil-activated Kir6.1/SUR2B current by 70.4 ± 7.5 % which was significantly greater than the reduction through rundown. Representative current and a bar graph showing the effect of 100 nM PMA are shown in **Figure 5.1**.

5.3 Consequences of PKC activation on Kir6.1 mutant containing channels

To investigate which residues on Kir6.1 are responsible for PKC phosphorylation, serine residues at 354, 379, 385, 391 and 397 were mutated by replacement with alanine. The constructions of the mutants and their DNA sequencing are described in section 2.9. HEK 293 cells expressing wild type or mutant Kir6.1 with SUR2B subunits were assessed for their electrophysiological response to the PKC activator (PMA 100 μ M). It was found that the percentage of transfected cells yielding pinacidil-activated current was variable, from 50% to 100%. Current density of the cells expressing WT and mutant channels was also variable. The highest current density was found for the Kir6.1S379 mutant whereas the lowest one was found for the Kir6.1S397 mutant. However, the current density was not different significantly between different mutants ($p=0.42$). Current density and proportion of cells responding to pinacidil with a current >100 pA is detailed in **Table 5.1**

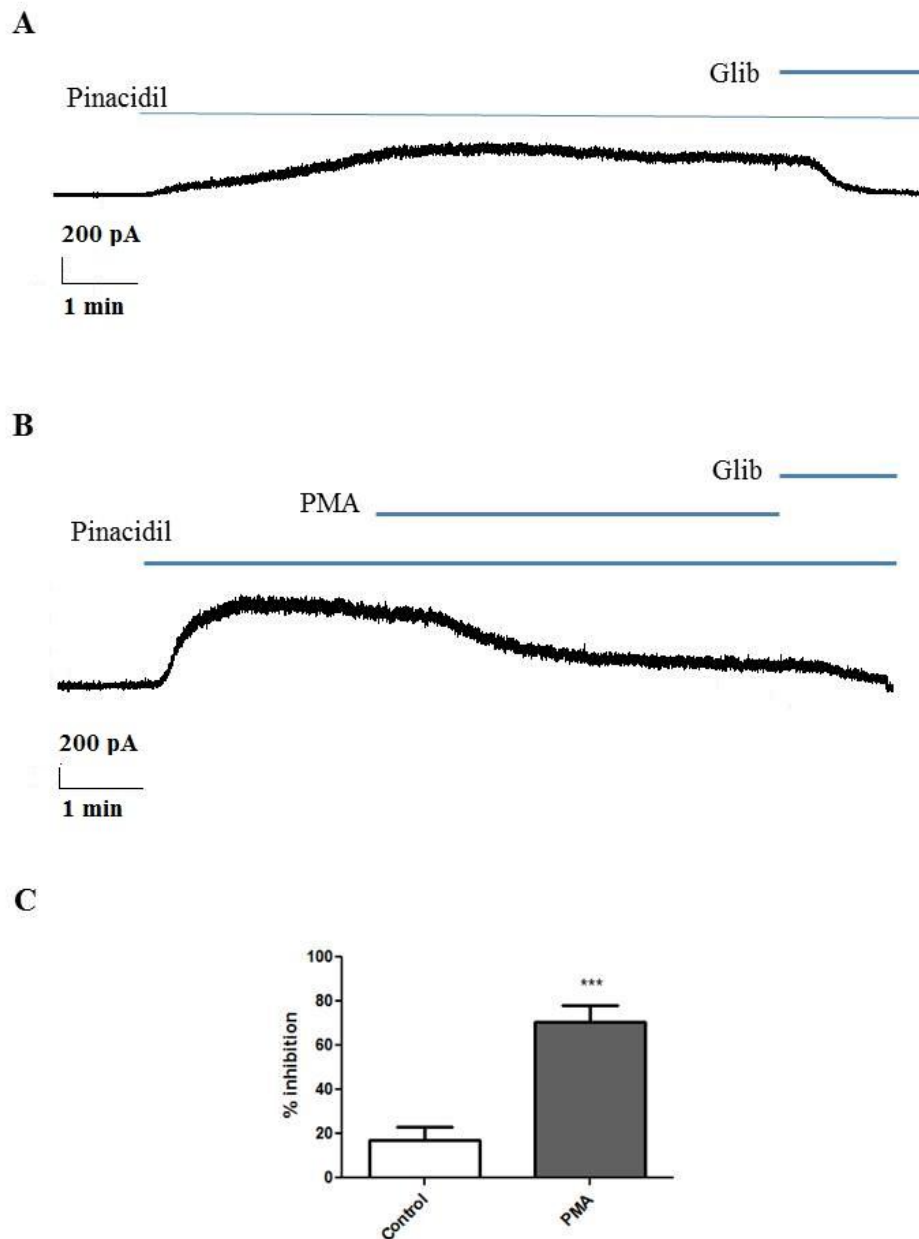


Figure 5.1 Effect of PMA stimulation on Kir6.1/SU2B channel activity. A) Representative current recorded from WT without PMA. A low rate of rundown was evident. B) Representative recording showing the effect of PMA on WT current. C) Bar graph showing the percentage of channel inhibition resulting from 100 nMPMA application which was statistically different from the rundown in the control ($p < 0.001$, $n = 7$ and 10 for control and PMA group respectively).

Table 5.1 Current density and proportion of cells expressing current > 100 pA when activated by pinacidil

Recombinant channel	Current density (pA/pF) (n)	% of cells yielding currents (n)*
Kir6.1/SUR2B (WT)	46.7 ± 10.7 (10)	50 (64)
Kir6.1S354A/SUR2B	40.1 ± 16.4 (4)	50 (14)
Kir6.1S379A/SUR2B	84.2 ± 32.2 (5)	53.3 (30)
Kir6.1S385A/SUR2B	62.7 ± 31.2 (4)	60.7(23)
Kir6.1S391A/SUR2B	63.5 ± 24.9 (5)	86.6 (15)
Kir6.1S397A/SUR2B	29.6 ± 16.2 (7)	100 (7)

5.3.1 Mutation of Kir6.1S354 does not affect PKC modulation of Kir6.1/SUR2B channel

Experiments reported in chapter 4 investigating functional response of the Kir6.1/SUR2A subunit combination to PKC activation showed that Kir6.1S354 was not a potential phosphorylation site. This result was supported by the *in vitro* phosphorylation study of Singh (2002), where Kir6.1 phosphorylation still occurred after mutagenesis of this residue. Shi et al (2008a) also suggested that Kir6.1S354 was not involved in PKC-mediated Kir6.1 phosphorylation. However, Shi indicated that the functional response of the Kir6.1/SUR2B subunit combination was altered when Kir6.1S354 was mutated. To confirm the potential of this site in contributing to the functional response to PKC, the response of Kir6.1S354A/SUR2B channel current to PKC activation was observed. Experiments conducted herein showed that inhibitory effect of PMA on Kir6.1S354A/SUR2B channels was not significantly different from that of WT channels. PMA inhibited the mutant current by $58.2 \pm 16.2\%$ whereas, it reduced WT current by $70.4 \pm 7.5\%$. **Figure 5.2** shows a representative current of Kir6.1S354A/SUR2A channels in the presence of PMA and comparison of channel inhibition by PMA in WT and the mutant. This study confirmed that S354 is not involved in PKC phosphorylation of Kir6.1.

5.3.2 Mutation of Kir6.1S379 reduces the inhibition of channel current by PKC

It was suggested that KirS379 was the most likely site for PKC phosphorylation. Singh found that phosphorylation of Kir6.1 did not occur only when the S379 residue was mutated (Singh, 2002). The experiments of Shi et al (2008a) also demonstrated

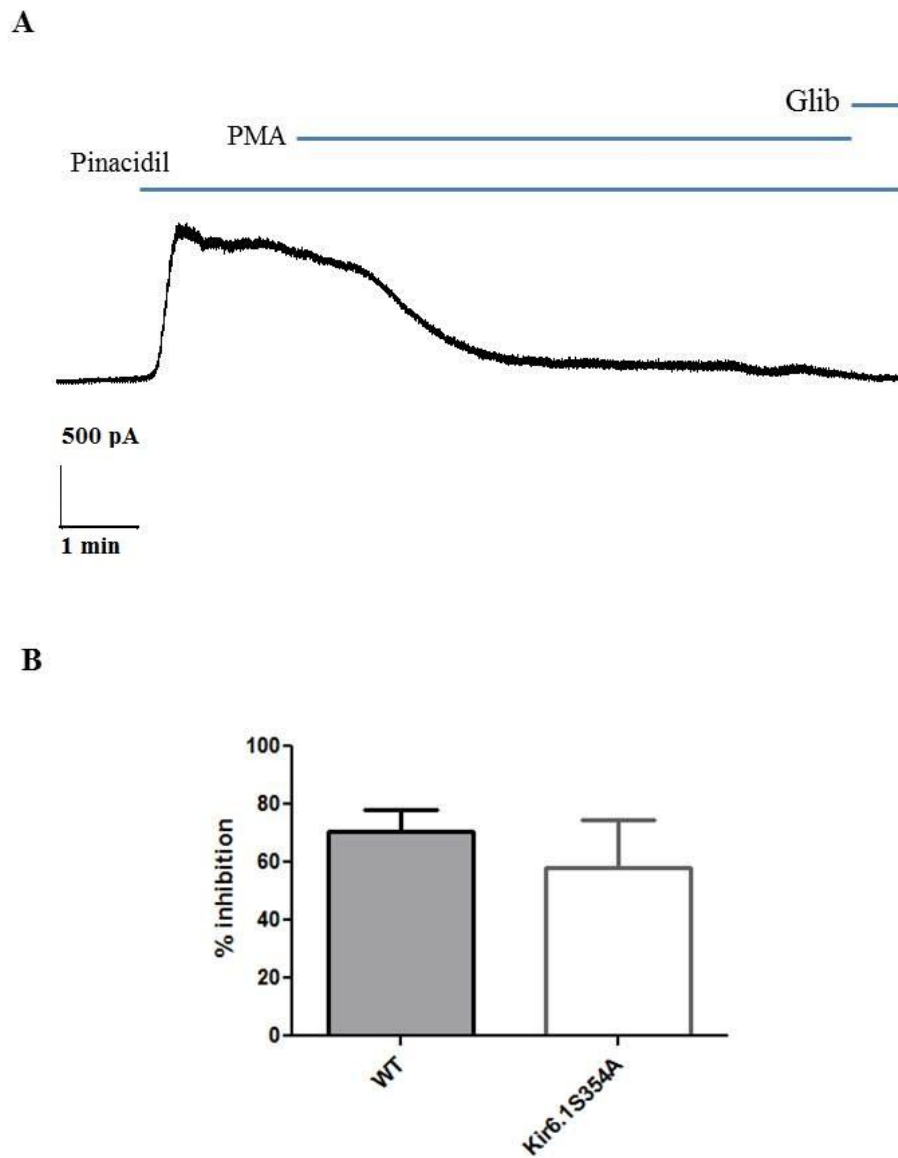


Figure 5.2 Effect of PMA on Kir6.1S354A/SUR2B channel currents. A) Representative recording showing the channel inhibition by 100 nM PMA. B) Bar graph showing the percentage of channel inhibition by 100 nM PMA in WT and Kir6.1S354 mutant containing channels, which was not significantly different ($n=10$ and 4 for WT and KirS354 respectively).

the vital role of the Kir6.1S379 residue, both in the functional response to PKC activation and in that *in vitro* phosphorylation study. However, the whole cell patch clamp study of Shi et al (2008a) indicated that mutation of Kir6.1S379 prevented the inhibitory effect of PKC on Kir6.1SUR2B channel currents to as the same extent as other mutants. This appeared to be contrary to the phosphorylation experiments of Singh (2002) indicating that Kir6.1S379 is the most likely site for PKC phosphorylation. As shown in chapter 4, the degree of loss of inhibitory response to PKC phosphorylation on mutation of Kir6.1 S379 to alanine when expressed with SUR2A, was the same as that of the Kir6.1 S385 to alanine mutation.

To clarify the role of S379 in functional response, the whole-cell patch clamp technique was performed in HEK 293 cells expressing Kir6.1S379/SUR2B. PMA was shown to inhibit Kir6.1S379A/SUR2B channel activity by $40.4 \pm 9.0\%$ which was significantly less than that of WT ($p = 0.02$, $n = 4$). This result confirmed that Kir6.1S379 was a substrate for PKC phosphorylation and contributes to Kir6.1-containing channel inhibition with both SUR2 subunits. A representative recording of the inhibition of Kir6.1S379A/SUR2B current by PMA was shown in **Figure 5.3**.

5.3.3 Mutation of Kir6.1S385 residue reduces inhibitory effect of PKC stimulation

Experiments in chapter 4 showed that the inhibitory response of Kir6.1/SUR2A channel to PKC was decreased when Kir6.1S385 was mutated. This result concurred with the result of Shi et al (2008a) from both their *in vitro* and electrophysiological studies. Conversely, Singh (2002) did not find the effect of Kir6.1S385 mutation on Kir6.1 phosphorylation suggesting that this residue is not a substrate for PKC.

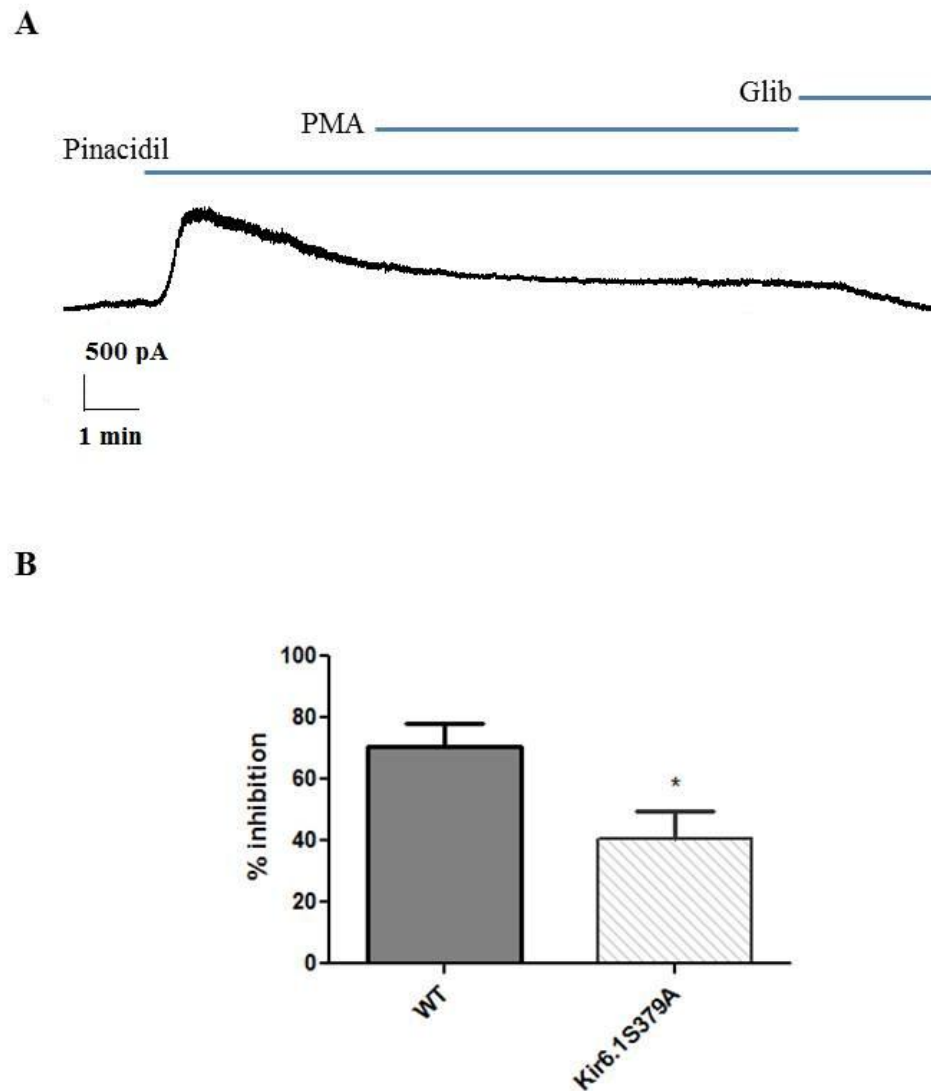


Figure 5.3 Inhibitory effect of PMA on Kir6.1S379A/SUR2B current. A) Representative recording of Kir6.1S379A/SUR2B current inhibition in the presence of PMA. Application of 100 nM PMA when the current was stable inhibited the current to some degree. B). Bar graph showing the inhibitory effect of PMA on the mutant channels, which was significantly less than that of WT ($p=0.02$, $n=10$ and 4 for WT and Kir6.1S379A channels, respectively).

To further clarify the role of Kir6.1S385, this residue was replaced with alanine and then the inhibitory response to PKC activation of the Kir6.1S385A/SUR2B channel was examined. Application of 100 nM PMA inhibited pinacidil-activated Kir6.1S385A/SUR2B current by $42.6 \pm 4.7\%$ which was statistically less than the inhibition of WT. This result indicated that S385 contributes to the inhibitory response of the channel to PKC stimulation. A representative of Kir6.1S385A/SUR2B current in the presence of PMA was shown in **Figure 5.4A**. **Figure 5.4 B** illustrates the comparison of channel inhibition between WT and the mutant. This study confirms the role of the Kir6.1S385 residue as a PKC phosphorylation site that influences channel inhibition.

5.3.4 Mutation of Kir6.1 S391 does not affect the inhibition of channel current on PKC stimulation

Experiments in chapter 4 showed that the inhibitory effect of PKC did not change when serine 391 of Kir6.1 was replaced with alanine in Kir6.1/SUR2A channels. This result was in contrast to Shi et al (2008a) who suggested a role for S391 in Kir6.1 phosphorylation from their experiments. Shi et al (2008a) investigated whether the Kir6.1S391 mutant responded differently to PKC. It was found that mutation of Kir6.1S391 resulted in a reduction of channel inhibition by PKC. The conflicting results of chapter 4 and Shi et al (2008a) indicate that the role of S391 in Kir6.1 phosphorylation is still unclear. To confirm a role of S391 in Kir6.1 phosphorylation, the whole cell patch clamp technique was used to investigate the effect of PKC on Kir6.1S391/SUR2B channel activity. It was found that PMA reduced the currents by

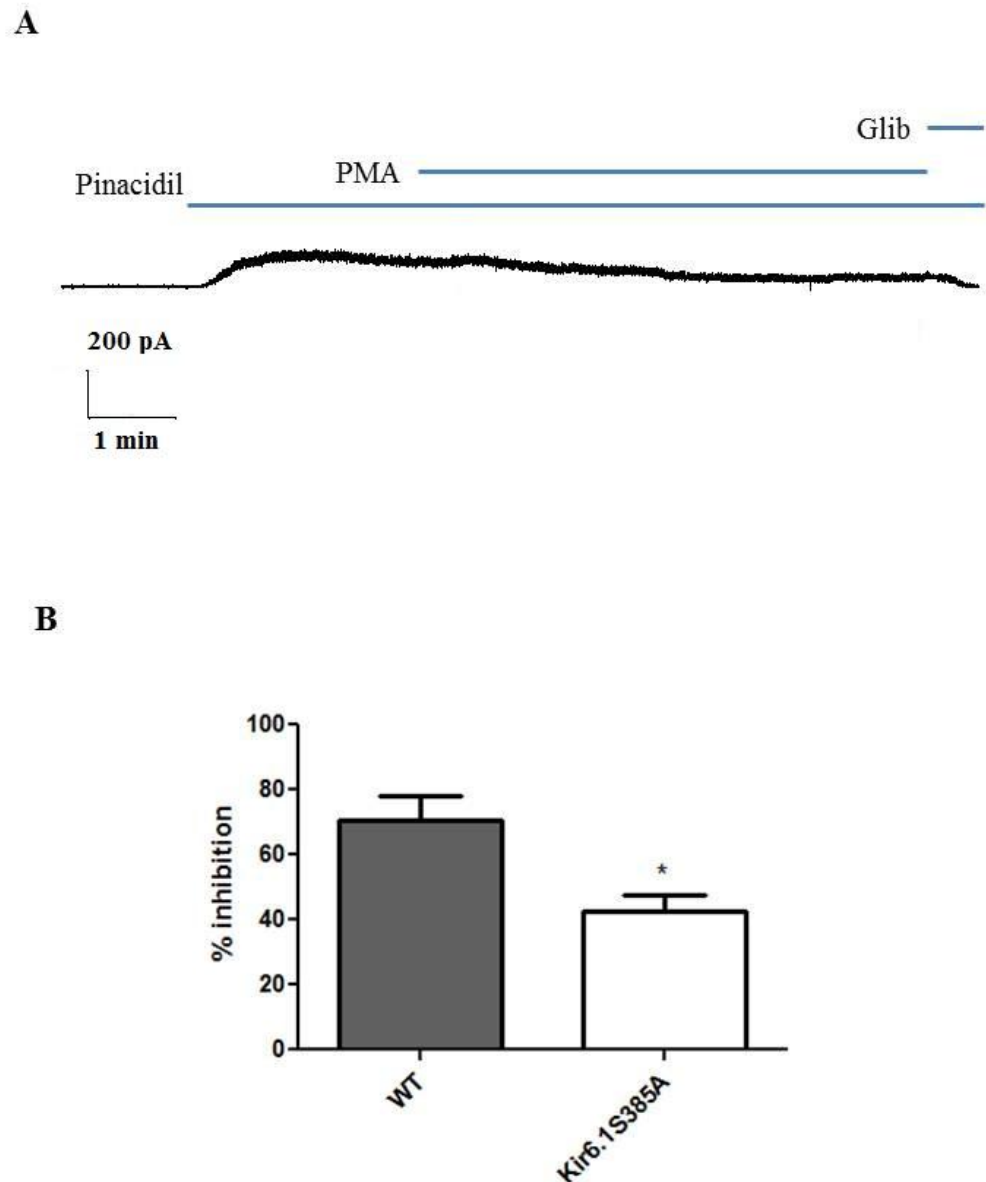


Figure 5.4 Inhibitory effect of PMA on Kir6.1S385A/SUR2B channel currents. A) Representative current showing the inhibitory effect of PMA on Kir6.1S385A/SUR2A channel activity. PMA can inhibit the channel to some degree. B) Comparison of channel inhibition by PMA in WT and Kir6.1S385A/SUR2B mutant channels. The percentage of inhibition was significantly different between WT and the mutant ($p=0.03$, $n=10$ (WT), 4(Kir6.1S385A)).

56.6 $\pm$ 8.8%. This inhibition was not different significantly from that of WT, therefore, this study excludes the role of S391 residue in PKC-mediated channel inhibition of Kir6.1 channels expressed with both SUR2B and SUR2A (chapter 4). The result of this study is shown in **Figure 5.5**.

5.3.5 PKC stimulation inhibits Kir6.1S397A/SUR2B channel current

The role of S397 in kir6.1 phosphorylation was also controversial. It was found that mutation of S397 residue did not affect the level of Kir6.1 phosphorylation (Singh, 2002). However, Shi et al (2008a) demonstrated that mutation of this site caused the reduction of the inhibitory effect of PKC the Kir6.1/SUR2B channel. The involvement of Kir6.1S397 was also confirmed in Kir6.1/SUR2A channel. Inhibitory effect of PMA was significantly reduced in the Kir6.1S397/SUR2A mutant although it was less than that of the Kir6.1S379 and Kir6.1S385 mutants with this accessory subunit. To clarify whether Kir6.1S397 contributes to inhibitory response of the channel to PKC phosphorylation when expressed with SUR2B subunit, channel activity was examined before and after PMA application. It was demonstrated that PMA inhibited the current by the same extent as it did to WT (62.5 $\pm$ 6.7% versus 70.7 $\pm$ 7.5%, for the Kir6.1397A/SUR2B mutant and WT respectively). The comparison of the percentage inhibition by PMA to WT is shown in **Figure 5.6**. This experiment showed that when expressed with SUR2B the Kir6.1S397 residue is not involved in PKC- mediated regulation of Kir6.1 containing channels. Although contrary to the finding of a slight inhibition via phosphorylation of this residue in Kir6.1/SUR2A channels, the results together indicate that any inhibitory influence

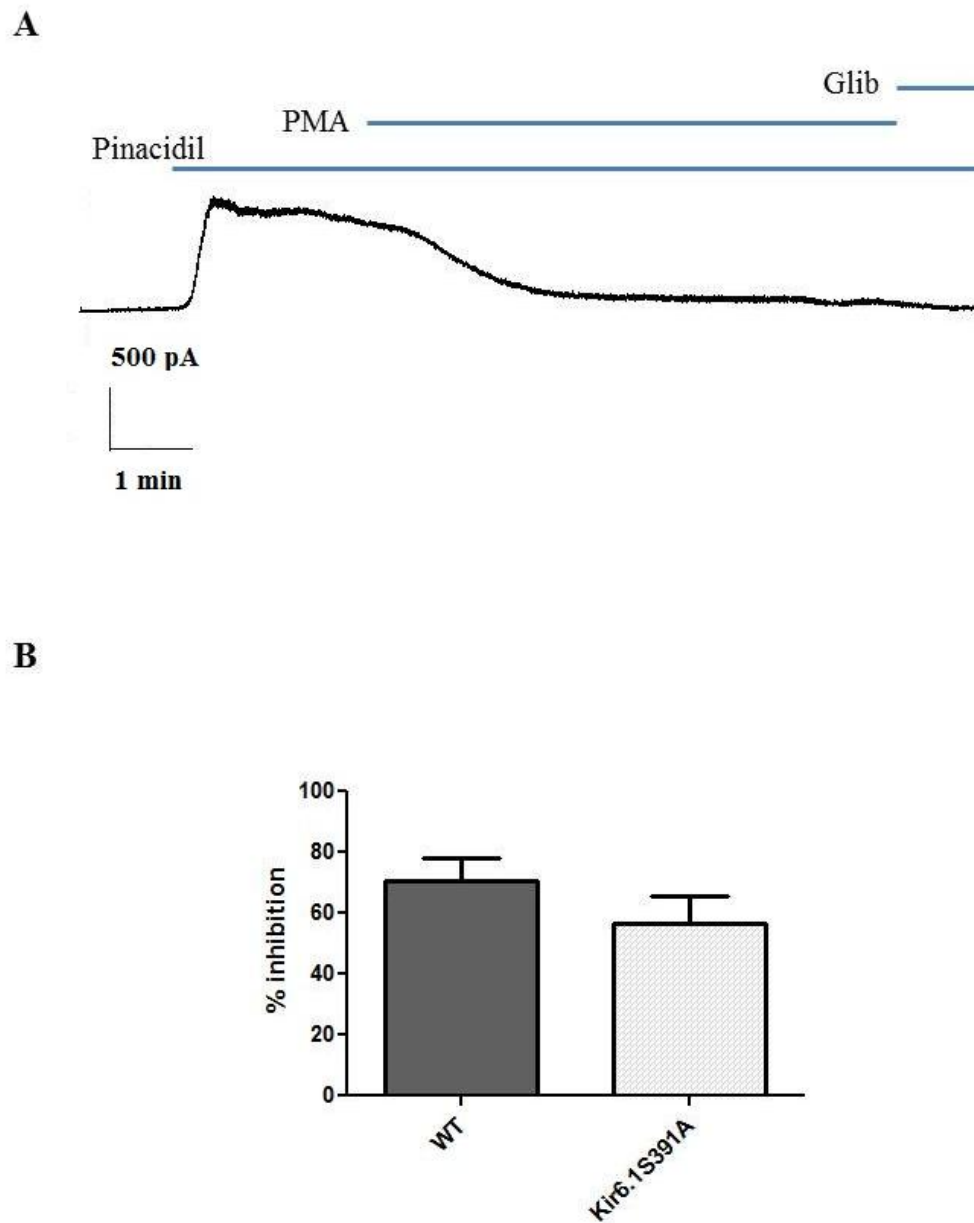


Figure 5.5 Effect of PMA on Kir6.1S391A/SUR2B channel current. A) recording showing the inhibitory effect of PMA. B) The inhibitory effect of PKC activation on Kir6.1S391A/SUR2B currents did not statistically differ from that of WT ($p=0.25$, $n=10$ (WT), 5(Kir6.1S391A)).

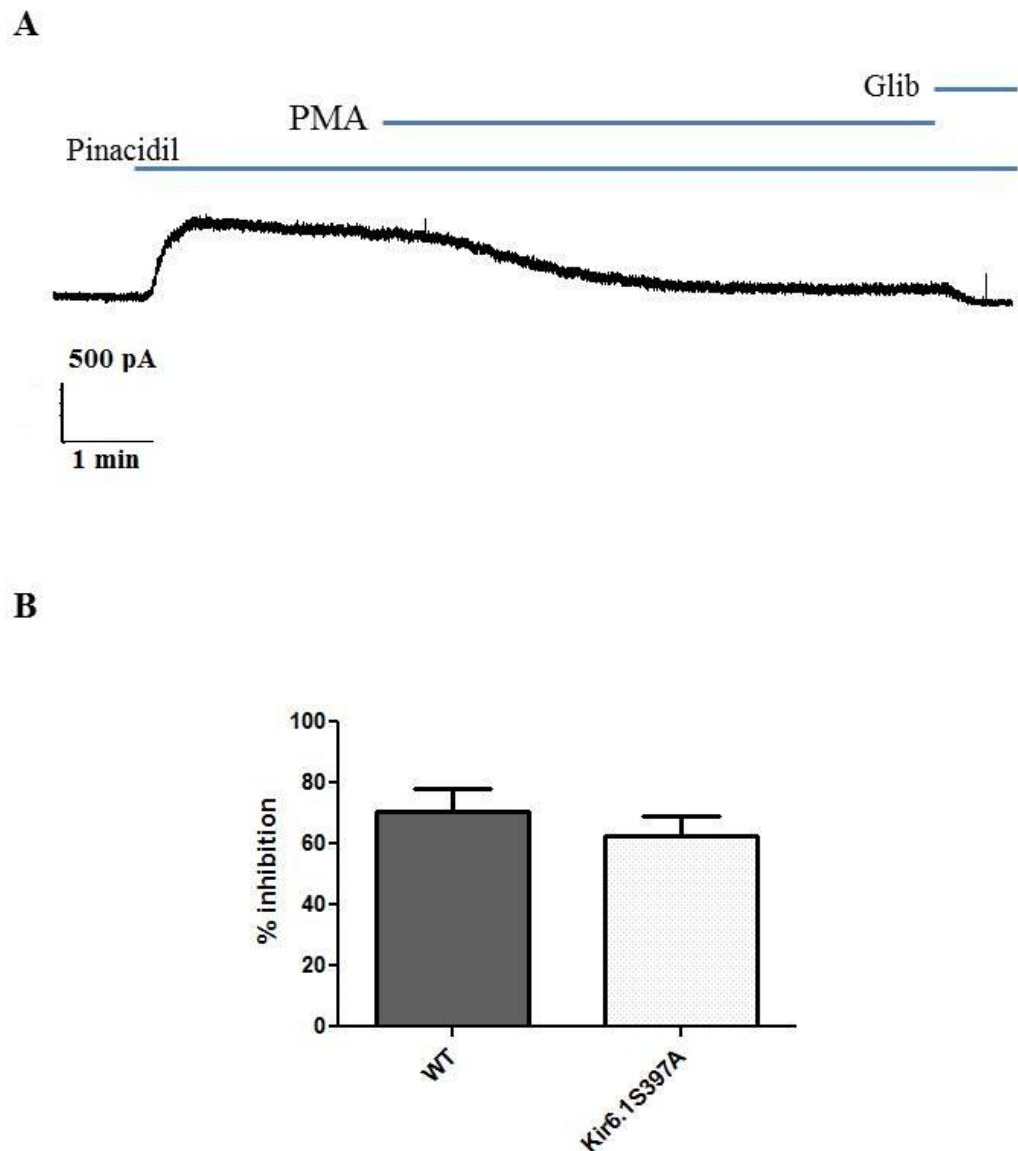


Figure 5.6 Effect of PMA on Kir6.1S397A/SUR2B channel current. A) Representative recording demonstrating the inhibitory effect of PMA on Kir6.1S397/SUR2B channel. B) Bar graph showing the percentage of channel inhibition by PMA in WT and Kir6.1S397A/SUR2B channel, which was not statistically different ($p = 0.46$, $n = 7$).

exerted through this residue is smaller than that of other residues (S379, S385) with either SUR2 isoform.

Summary

HEK cells expressing Kir6.1/SUR2B recombinant channels showed no activity while membrane potential was clamped at 0 mV. The channels could be activated by 100 μ M pinacidil and blocked by 100 μ M glibenclamide. However, only 50% of transfected cells yielded pinacidil activated currents and these with only relatively low current density. PKC activation led to WT channel inhibition. Mutation of S379 and S385 resulted in a significant reduction of the inhibitory effect of PKC. Conversely, S354, S391 and S397 mutants did not show a significant alteration in channel inhibition by PKC. The results of this chapter indicate that Kir6.1S379 and S385 are responsible for PKC-mediated inhibition of Kir6.1/SUR2B channels. The absence of effect of mutation of Kir6.1S397 on PKC mediated channel inhibition in Kir6.1/SUR2B channels was different to this residue observed for Kir6.1/SUR2A channels. This result suggests that this inhibitory influence via this residue is modulated by the nature of specific SUR isoform. Whatever, even in the presence of SUR2A, the inhibitory effect through this residue was relatively small compared to Kir6.1S397, S385 indicating that phosphorylation of these residues is of main action in bringing about Kir6.1 containing channel inhibition. The effect of PKC activation on WT and mutants of Kir6.1/SUR2B and Kir6.1SUR2A channels is summarized as in **Table 5.2**.

Table 5.2 PKC-mediated channel inhibition of WT and Kir6.1 mutants in combination with SUR2A or SUR2B channels

Kir6.1 subunit	% channel inhibition by PKC activation in the channels expressed with different SUR subunits	
	SUR2A	SUR2B
Kir6.1	65.1 $\pm$ 5.5	70.4 $\pm$ 7.5
Kir6.1S354	68.5 $\pm$ 7.4	58.2 $\pm$ 1.6
Kir6.1S379	31.4 $\pm$ 3.3 *	40.4 $\pm$ 9.0 *
Kir6.1S385	26.0 $\pm$ 5.7 *	42.6 $\pm$ 4.7 *
Kir6.1S391	65.6 $\pm$ 3.8	56.6 $\pm$ 8.8
Kir6.1S397	48.1 $\pm$ 2.7 *	62.5 $\pm$ 6.7

* indicates statistically different from that of WT of each subunit combination

Chapter 6: Cellular trafficking of Kir6.1 subunit containing K_{ATP} channels in response to protein kinase C activation

There is evidence showing that PKC activates cardiac and pancreatic K_{ATP} channels, whereas it inhibits vascular type K_{ATP} channels (Light, 1996, 2000, Hu, 2003, Jiao, 2008, Shi 2008a). In this study, the inhibitory effect of PKC was demonstrated on a novel recombinant K_{ATP} channel subunit combination, kir6.1/SUR2A channel (see chapter 4). It was proposed that PKC may modulate the K_{ATP} channel activity by affecting channel open probability or number of the surface channels. Light et al (1996, 2000) found that PKC increased open probability of Kir6.2/SUR2A, whereas some studies have indicated the internalization of Kir6.1/SUR2B promoted by PKC (Jiao, 2008). However, little is known about the mechanism by which PKC affects Kir6.1/SUR2A channel. In this study, it was shown that mutation of endosome target residues on the Kir6.1 subunit resulted in attenuation of inhibitory effect of PKC on Kir6.1/SUR2A channel (see section 4.2.6). Therefore, it was proposed that PKC affects Kir6.1/SUR2A activity by alteration of the channel trafficking rather than channel kinetics. The objective of this sub-study was to elucidate whether PKC affects the surface channel density of Kir6.1/SUR2A channels. The presence of this channel on the cell surface was detected by using an immunofluorescence staining technique for an epitope tag and then fluorescence intensity was evaluated by fluorescence plate reader or observed by confocal microscopy.

6.1 Optimization of surface Kir6.1/SUR2A channel labelling

6.1.1 Kir6.1/SUR2A internalization could not be detected by fluorescence intensity measurement.

Immunofluorescence assay has been described as a rapid and convenient method to investigate steady state cell surface ion channel density and endocytosis. In an attempt to investigate the internalization of Kir6.1/SUR2A channel, HEK 293 cells expressing Kir6.1 containing an extracellular hemagglutinin (HA) tag with SUR2A were treated with 100 nM PMA at 37°C for 5 or 30 minutes and then fixed and labelled with anti-HA tag primary antibody and FITC-conjugated secondary antibody. Fluorescence intensity was measured with a microplate reader and the relative intensity between non treated and treated group was compared (see section 2.10). In addition, cells transfected with SUR2A alone was also studied to determine the background of the assay and was used to normalize the intensity obtained from both non-treated and treated groups.

In initial experiments, it was difficult to observe a difference of normalized fluorescence intensity between treated and non- treated group because of high background of the test (1 versus 1.1 ± 0.1 versus 1.1 ± 0.1 for background, control and PMA group respectively, n=5 experiments). The background accounted for 88% of the highest intensity. A bar graph showing normalized fluorescence intensity of the groups is shown in **Figure 6.1**.

To decrease noise and gain more signal, the amount of subunit DNA transfected and the dilution of primary antibody used in HA-tag detection were varied. It was found that the best titration was at 1:250 of primary and secondary antibodies. However, it did not reduce the background further (as shown in **Figure 6.2 A**). Increasing the

amount of DNA of each subunit transfected to 3 µg did not improve the specific signal or allow the background to be better distinguished from the test (**Figure 6.2 B**). The time course of PMA incubation was also questioned and so the variation in immunofluorescence staining as a function of intensity PMA incubation duration was studied. It was found that, in the presence of the high background fluorescence, fluorescence intensity of control, PMA 5 min, 10 min, 30 min and 60 min were not different to each other (**Figure 6.2 C**). In summary, it was shown that immunofluorescence used to quantify surface Kir6.1/SUR2A channels using a microplate reader was not sensitive enough to distinguish the signal from the noise. Therefore, detection of immunofluorescence by confocal microscopy was considered.

6.1.2 Optimization for confocal microscopic study of trafficking of Kir6.1/SUR2A channels

The purpose of this experiment was to compare the relative surface density of Kir 6.1/SUR2A channels in non-treated and PMA treated cells. Confocal microscopy was considered superior for this purpose. Generally, immunostaining for confocal microscopy requires fixation, permeabilization and labelling of cells. However, this study aimed to study surface Kir6.1/SUR2A channels using an extracellular epitope, therefore, permeabilization was not necessary.

6.1.2.1 Membrane Kir6.1/SUR2A labelling by immunofluorescence confocal microscopy

To test whether PKC promotes internalization of Kir6.1/SUR2A channels, HEK 293 cells transfected with Kir6.1-HA tag /SUR2A were used in this experiment. The

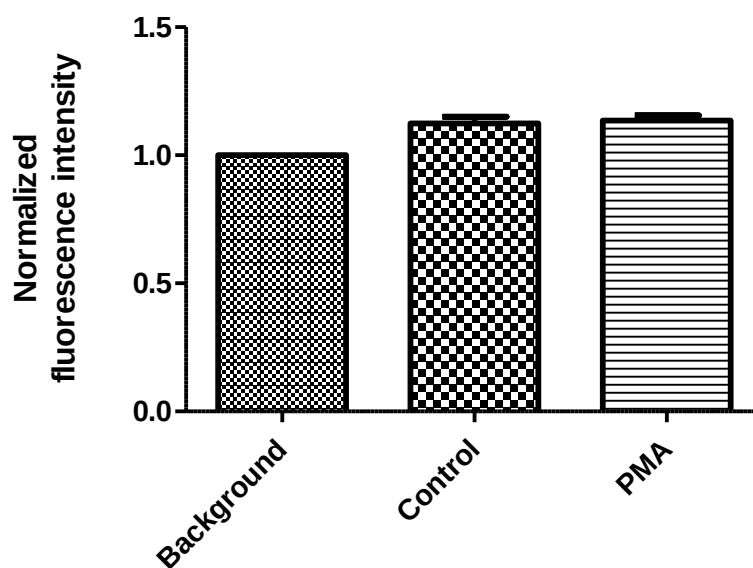


Figure 6.1 Effect of PMA on fluorescence intensity. Bar graph comparing the fluorescence intensity between background, control and PMA treated cell groups (obtained from 5 experiments with a 2 μ g each DNA transfection, 1:250 dilution of primary and secondary antibodies) which showed no difference between the control and PMA groups. It was noted that the fluorescence intensity from cells transfected with SUR2A alone, background group, is about 88% of the highest intensity.

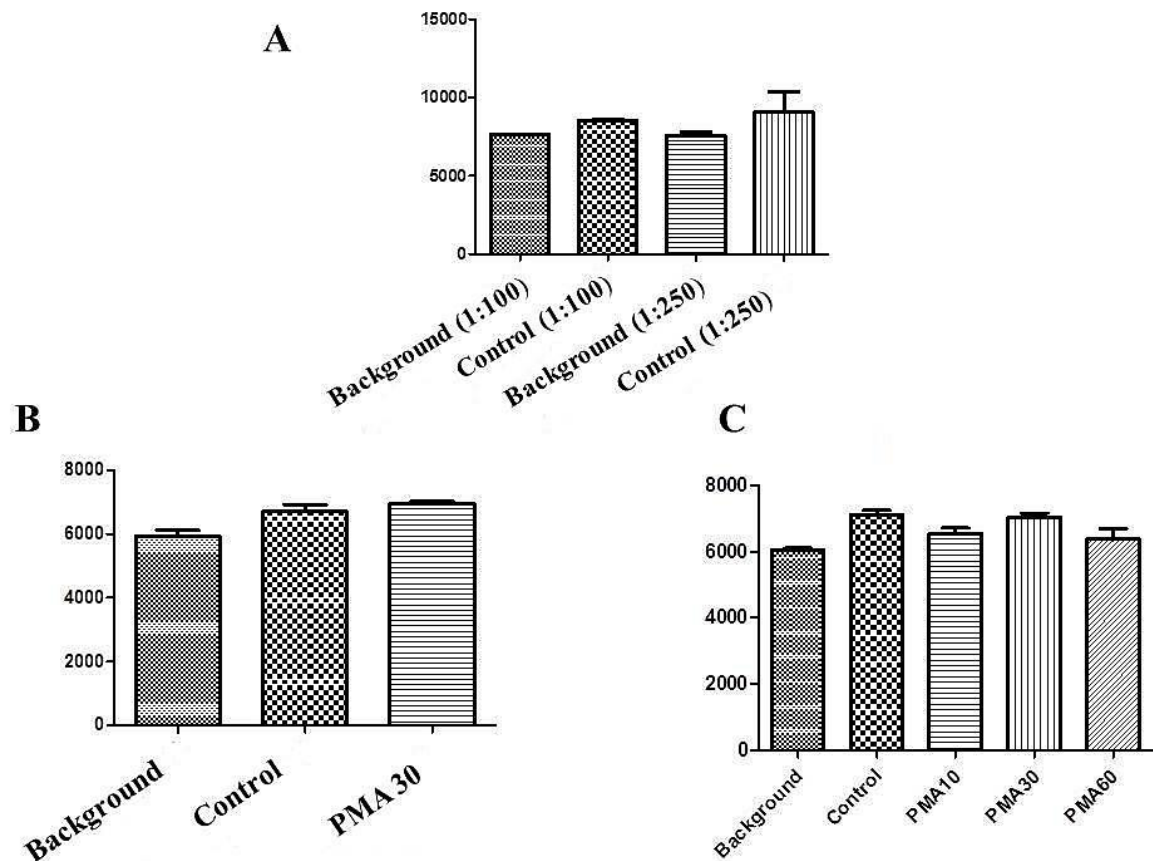


Figure 6.2 Optimization for Kir6.1/SUR2A detection by immunofluorescence. A) Titration of primary antibody dilution. Titration primary antibody from 1:100 to 1:250 (with 1:250 secondary antibody) did not lower the background fluorescence ($n=2$). B) Increasing the DNA amount of subunit DNA transfected from $2\mu\text{g}$ to $3\mu\text{g}$ each did not permit background and signals to be distinguished (the experiment was carried out at 1:250 dilution of primary and secondary antibodies, $n=2$). C) Effect of time course of PMA incubation on fluorescence intensity. There was no difference between groups ($n=4$). The background was still high.

transfection and labelling protocol used in this experiment was the same as that in the fluorescence intensity measurement protocol, assigned as **protocol I** (see section 6.1.1). After cell staining, image processing was performed with a confocal microscope. After that, single cells were quantified for fluorescein intensity by using image processing software. It was shown that co-transfection of the cells with mCherry-F resulted in red label of cell membranes (**Figure 6.3**) which was detected as a border of staining around the cells. The Kir6.1-containing channel was also successfully labelled with primary and FITC- conjugated secondary antibody resulting in a bright green fluorescence ring locating at the cell membranes. As expected, intracellular Kir6.1 could not be detected (**Figure 6.3**).

Although the images were clear for detection of cell membranes and Kir6.1-containing channel at the surface, it was difficult to determine any effect of PMA. It was not possible to compare membrane Kir6.1 fluorescence intensity level between control and PMA treated cells appropriately because of the difference in background of each picture and the unavoidable setting of different parameters used in confocal microscope to obtain images.

It was noticed that the number of cells labelled green only was higher in PMA 5 min and PMA 30 min groups. In control group, 95% of cells were successfully transfected with both Kir6.1/SUR2A and mCherry-F with 1.6% red only (mCherry-F) and 3.4% green only (Kir6.1-HA) cells. It was noteworthy that, in PMA group, the number of cells showing green fluorescence only was higher than that of control group (14% in PMA 5 min and 15% in PMA 30 min). Comparison of the percentage of cells representing red only, green only and both red and green in control and PMA treated group is shown in **Figure 6.4**.

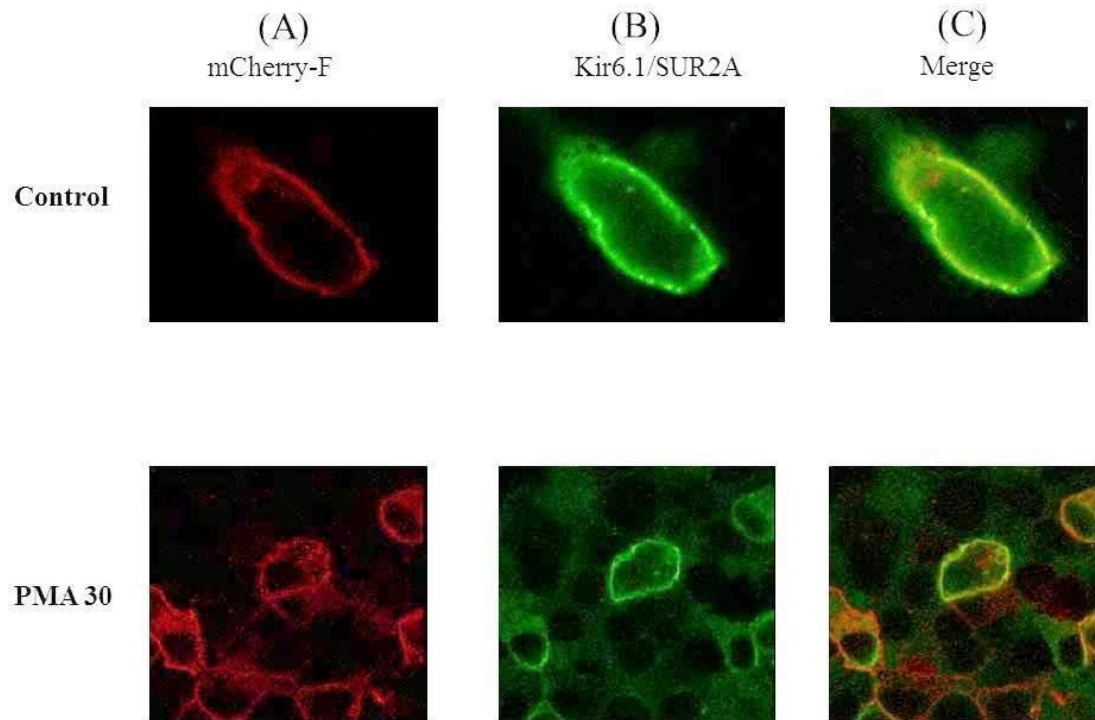


Figure 6.3 Confocal microscopy of immunofluorescently-labelled Kir6.1/SUR2A channels. Images of HEK cells expressing Kir6.1/SUR2A channels in non-treated and PMA treated group using **protocol I**. A) Representative image showing that plasma membrane was labelled with mCherry-F (red). B) Image of HEK cells expressing Kir6.1 containing channels labelled with anti-HA antibody and FITC-secondary antibody(green). C) The merged image of A and B indicated that Kir6.1/SUR2A channels located at the plasma membrane. The internalization of Kir6.1/SUR2A channels could not be observed in both non-treated and 100 nM PMA treated cells using this protocol.

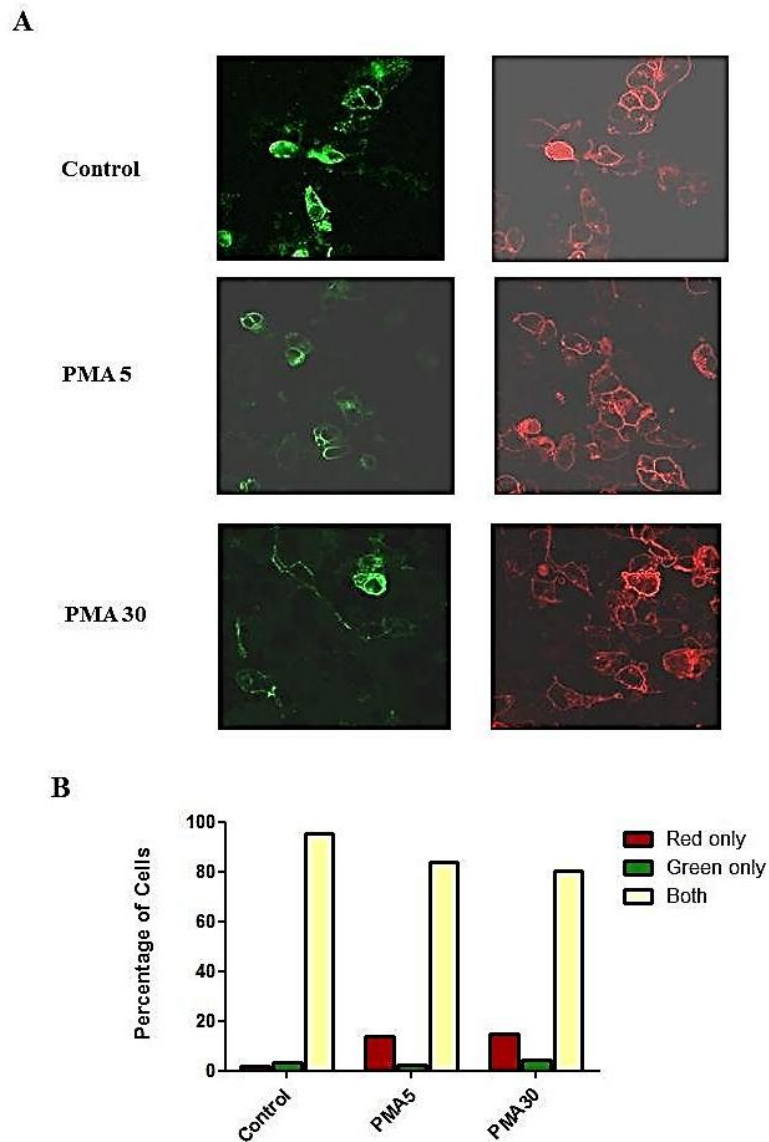


Figure 6.4 Comparison of the percentage of cells expressing mCherry-F and Kir6.1/SUR2A channels on the plasma membrane between control and PMA – treated cells. A) Images showing HEK cells expressing mCherry (red) and Kir6.1 protein (green) in the absence or presence of 100 nM PMA. In the presence of PMA, the number of cell expressing red fluorescence only was higher than that of control (control=3.4%, n=114, PMA 5 min=13%, n=107, PA 30 min=15%, n=311). The increase in the number of red fluorescence only cells suggests that there might be internalization of Kir6.1 protein (green) so that only red was found on the plasma membrane.

It was shown that the immunofluorescence protocol for confocal microscopic study provided clear images of Kir6.1/SUR2A channels at the plasma membrane. To make conclusion regarding trafficking/internalization of images required visualization of movement of Kir6.1/SUR2A. Given that it was not possible to discern a difference in the fluorescence intensity of labelled Kir6.1-containing channels at the surface of cells after PMA treatment, the protocol was further modified to assay for the presence of intracellular Kir6.1 subunits before and after a period of incubation at a permissive temperature. Briefly, Kir6.1-containing channels on the surface of HEK 293 cells were labelled with primary and secondary antibodies, treated with 100 nM PMA, finally fixed and then visualized. Using this protocol (namely, **protocol II**), it was found that it was possible to detect internalisation of surface-labelled channels. Intracellular Kir6.1/SUR2A channels associated fluorescence was able to be observed after a period to permit internalization as shown in **Figure 6.5**.

6.2 PKC promotes internalization of Kir6.1/SUR2A channel

To study the hypothesis that Kir6.1/SUR2A channels are internalized on PKC stimulation, HEK cells expressing Kir6.1-HA-tag/SUR 2A channels were blocked and labelled with rat anti HA antibody and FITC conjugated secondary antibody and then incubated with 100 nM PMA at 37°C for 5 or 30 min. The cells then were fixed and visualized by confocal microscopy. It was found that in control cells which were kept at 4°C all time during procedures, the Kir6.1/SUR2A was detected at the cell surface. Incubation the cells at 37°C for 30 minutes resulted in endocytosis in some cells. In some cells treated with PMA for 5 minutes, internalization was apparent but did not

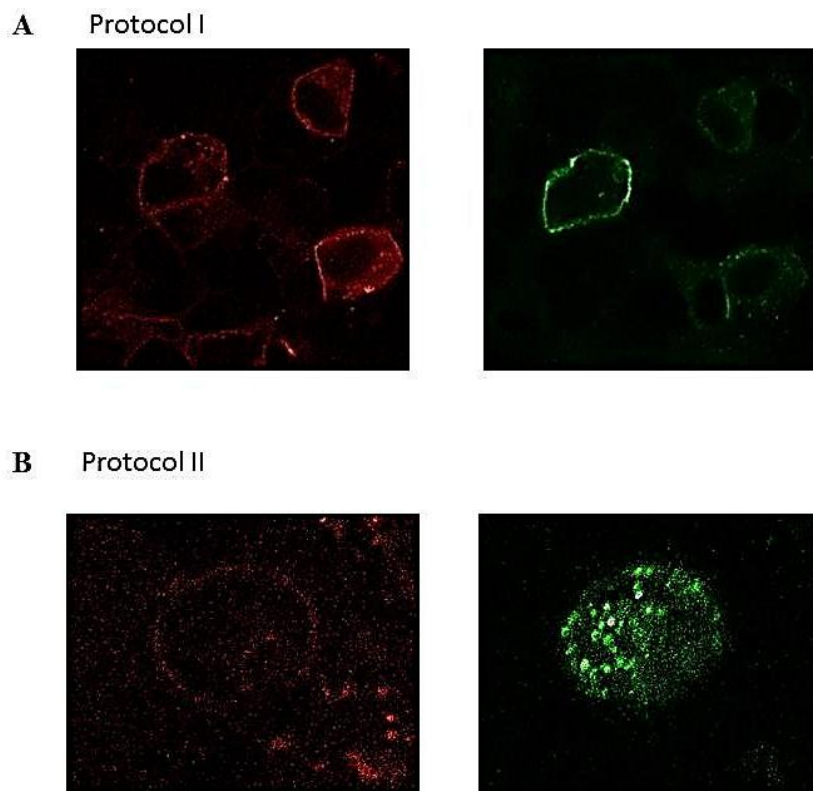


Figure 6.5 Comparison of images obtained from the immunofluorescence labelling protocol I and II A) Image of cells treated with 100 nM PMA showing Kir6.1/SUR2A localization at the membranes using protocol I which was performed by treating the cells with PMA, then fixing and finally labelling. B) Visualization of Kir6.1/SUR2A channel internalization using protocol II where HEK 293 cells expressing Kir6.1-HA/SUR2A and mCherry-F were labelled first, then treated with PMA and finally fixed. There are some punctae seen inside the cells. Red (mCherry-F) indicated cell membranes, green indicated Kir6.1/SUR2A channel.

reach statistical significance for the whole population. Significant trafficking of Kir6.1/SUR2A, as shown by punctate intracellular labelling, was found in cells treated with 100 nM PMA for 30 minutes (**Figure 6.6**). To quantify intracellular Kir6.1/SUR2A channel, the green intensity was measured by using image processing software. To avoid the confounding effect of the background, the mean intracellular intensity of each cell was normalized to the mean background intensity of the image. Then, the normalized green intensity was compared between groups. It was shown that the mean intensity of intracellular green fluorescence of PMA 30 min group was significantly higher than that of PMA 5 min and control groups at 37⁰C (40.4 ± 7.4 , 18.9 ± 9.1 , 8.2 ± 2.5 , n= 14, 9, 16 cells from 3 experiments). However, the intensity of PMA 5 min was not different statistically compared to cells incubated at 37⁰C. The comparison of mean fluorescence intensity between groups is shown in **Figure 6.7**. According to quantitative analysis of internalization of Jiao et al (2008), in which HEK cells were considered as positive (> 5 punctae) or negative (< 5 punctae) for internalization was applied and then the number of positive cells was counted. It was found that 68% of cells treated with PMA 30 min (n=31, 3 experiments) showed internalization whereas the percentage of internalization of cells treated with PMA for 5 min group and control group at 37⁰C was 19% and 33% respectively (n=37 and 21, respectively, from 3 experiments).

In summary, it was found that a basal level of internalization of Kir6.1/SUR2A occurred to some degree at 37⁰C. Incubation of the cells with PMA for 30 minutes significantly promoted internalization of the channels. However, treating the cells with PMA for 5 minutes did not affect channels internalization significantly.

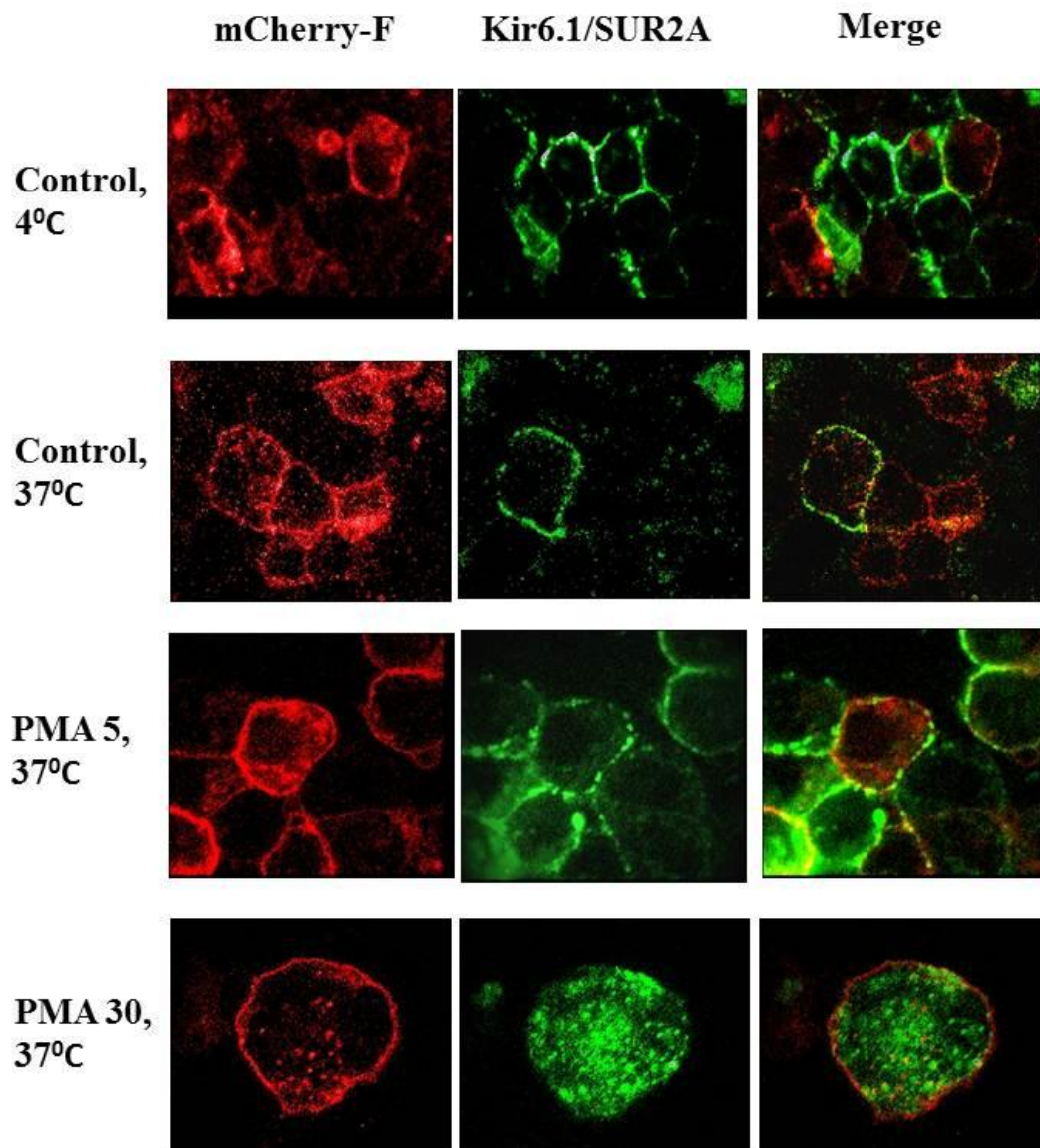


Figure 6.6 Effect of PMA on internalization of Kir6.1/SUR2A channels. *HEK 293* cells expressing *Kir6.1-HA tag/SUR2A* and *mCherry* were labelled for plasma membrane (red, left column) and *Kir6.1* protein (green, middle column). Images in two upper rows showed no internalization of *Kir6.1/SUR2A* channel in non-treated cells. Incubation the cells with 100 nM for 5 minutes did not promote the internalization. Intracellular punctae were seen in cells treated with PMA for 30 minutes (bottom row image).

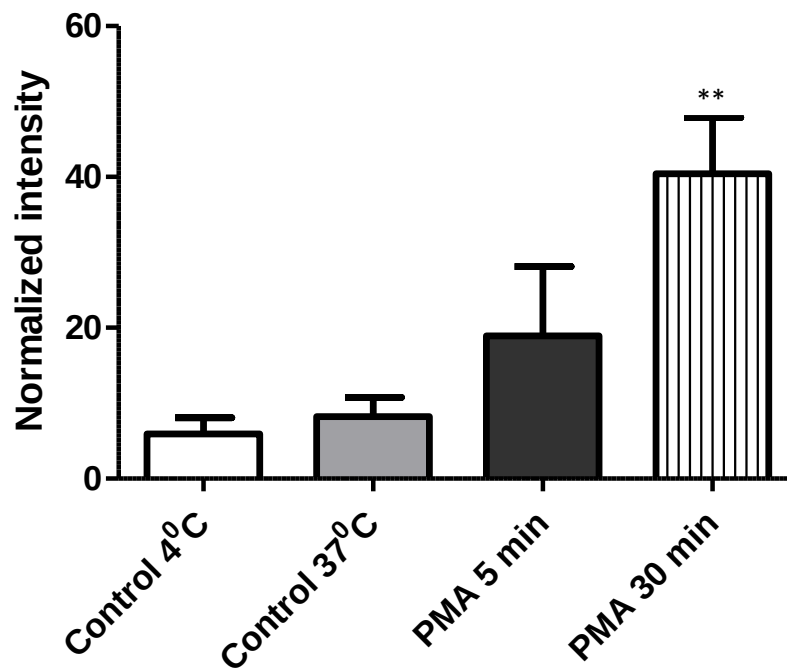


Figure 6.7 Comparison of normalized intracellular green fluorescence intensity between control and PMA groups. The normalized intensity of PMA 30 min group was significantly higher than that of control 37°C group ($p=0.003$, $n=14$ and 9 for PMA 30 min and control group from 3 experiments).

6.3 PKC mediated Kir6.1/SUR2A internalization depends on dynamin

The mechanism of K_{ATP} trafficking is still unclear. There is evidence suggesting that it is dynamin dependent. Hu et al (2003) found a dynamin dependent effect of PKC on action potential duration of rat ventricular myocytes. Jiao et al (2008) demonstrated that the inhibitory effect of PKC on Kir6.1/SUR2B currents was reduced in HEK cells co-transfected with the dynamin mutant K44E by 25%. Confocal microscopic study also showed that the internalization of Kir6.1/SUR2B induced by PKC was blocked by a dominant negative dynamin mutant (Jiao et al, 2008). However, to date there is no evidence concerning the mechanism of Kir6.1/SUR2A internalization. This study aimed to investigate the hypothesis that PKC activation stimulates internalization of Kir6.1/SUR2A in a dynamin dependent manner. HEK cells expressing Kir6.1/SUR2A were studied by immunofluorescence microscopy. The cells were treated with 100 nM PMA or 100 nM PMA and 60 μ M dynasore, a cell permeable, inhibitor of dynamin GTPase activity for 30 minutes and then labelled and visualized by confocal microscopy.

It was found that treatment the cells with the dynamin inhibitor could block the internalization of Kir6.2/SUR2A to some degree. There were less punctae in cells treated with dynasore than that of the PMA alone group. There were also less number of cells scored as positive result (>5 punctae), 17.5% in control group versus 68% of PMA 30 min group (n=40 from 2 experiments for dynasore, n=31 from 3 experiments in PMA group). Fluorescence image showing the effect of dynamin inhibitor is shown in **Figure 6.8**.

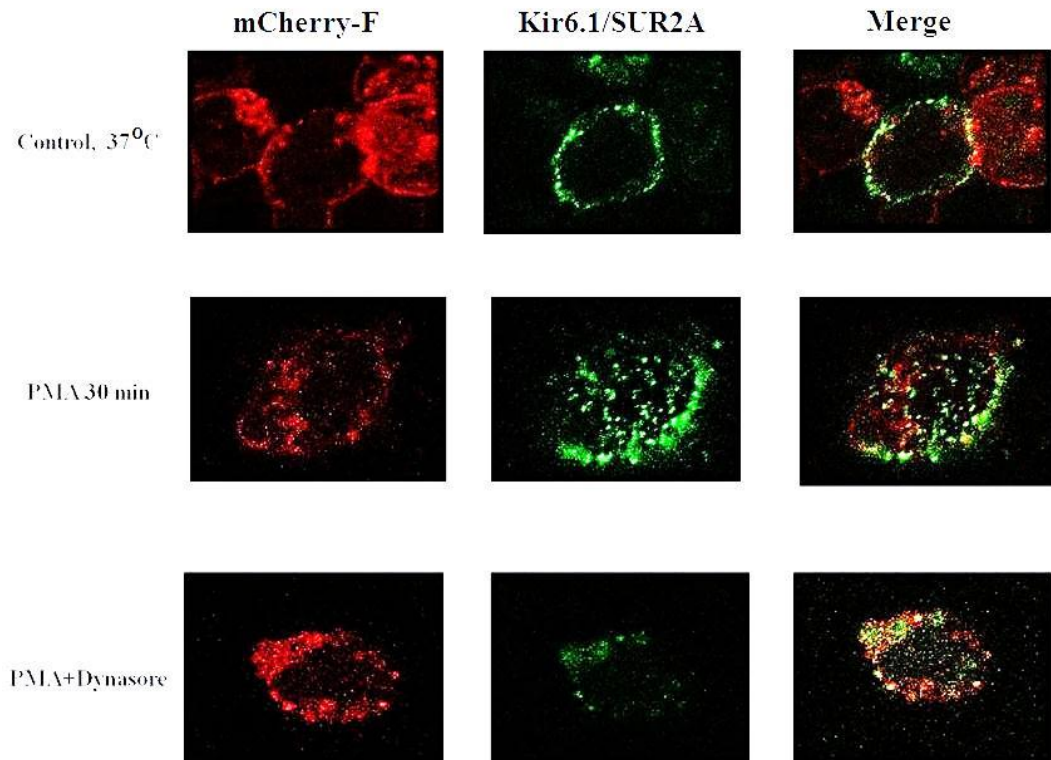


Figure 6.8 Effect of dynamin inhibitor on Kir6.1/SUR2A internalization. *PMA caused internalization of Kir6.1/SUR2A channel resulting in visualization of intracellular punctate (middle row images) when compared to non-treated cells (upper row image). Dynasore, a dynamin inhibitor, inhibited the internalization of Kir6.1/SUR2A mediated by PKC (bottom row images).*

These data indicated that PKC-mediated internalization of Kir6.1/SUR2A is dynamin dependent. However, more experiments are required to confirm the effect of dynamin on Kir6.1/SUR2A internalization.

Summary

Internalization of Kir6.1/SUR2A can be assessed by immunofluorescence with confocal microscopy. In HEK cells transfected with Kir6.1/SUR2A, incubation of cells with PMA for 30 minutes resulted in internalization of Kir6.1/SUR2A channels. PMA treatment for 5 minutes did not promote the internalization. The PKC activated Kir6.1/SUR2A trafficking was reduced by dynasore, the dynamin inhibitor, and was concluded therefore to be dynamin dependent.

Chapter 7: Identification of PKC isoforms responsible for adenosine-induced Kir6.1 phosphorylation in rat ventricular myocytes

The studies in chapter 4 and 5 demonstrated that PKC could reduce Kir6.1-containing channel current. It is proposed that phosphorylation of kir6.1 by PKC leads to alteration of the channels. However, it is not clear now which PKC isoforms are responsible for this phenomenon. The objective of this sub-study was to confirm the role of A₁AR activation on Kir6.1 phosphorylation and to clarify which PKC isoforms are responsible for the phosphorylation. It is hypothesized that A₁AR activation can bring about the phosphorylation of kir6.1 and that PKC ϵ and PKC δ may play a role in Kir6.1-containing K_{ATP} channel phosphorylation.

7.1 A₁AR mediates Kir6.1 phosphorylation in rat cardiomyocytes

Experiments in chapter 4 and 5 have shown the effect of PKC on Kir6.1-containing K_{ATP} channel activity which is thought to be a result of Kir6.1 phosphorylation. It was hypothesized that application of A₁AR agonist should also result in Kir6.1 phosphorylation. To investigate whether A₁AR stimulation induces K_{ATP} phosphorylation, male Wistar rat ventricular myocytes pre-incubated with ³²P orthophosphate to label the ATP pool were treated with adenosine A₁ receptor agonist, 2-Chloro-N⁶-cyclopentyladenosine (CCPA), and then the phosphorylated Kir6.1 was detected and analysed using an immunoprecipitation protocol (see section 2.4.2) Incorporation of ³²P into Kir6.1 was quantified by measuring the relative absorbance of bands corresponding to Kir6.1 in autoradiogram of immunoprecipitated ³²P-Kir6.1 separated by SDS-PAGE. It was found that in the absence of CCPA, Kir6.1 phosphorylation still occurred. However, CCPA further stimulated Kir6.1

phosphorylation significantly. The normalized basal phosphorylated Kir6.1 level was statistically different from that stimulated by CCPA (1 versus 2.3 ± 0.6 , respectively, $n = 9$ experiments, $p = 0.003$, **Figure 7.1**). This confirms that A₁AR activation leads to Kir6.1-containing K_{ATP} channel phosphorylation in isolated rat ventricular myocytes.

7.2 PKC isoforms and adenosine-mediated Kir6.1 phosphorylation

There is evidence showing that specific PKC isoform play a role in K_{ATP} channel regulation. Jiao et al (2008) found that PKC ϵ inhibitor peptide (ϵ V1-2) inhibited the PKC-mediated inhibitory effect of Kir6.1/SUR2B recombinant channel. In addition, it was also demonstrated an increased level of Kir6.2 phosphorylation following IPC and PKC ϵ -agonist mediated preconditioning (Raval et al, 2007). By using fluorescence microscopy, Garg et al (2007) demonstrated that in a COS-7 cell line transfected with Kir6.2/SUR2A, mitoplast localization of Kir6.2 was higher after treating the cells with PMA and it was blocked by PKC ϵ peptide inhibitor (PKC ϵ V1-2). Immunoblot analysis also confirmed the alteration of mitochondrial Kir6.2 level by PMA and the inhibitory effect of PKC ϵ (Garg et al, 2007).

There are some studies that mention other specific PKC isoforms responsible for IPC. Translocation from cytosol to membrane of PKC δ and PKC ϵ after IPC was found, whereas Hudman et al (2004) reported the translocation of PKC α , β , γ , δ , ι , λ/ζ in response to PKC activator, PMA. Mitchell et al (1995) demonstrated translocation of PKC α and δ in IPC heart. It is still unclear which specific PKC isoforms are responsible for IPC and K_{ATP} activation.

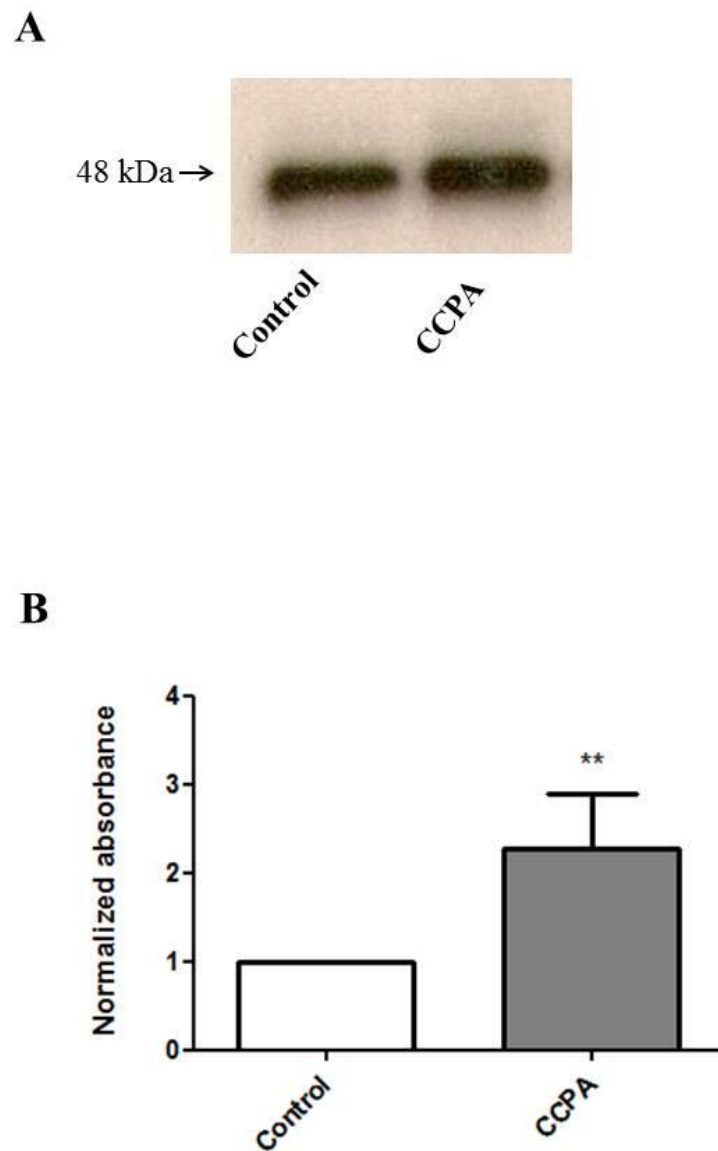


Figure 7.1 Comparison of phosphorylated Kir6.1 amount between control group and CCPA group. A) A bar graph showing the normalized absorbance for ^{32}P kir6.1 in control and CCPA treated group. The level of phosphorylated Kir6.1 was significantly higher in the CCPA group ($p = 0.003$, $n = 9$ experiment). B) Representative autoradiogram showing the different phosphorylated Kir6.1 level between control and CCPA group.

Therefore, the aim of the present study was to investigate the role of specific PKC isoforms on A₁AR-stimulated Kir6.1 phosphorylation. To do this, rat cardiomyocytes were treated with Tat conjugated-PKC inhibitors for five PKC isoforms, Tat-PKC α inhibitor (α C2-4), Tat- PKC β inhibitor (β C2-4), Tat-PKC γ inhibitor (γ V5-3), Tat-PKC δ inhibitor (δ V1-1) and Tat-PKC ϵ inhibitor (ϵ V1-2) and their effect on A₁AR stimulated phosphorylation was observed. The protocol used in this experiment was described in the phosphorylation protocol (see section 2.4.2). However, the cells were incubated with 100 nM of PKC inhibitors for 10 minutes prior to stimulation with CCPA.

7.2.1 PKC α , β , γ and δ are not involved in A₁AR-induced Kir6.1 phosphorylation

Comparison of the normalized absorbance for ³²P-Kir6.1 from ventricular myocytes treated with Tat-PKC α , Tat-PKC β , Tat-PKC γ and Tat PKC δ inhibitors found no significance difference to CCPA control samples, although a trend towards inhibition was observed in each inhibitor (0.6 ± 0.1 , 0.80 ± 0.2 , 0.7 ± 0.1 , 0.6 ± 0.1 , respectively, n=7, **Figure 7.2**).

7.2.2 PKC ϵ is responsible for A₁AR-induced Kir6.1 phosphorylation

It was demonstrated that application of PKC ϵ inhibitor reduced A₁AR- induced Kir6.1 phosphorylation. There is significant difference in normalized absorbance for ³²P-Kir6.1 of Kir6.1 phosphorylation between control group and PKC ϵ inhibitor treated group (0.4 ± 0.1 versus 1, n=7, p<0.05) as shown in **Figure 7.2**.

7.2.3 Effect of specific PKC activators on A_1AR -induced Kir6.1 phosphorylation

To confirm the role of a proposed specific isoform of PKC responsible for Kir6.1 phosphorylation, the effect of specific PKC activators was investigated. $\Psi\delta$ RACK and $\Psi\epsilon$ RACK were used to activate PKC δ and ϵ . RACK is the abbreviation for receptors for activated C-kinase, which is believed to be a family of isozyme specific intracellular anchoring proteins. It was identified by Mochly-Rosen's group in 1991. $\Psi\epsilon$ RACK is an 8 amino acid sequence which is homologous to the PKC ϵ binding site on ϵ PKC RACK (Mochly-Rosen, 1991, Mackay, 2001). It was found that introduction of $\Psi\epsilon$ RACK can activate PKC ϵ in mouse heart (Dorn, 1999). Result shown in Figure 7.2 demonstrated that PKC ϵ inhibitor could diminish A_1AR -induced Kir6.1 phosphorylation. Therefore, it was hypothesized that introduction of Tat-conjugated $\Psi\epsilon$ RACK to rat cardiac myocytes should promote of Kir6.1 phosphorylation.

To investigate the effect of specific PKC activators, after ^{32}P incorporation, rat cardiomyocytes were separated in to 3 groups, Control (no CCPA), $\Psi\delta$ RACK, $\Psi\epsilon$ RACK treated group which were incubated with 100 nM $\Psi\delta$ RACK or $\Psi\epsilon$ RACK for 30 minutes then cells were proceeded according to the phosphorylation protocol in section 2.4.2 The absorbance of each group was determined and normalized to that of control.

The densitometric analysis showed that $\Psi\epsilon$ RACK, but not $\Psi\delta$ RACK potentiated Kir6.1 phosphorylation. The normalized absorbance of cells treated with PKC ϵ activator was significantly higher than that of control (2.2 ± 0.4 versus 1), whereas there was no significant difference between $\Psi\delta$ RACK and control group (1.8 ± 0.4 versus 1, n=6). The comparison among groups is shown in **Figure 7.3**.

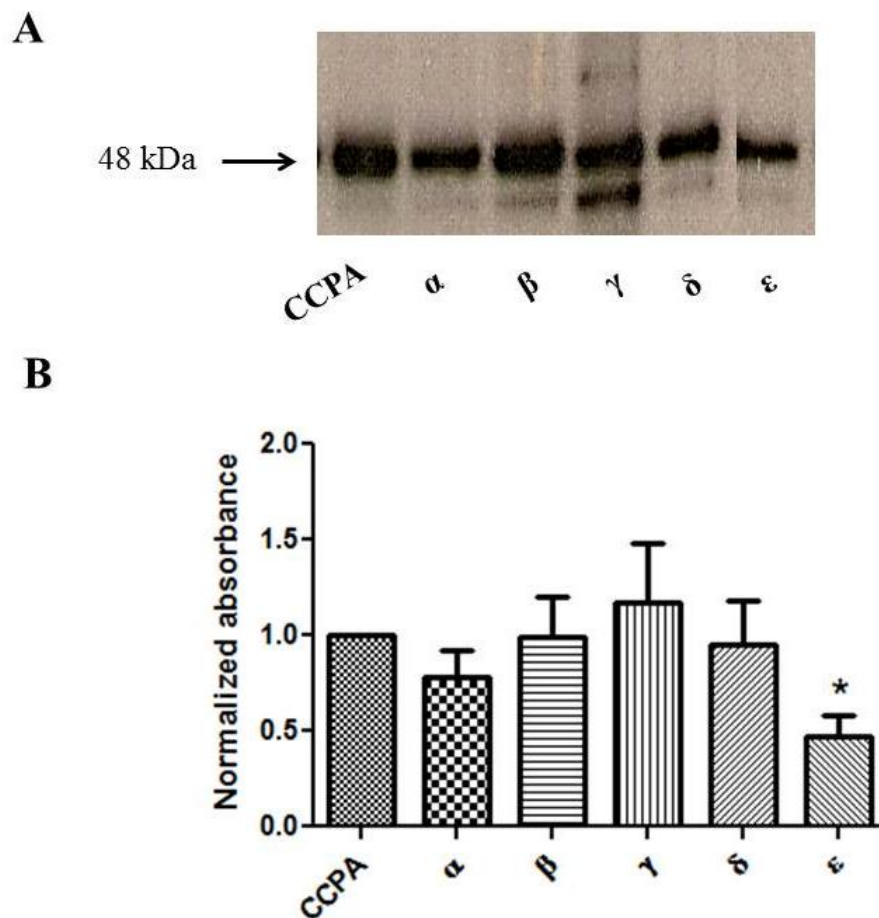


Figure 7.2 Comparison of the level of phosphorylated Kir6.1 in the presence and absence of PKC inhibitor peptides. Phosphorylation of Kir6.1 was stimulated by CCPA in the presence and absence of PKC inhibitor peptides. The effect of each isoform specific PKC inhibitors was then determined by densitometric analysis. A) A representative experiment band showing the autoradiogram of phosphorylated kir6.1 level in control and PKC inhibitor treated groups. B) A bar graph comparing absorbance among CCPA alone and PKC inhibitor treated groups. The absorbance of each group was normalized by that of CCPA group. The normalized absorbance of PKC ϵ inhibitor group significantly lower than that of CCPA ($p < 0.05$, $n = 7$).

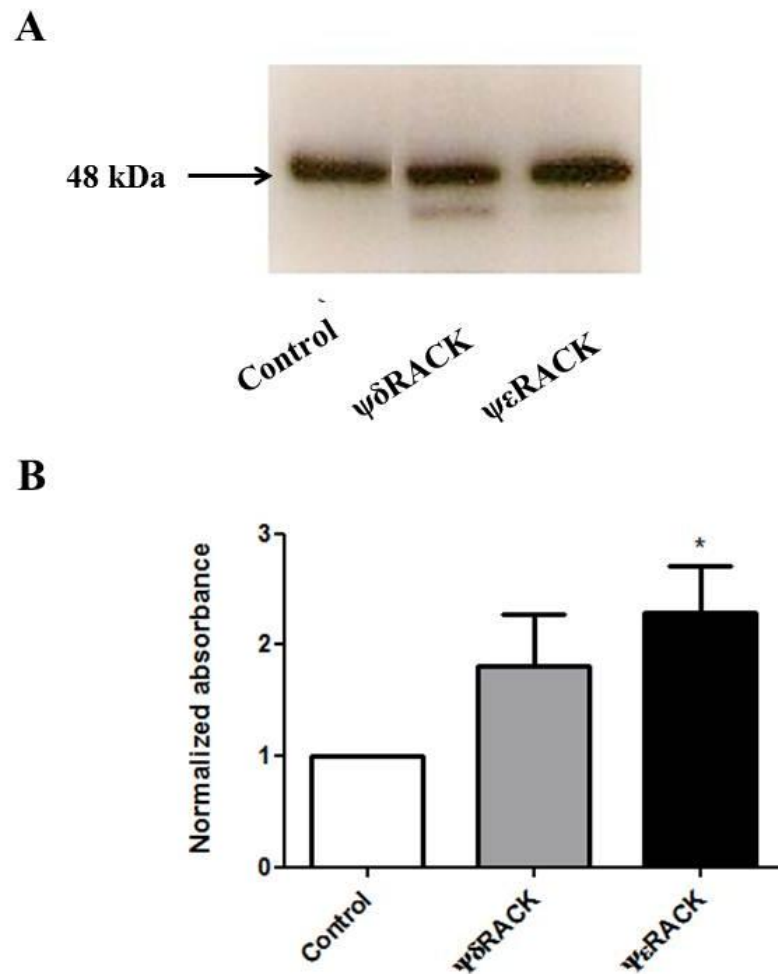


Figure 7.3 Comparison of the level of phosphorylated Kir6.1 in the absence and presence of PKC activator peptides. A) A representative experiment showing phosphorylated Kir6.1 amount in control, $\psi\delta$ RACK and $\psi\epsilon$ RACK groups. B) A bar graph representing the effect of PKC activators, $\psi\delta$ RACK and $\psi\epsilon$ RACK. The phosphorylation of Kir6.1 in $\psi\epsilon$ RACK group was significantly higher than that of control group whereas that of $\psi\delta$ RACK was not ($n=6$).

A concern was about the effect of cell penetrating peptide, Tat, alone, which is conjugated to PKC inhibitors and activators. Tat, or arginine –rich cell-penetrating peptides, can transport cargo molecules across cell membrane in an active and functional form (Dietz, 2004). There is no report about the effect of Tat on K_{ATP} phosphorylation. However, to avoid this potential confounding effect, Tat alone was also tested for its influence on Kir6.1 phosphorylation. It was found that when the cells were treated with 100 nM Tat + CCPA, the Kir6.1 level did not differ significantly from that of CCPA alone group (0.6 ± 0.2 versus 1, n=7, data not shown). This implied that Tat did not alter the phosphorylation of Kir6.1.

Summary

In the rat isolated myocytes, A₁AR agonist, CCPA, was shown to stimulate phosphorylation of Kir6.1. It is found that administration of PKC ϵ inhibitor peptide could attenuate CCPA-induced Kir6.1 phosphorylation, whereas PKC α , PKC β , PKC γ and PKC δ had no effect. Isoform specificity was further confirmed by the demonstration that $\Psi\epsilon$ RACK, a PKC ϵ activator, could increase the level of phosphorylated Kir6.1, whereas $\psi\delta$ RACK was not. Together the results for Tat-PKC ϵ inhibitor peptide and $\Psi\epsilon$ RACK activator peptide suggest a specific role for PKC ϵ in the phosphorylation of Kir6.1 in response to CCPA stimulation.

Chapter 8: Discussion

PKC is thought to be central in ischaemic preconditioning pathways whereas K_{ATP} channels are believed to be the end effectors of this mechanism. It was proposed that PKC mediated cardioprotection by opening $sarK_{ATP}$ channels resulting in shortening of action potential duration leading to reduction in intracellular calcium accumulation and contraction (Zaugg et al, 2003). The finding that the protective consequences of IPC did not correlate to shortened action potential duration, as well as the blockade of IPC by 5 HD, a specific $mitoK_{ATP}$ channel blocker, urged researchers to pay attention to the role of $mitoK_{ATP}$ channels in IPC. The pharmacological properties of the $mitoK_{ATP}$ channel have been well studied whereas electrophysiological property and molecular structure is still not clear. One of the proposed subunit combinations for the $mitoK_{ATP}$ was Kir6.1, contributing the pore forming subunit, and SUR2A providing the regulatory subunit (Singh et al, 2003). By linking PKC and $mitoK_{ATP}$ channels, it was hypothesized that, this type of K_{ATP} channel is modulated directly by PKC during IPC. Although there is evidence showing that Kir6.1 is phosphorylated by PKC *in vitro*, the effect of PKC on Kir6.1/SUR2A channel activity, including its mechanism, have not been studied. There have been two *in vitro* studies mapping PKC phosphorylation sites on Kir6.1 (Singh, 2002, Shi, 2008a). Involvement of these sites has yet to be confirmed for a role in PKC-mediated functional response of Kir6.1/SUR2A channel.

It was hypothesised that in IPC, direct PKC-mediated phosphorylation of Kir6.2/SUR2A channels in cardiac sarcolemma results in channel activation and,

thereby, reduced membrane excitation, as part of the cardioprotective mechanism. It was further hypothesised that PKC-stimulated Kir6.1/SUR2A channel activation in mitochondria also contributes to a cardioprotective phenotype. The main objectives of this study were to investigate whether direct PKC phosphorylation could regulate Kir 6.1/SUR2A channel activity and whether proposed phosphorylation sites on the Kir6.1 subunit account for the functional response of Kir6.1-containing channels to PKC. It was found that PKC activation modulated recombinant Kir6.1/SUR2A and Kir6.1/SUR2B channels by reducing channel current in the plasma membrane of HEK 293 cells. Three residues were found (S379, S385 and S397) to be responsible for functional modulation of Kir6.1/SUR2A channel by PKC, whereas two of these residues (S379, S385) appeared to be responsible for Kir6.1/SUR2B channel. Given the slow time course of channel inhibition by activated PKC, whether PKC inhibited Kir6.1/SUR2A channel by affecting their trafficking was also investigated.. It was found that PKC lost its inhibitory effect when tested on Kir6.1364IL365AA/SUR2A, a channel containing an endosome targeting mutant in the Kir6.1 subunit. An effect in channel trafficking was further clarified by confocal microscopic study, which showed that incubating cells expressing Kir6.1/SUR2A with PMA for 30 minutes resulted in internalization of Kir6.1. This effect was blocked by dynasore, a dynamin GTPase inhibitor. From these experiments, it was suggested that PKC inhibits Kir6.1/SUR2A channel activity by accelerating the dynamin dependent trafficking of the channel away from the plasma membrane.

A further main objective of this study was to clarify which PKC isoforms are responsible for PKC-mediated Kir6.1 phosphorylation in cardiomyocytes. It was found that PKC ϵ only was the main isoform responsible for Kir6.1 phosphorylation.

8.1 Expression of recombinant Kir6.1/SUR2A channels in HEK cells.

To elucidate the effect of PKC on a novel K_{ATP} channel, Kir6.1/SUR2A channel, HEK 293 cells were transfected with pIRES2-EGFP-F-Kir6.1 and pIRES-EGFP-F-SUR2A. The transfection was optimized to obtain the maximum transfection efficiency and minimum cell toxicity. In addition, the protocol was optimized to obtain a measurable level of the functional channel. It was found that HEK cells can express functional Kir6.1/SUR2A channel with low and varied current density when compared with Kir6.2/SUR2A. Results in this study are consistent with those of Kono et al (2003) who found higher current in Kir6.2/SUR2A recombinant channel.

It was found that, even though the Kir6.2/SUR2A can be transfected in the HEK cells using 1 μ g of each DNA, the channels did not express functionally under these conditions. Small currents were obtained from cells transfected with 1 μ g each of Kir6.1 and SUR2A DNA in response to application of P1075. This result suggests that both subunits were expressed but that the amount of functional channels incorporated at the plasma membrane was low. It has been reported that recombinant Kir6.1/SUR2A channels were not able to produce spontaneous current (Kono, 2000). However, Liu et.al (2001) showed that channels formed from this recombinant subunit combination can be opened by P1075 and pinacidil with a current density around 130 pA/pF (Liu et al, 2001). Consistently, the present experiments showed that Kir6.1/SUR2A channels could be activated by P1075 and blocked by glibenclamide. However, the current density found in our experiment was smaller which may be due to the smaller amount of DNA used in these experiments.

Although spontaneous channel activation was not seen under symmetrical K^+ condition, Kono et al (2000) had reported that the presence of UDP was necessary to obtain spontaneous opening of Kir6.1/SUR2A channels (Kono, 2000). In the present study, it was shown that, in the presence of UDP, the P1075 activated Kir6.1/SUR2A current and current density was significantly higher than that of UDP absence. This result confirmed the importance of UDP in channel regulation.

The current-voltage relationship found in the present experiment did not show an obvious inward rectification property. It is noteworthy that similar observations with Kir6.1/SUR2B recombinant channel have been reported (Shi, 2008a). Rundown of the K_{ATP} channel current occurred in this series of experiments. A paper addressed the importance of MgADP in slowing rundown of Kir6.2/SUR2A (Tammaro, 2007). In the present experiment, the MgADP was added in the intracellular solution. However, the rundown still occurred. In the study of Shi et al (2008a) in Kir6.1/SUR2B, the rundown was not mentioned when the channel was stimulated with pinacidil (Shi et al, 2008a). Another possibility is that this rundown may be associated with the particular action of the activator itself. It was found that pinacidil activated Kir6.1/SUR2A current has less rundown than that of activated with P1075, suggesting that properties of rundown are, in part, determined by the nature of the channel activator used.

8.2 Functional responses to protein kinase C phosphorylation of wild type and mutant Kir6.1/SUR2A channels

This study was the first to investigate the regulatory effect of PKC on Kir6.1/SUR2A channel. By using the whole cell patch clamp technique, it was demonstrated that pinacidil-activated kir6.1/SUR2A channel current was inhibited by PKC activator by 65%. The effect of PKC through phosphorylation was confirmed by use of a PKC inhibitor, which was able to block this effect. In contrast, PKC activation by PMA was unable to reduce Kir6.2/SUR2A current indicating that the effect of PKC depends on the nature of the Kir subunit present rather than that of the SUR subunit. This was also observed in Kir6.1-containing channels. Shi et al demonstrated that PKC can inhibit Kir6.1/SUR2B current but not that of Kir6.2/SUR2B (Shi et al, 2008a). The regulation of Kir6.1-containing channel was thought to be the result of direct PKC phosphorylation of the Kir6.1 subunit, which was supported by the finding that there are 7 consensus phosphorylation sites on the Kir6.1 subunit and none on the SUR subunit.

To investigate the contribution of individual PKC phosphorylation sites in the inhibition of Kir6.1/SUR2A activity, serine residues at five proposed sites were mutated individually to the non-phosphorylated amino acid, alanine. The functional response of the each mutant to PKC activation was examined by patch clamp recording. When expressed with SUR2A, it was found that the inhibitory effect of PKC was apparently diminished in Kir6.1S379A (34% decrease) and S385A (40% decrease) whereas the inhibition was unaffected in Kir6.1S354A and Kir6.1S391A

containing channel. In Kir6.1S397A mutant containing channel, a reduction in PKC inhibition was shown but this was not as much as that of Kir6.1S379A and Kir6.1S385A mutant containing channels (17% decrease). These experiments suggested that three residues, S379, S385 and S397, are responsible for PKC phosphorylation mediated inhibition. However, S379 and S385 seem to be more critical sites than S397. These results were contrary to an *in vitro* phosphorylation experiment of Singh et al (2002), which found that among the five mutants, only the Kir6.1S379A mutant was crucial because mutation of this site only resulted in no Kir6.1 phosphorylation at all. In contrast, an *in vitro* phosphorylation study of Shi (2008a) showed that S379, S385, S391 and S397 but not S354 could be phosphorylated by PKC. However, Shi's protocol was different from that of Singh in terms of the mutation strategy employed. A combined multiple site mutation strategy was used in Shi et al's experiments. Shi et al showed a difference in ^{32}P incorporation between a 3 site (S385A, S391A, S397A), and a 4 site (S379, S385, S391, S397) combination. A much reduced ^{32}P incorporation into 4 site mutant was interpreted to that S379 is a critical site for PKC mediated phosphorylation. However, this suggestion may be argued since the other models of 3 and 4 site mutation were not tried. An involvement of the S354 residue was excluded after the finding that the level of phosphorylation of 4 (S379+S385+S391+S397) and 5 (S354, S379, S385, S391 and S397) site mutants was not different (Shi et al, 2008a). Whole cell patch clamp technique in symmetrical potassium solution revealed that the effect of PKC was reduced by 20% in each of Kir6.1S354A, Kir6.1S385A, Kir6.1S391A, and Kir6.1S397A mutants (Shi et al, 2008a). This finding was different from result of the present study which showed that the size of the reduction of PKC-mediated channel

inhibition was not the same for all mutants. This may be a result of the investigation from the different combination and also experiment protocol used.

8.3 Functional responses to protein kinase C phosphorylation of wild type and mutant Kir6.1/SUR2B channels

To investigate the discrepancies with Shi et al's result and whether phosphorylation of Kir6.1 combined with a different SUR2 subunit resulted in different functional response of the channels to PKC, recombinant Kir6.1/SUR2B channel including Kir6.1 mutants were studied. It was found that PKC reduced Kir6.1/SUR2B current by about 70%. This finding correlated to that of Shi et al and Jiao et al who found the inhibitory effect of PKC on Kir6.1/SUR2B channel about 70 and 50 %, respectively (Shi et al, 2008a, Jiao et al, 2008). Further investigation was made herein to clarify the inhibitory potential of Kir6.1 phosphorylation sites on Kir6.1/SUR2B activity in response to PKC. It was found that mutation of S379 and S385 altered the functional response of the channel to PKC. The inhibitory effect of PKC was diminished by about 30% indicating that PKC phosphorylation was effective at these sites. However, mutation of S397, which was demonstrated to transmit a small effect of Kir6.1/SUR2A channel, did not produce any change in PKC-mediated inhibition in the Kir6.1/SUR2B channel. Apparently, S354 and S391 were not involved in phosphorylation of Kir6.1/SUR2B channels, since no change in inhibition was observed. The discrepancy of PKC effect between Kir6.1/SUR2A and Kir6.1/SUR2B channel may be from uncontrollable conditions, for example, the difference of current density between these two groups. It was found that the current density and also the current amplitude of recombinant Kir6.1/SUR2B channels were lower than that of

Kir6.1/SUR2A channels. Interpretation of small changes in current was more difficult in the low current group, since a little change in current was likely to produce large percentage changes. The results are clear, however, that S379 and S385 were both responsible for PKC phosphorylation current inhibition.

8.4 PKC activation inhibits Kir6.1 containing channels by alteration channels trafficking

To understand how PKC activation inhibits the Kir6.1/SUR2A channel, the current expressed by an endosome targeting mutant of Kir6.1, Kir6.1364IL365AA/SUR2A, was observed during exposure to PKC activator. It was found that the inhibitory effect of PKC was abolished in this mutant channel indicating the involvement of Kir6.1 trafficking in response to PKC. Further investigation by confocal microscopy demonstrated that Kir6.1 was internalized after incubation of cells with PMA for 30 minutes, while, only some cells stimulated by PMA in 5 minutes showed punctate internalization in the cells. This confirmed that PKC induced internalization of Kir6.1/SUR2A, which is compatible to the result of other studies on Kir6.1/SUR2B channel. Jiao et al (2008) demonstrated that Kir6.1/SUR2B current was inhibited by PMA and that the effect did not occur when a dynamin mutant K44E was co-expressed. Further investigation by immunofluorescence in Jiao et al's study also demonstrated the internalization of the channel after HEK cells expressing Kir6.1/SUR2B were incubated with PMA for 15 minutes. Increased intracellular punctuate labeling was observed in the PMA treated WT cells. This punctuate labeling disappeared in the PMA treated dynamin mutant group.

The inhibitory effect of PKC was shown in cells treated with PMA for 5 minutes in the present study and in the experiment of Shi et al. However, immunofluorescence images after only 5 minute exposure to PMA showed internalization of the channel in only some cells. The conflict between the current inhibition and the internalization of the channel at 5 minute after treatment with PMA may be from the temperature condition. In patch clamp experiment, the temperature was controlled at 25-30°C throughout the procedure, while in trafficking experiment the cells were immediately taken from 4°C fridge to 37°C incubator to be treated with PMA for 5 minutes. Therefore, the temperature may not reach 37°C at the end of 5 minute which interrupted PMA action. The finding that the number of positive cells (> 5 punctae) incubated at 37°C without PMA treatment for 30 minutes was higher than that of treated with PMA for 5 minutes supported this idea.

Although some studies suggest an effect of PKC phosphorylation on channel trafficking only, the effect of PKC on channel kinetics was still an issue. Thornloe et al (2002) also showed the inhibitory effect of PKC activator on Kir6.1/SUR2B channel with alteration in open probability of the channel. Therefore, it is possible that PKC activation may modulate the channel directly or promote the internalization of the channels. Similarly, Liu et al (2000) also demonstrated that PKC increased open probability of Kir6.2/SUR2A channels.

In this study, the mechanism by which PKC induced internalization was explored. Using confocal microscopy, it was shown that, dynasore, a dynamin inhibitor could block internalization of Kir6.1/SUR2A channel in response to PKC. Similarly, Jiao et

al (2008) also demonstrated that PKC mediated Kir6.1/SUR2B internalization was dynamin dependent. However, some caution is required in the interpretation of the experiments herein as only 2 experiments were conducted. Further experiment required to permit a conclusion.

8.5 Proposed role of Kir6.1/SUR2A channels in cardioprotection

The question that remains is how alteration in Kir6.1/SUR2A channel activity may result in cardioprotection. The role of this channel was proposed according to its localization. Firstly, Kir6.1 was found in cardiac sarcolemmal membrane, but not abundantly (Singh et al, 2003). If Kir6.1/SUR2A is present at cell membrane, the inhibitory effect of PKC on this channel may help in reducing deleterious effect of excess opening of Kir6.2/SUR2A. It is hypothesized that cardioprotective effect of IPC is a result of opening of cardiac sarcK_{ATP} channel. The majority of sarcK_{ATP} channel was shown to be the Kir6.2/SUR2A subunit combination (Seino, 1999), opening of 1% of this sarcK_{ATP} channel is enough to shorten action potential duration (Tammaro, 2006), leading to less Ca²⁺ influx and therefore spare energy and reduce cell damage (Light et al, 2001). Light et al (2000) demonstrated that constitutively active PKC increased Kir6.2/SUR2A activity by about 390%. It was shown that K_{ATP} current in cells expressing Kir6.1ΔC26 alone was still activated by PKC implying the role of Kir6.2, but not SUR2A subunit, in this stimulation. However, shortening action potential duration is arrhythmogenic (Janse and Wit, 1998). Miki et al (2002) demonstrated that Kir6.1 expressed in mouse heart. Kir6.1 null mice exhibited sudden death and abnormalities in electrocardiogram, spontaneous ST elevation including atrioventricular block. It is possible, therefore, that inhibition of Kir6.1/SUR2A may counterbalance the effect of Kir6.2/SUR2A activation by PKC.

Secondly, if Kir6.1/SUR2A exists in the mitochondria, it is possible that PKC activation promotes channel trafficking from cell membrane to mitochondria, which results in greater abundance of mitoK_{ATP} channel. Activation of mitoK_{ATP} channels resulted in influx of K⁺, increased matrix volume, decreased intermembrane space as well as decreased Ca²⁺ influx, all of which preserve mitochondria function (Testai et al, 2007). It may be argued that activation of PKC has been shown to inhibit this channel rather than opening the channels. A possible explanation is that the characteristic of mitoK_{ATP} (Kir6.1/SUR2A) in response to PKC may be different from that of present at cell membranes. The inhibitory effect of PKC in this study was a result of the internalization of the channel rather than the closure of the channels and it is possible that internalized channels are still functional. In support of this hypothesis, Garg et al also reported the PKC induced import of Kir6.2-containing channel from the cytosol to mitochondria (Garg et al, 2007).

8.6 Identification of PKC isoforms responsible for adenosine-induced Kir6.1 phosphorylation in rat ventricular myocytes

This sub study was performed to investigate which PKC isoforms are responsible for adenosine-induced Kir6.1 phosphorylation in rat heart model. It was found that the A₁AR agonist, CCPA, induced phosphorylation of Kir6.1 and specific isoform PKC, PKC ϵ , is involved whereas the other isoforms did not show a role in Kir6.1 phosphorylation. Tat-PKC ϵ attenuated CCPA-induced Kir6.1 phosphorylation whereas $\Psi\epsilon$ RACK, PKC activator, increased the level of phosphorylated Kir6.1. These results suggested a role for PKC ϵ in the regulation of Kir6.1-containing K_{ATP} channels in the heart.

This study confirmed the role of the A_1AR stimulated pathway in Kir6.1 phosphorylation. It was found that level of phosphorylated Kir6.1 was significantly higher in CCPA treated cell cells compared to that of control cells. Phosphorylated Kir6.1 protein was also observed in the control group. The reason for this is that there may be a basal level of PKC phosphorylation or other triggers, for example, bradykinin and also endogenous adenosine, released during harvesting the heart for these experiment. However, the consequence of exogenous A_1AR stimulation was clear when compare to that of background.

In this study, it was shown that PKC ϵ inhibitor was able to block Kir6.1 phosphorylation. This suggests that A_1AR mediated-phosphorylation involves, at least in part, PKC activation. Mechanism by which A_1AR mediates cardioprotection was thought to involve in PKC and K_{ATP} channels (Liang et al, 2000). The data about the ability of A_1AR to modulate K_{ATP} channel was conflicting. However, the link between PKC and A_1AR is almost clear. It was found that PKC inhibitors could block A_1AR agonist effect. There is evidence suggesting that A_1AR activation promotes the translocation of PKC ϵ from cytosolic to membrane fraction (Fenton, 2009, Yang 2009). Therefore, in this current study it is possible that CCPA induced the translocation of PKC ϵ from cytosolic to membrane and then mediated Kir6.1 phosphorylation. On the other hand, some studies indicated the translocation of PKC ϵ from cytosol to mitochondria in ischemic reperfusion (Budas, 2010). Therefore, A_1AR may promote PKC ϵ translocation from cytosol to mitochondria and then phosphorylate Kir6.1 substrate.

8.7 Conclusion

The results of this study demonstrated that PKC activation inhibits a novel K_{ATP} channel subunit combination, Kir6.1/SUR2A. Inhibition occurred over a time scale of minutes and was shown to be due to internalisation from the cell surface in a dynamin-dependent manner. Serine residues Kir6.1S379 and 385 were shown to be the most critical residues for the PKC-mediated channel inhibition.

^{32}P phosphorylation experiments in the isolated rat ventricular myocytes showed the ability of A_1AR agonist to stimulate phosphorylation of Kir6.1 protein. The A_1AR agonist effect was abolished by $PKC\epsilon$ inhibitor, not $PKC\alpha$, $PKC\beta$, $PKC\gamma$ or $PKC\delta$ inhibitors. $PKC\epsilon$ activator, $\psi\epsilon RACK$, was shown to stimulate Kir6.1 phosphorylation. It is summarized that $PKC\epsilon$ is involved in A_1AR -induced Kir6.1 phosphorylation.

8.8 Future work

1. It is suggested that further study should be performed in order to explore the role of Kir6.1-containing channel in animal model with ischaemia. Electrophysiological experiments of Kir6.1 mutated ventricular myocytes in response to ischaemia and PKC should be investigated.
2. To ensure that Kir6.1/SUR2A confers $mitoK_{ATP}$ channel characteristics, pharmacological properties (response to diazoxide, 5-HD, other KCOs) of this channel assessed by patch clamp technique is required.

3. The study of trafficking of Kir6.1 in response to ischaemia and IPC in animal model is suggested. Live cell confocal microscopy to assess dynamic distribution of Kir6.1 may be useful. Labelling of mitochondria and related organelles is required. To study the mechanism of Kir6.1 internalization more experiments for assessing role of dynamin and caveolin using dynamin mutant or inhibitors of caveolin is suggested.
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