Philadelphia College of Osteopathic Medicine
DigitalCommons@PCOM

PCOM Biomedical Studies Student Scholarship   Student Dissertations, Theses and Papers

5-2014

Cyclic Nucleotide-Dependent Phosphorylation Regulates BK-Ca Channel Activity in Human Coronary Artery Smooth Muscle Cells

Raeonda Williams
Philadelphia College of Osteopathic Medicine, raeondawi@pcom.edu

Follow this and additional works at: http://digitalcommons.pcom.edu/biomed

 Part of the Cardiology Commons, Medical Biochemistry Commons, and the Medical Physiology Commons

Recommended Citation

Georgia Campus- Philadelphia College of Osteopathic Medicine

The Biomedical Sciences Program

Basic Sciences Department

Cyclic Nucleic-Dependent Phosphorylation Regulates BK$_{Ca}$ Channel Activity in

Human Coronary Artery Smooth Muscle Cells

A Thesis in Biomedical Sciences by Raeonda Williams

Submitted in Partial Fulfillment of the Requirement for the Degree of Master of

Science in Biomedical Sciences May 2014

Biomedical Sciences Degree Programs
Thesis Signatory Page

We approve the thesis of Raeonda Melcenia Williams

Richard E. White, PhD   Date   5-27-14
Professor of Neuroscience, Pharmacology,
and Physiology
Thesis Advisor

Mary P. Owen, PhD, JD   Date   5/27/14
Professor, Pharmacology

Shu Zhu, MD, PhD   Date   5-27-14
Assistant Professor of Neuroscience,
Pharmacology, and Physiology

Brian M. Matayoshi, PhD   Date   5-27-2014
Professor, Physiology
Biomedical Sciences Program Director

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

List of Abbreviations

°C	Celsius
A/D	Analog/digital
Akt	Protein kinase B
ATP	Adenosine triphosphate
BK_{Ca}	Large conductance calcium-activated potassium
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CASMC	Coronary artery smooth muscle cells
cGMP	Cyclic guanosine monophosphate
cm^2	Centimeters squared
COOH-end	Carboxy terminal end
CPT-cAMP	Chlorphenylthio-cAMP
CVD	Cardiovascular disease
DHT	dihydrotestosterone
DM	Dissociation medium
DPBS	Dulbecco's Phosphate Buffer Saline
DTT	dithiothreitol

EDRF	Endothelium-derived relaxing factor
E_m	Membrane potential
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GPER	G-protein coupled estrogen receptor
HCASMC	Human coronary artery smooth muscle cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
HRT	Hormone Replacement Therapy
I-V	Current-voltage
IK_{Ca}	Intermediate conductance calcium-activated potassium
K^+	Potassium ion
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
kHZ	10^3 Hertz; kilohertz
mg	milligram
$MgCl_2$	Magnesium chloride
min	minutes

mL	milliliter
mm	millimeter
mmHg	Millimeters of Mercury
mmol	millimole
mV	10^{-3} volt; millivolt
MΩ	10^6 ohm; megaohm
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO$_3$	Sodium Bicarbonate
nM	10^{-6} meter; nanometer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOHA	N$^\omega$-hydroxy-L-arginine
NOS	Nitric oxide synthase
NP$_0$	Mean open channel probability
OA	Okadaic acid
p-Akt	Phosphorylated- protein kinase B
pA	10^{-12} amp; picoamp
PDE	Phosphodiesterase

P_i	Pyrophosphate
PI3-kinase	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKG	Protein kinase G
PP2A	Phosphoprotein phosphatase 2A
pS	10^{-12} Sieman; picosieman
ROS	Reactive oxygen species
rpm	Rotations per minute
SK_{Ca}	Small conductance calcium-activated potassium
SNP	Sodium nitroprusside
TEA	tetraethylammonium
VSM	Vascular smooth muscle
WHO	World Health Organization
µmol/L	Micromole/ liter

Abstract

Cardiovascular diseases (CVD) can induce dysfunction in organ systems by attenuating normal blood flow. Gonadal steroids are vasoactive hormones, but their role in contributing to cardiovascular function remains controversial. We have demonstrated that gonadal steroids can relax coronary arteries by opening the large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channel in smooth muscle cells by increasing cyclic nucleotide levels; however, the signaling pathways involved remain to be elucidated. The purpose of this study was to identify how phosphorylation (via cAMP- and cGMP-dependent protein kinases) / dephosphorylation (via phosphoprotein phosphatase 2A, PP2A) regulates BK_{Ca} channel activity in human coronary artery smooth muscle cells (HCASMC). BK_{Ca} channel activity was recorded from single HCASMC (Lonza/Clonetics) via single-channel and whole-cell patch-clamp. Channel activity was stimulated by increasing intracellular calcium levels or by increasing either cAMP or cGMP, but the stimulatory effect was mediated predominately via the cGMP-dependent protein kinase. In addition, inhibition of PP2A decreased channel activity. These findings demonstrate that cyclic nucleotide-dependent vasodilators can regulate arterial function via phosphorylation of BK_{Ca} channels, and that dephosphorylation of the channel (via PP2A) may play a role in channel activation. We propose that modulation of BK_{Ca} channels via hormone- or drug-induced phosphorylation could be a novel therapeutic means of helping to lower the risk of CVD in both males and females.

Acknowledgments

1. INTRODUCTION

1.1 Heart Disease

Heart disease has long been a major issue plaguing the American population. Data on death rates in the US revealed that a death attributable to cardiovascular disease (CVD) occurs every forty seconds (1). This issue is not confined to the US as CVD is the leading cause of mortality worldwide. Statistical analyses of World Health Organization (WHO) and United Nations data show increased CVD mortality rates in low and middle-income countries in comparison with high-income countries (2). The establishment of low-to high-income countries comes from the World Bank's use of gross national income per capita to classify countries (3). The US is considered a high-income nation and follows the trend of declining mortality caused by CVD. However, this does not erase the need for further improvements in curtailing this disease, as it still remains the number one cause of death in America (2).

CVD encompasses a multitude of pathophysiological complications obstructing normal activity of blood distribution throughout the cardiovascular system. Symptoms such as compromised heart and peripheral vessel function impact all body systems and include, but are not limited to heart attacks, ischemic strokes, arrhythmia, and valvular complications (4). Atherosclerosis and hypertension are two common precursors to CVD that cause restriction of blood flow (5). Atherosclerosis is the build-up of fat, calcium, and cholesterol to form plaque in vessels (6). Plaque occludes blood flow by protruding into the

vessel lumen as it grows or by pieces of it breaking off into the lumen (7).

Hypertension is a condition in which a person's blood pressure remains above

the accepted value of 139 mmHg systolic and 89 mmHg diastolic. It is important

to note that this accepted value for blood pressure is not definitive for each

person. However, the more one deviates from this value into higher readings, the

more cardiac events are likely to occur (8). According to the American Heart

Association statistical studies, one in three adults above the age of twenty are

living with hypertension. This shows that this condition is still fairly prevalent

even in a society with a declining mortality rate of deaths attributable to CVD

(1).

1.2 Hormones and Heart Disease

The impact of sex hormones on vascular health has been widely accepted,

however, their exact effect on the cardiovascular system is still debatable due to

a wide array of experimental results. The *Fogelberg et al*. (9) study showed that

inhibition of testosterone aromatization into estrogen promoted atherosclerosis.

Another study performed in orchidectimized mice revealed similar findings of

inhibition of aromatization yielding plaque formation. These findings support

estradiol attenuation of atherosclerotic conditions (10). Observational studies

have also shown that low levels of bioavailable testosterone correlate with lower

aortic atherosclerotic manifestation in men $\geq$55 years (11). Sex hormones also

affect vascular tonicity according to experimental data. Estrogen is thought to

lower hypertension by inducing relaxation of vascular smooth muscle in arteries

(12). The growing body of data led many to believe that the introduction of sex hormones could yield positive results against CVD.

Negative effects of sex hormones are demonstrated with the use of hormone replacement therapy (HRT) to supplement low estrogen levels during menopause. Women have a lower risk for CVD than men prior to menopausal age. This decline in advantage after menopause can be attributed to the diminished level of estrogen production in women after this point (13). Women who experience menopause at an earlier age are also more likely to suffer from an ischemic stroke. This implies that the sooner the onset of estrogen decline, the more likely the woman is to develop CVD (14). This along with other data signifying positive effects of hormones led many to believe that the use of HRT to raise estrogen levels would help combat CVD. However, when HRT was introduced in post-menopausal women, their risk for CVD actually worsened. For example, the Women's Health Initiative clinical trial employed 16,608 postmenopausal women who were given conjugated equine estrogens showed that the incidence of CVD increased in comparison to the placebo group. In fact, the study was cut short due to the strong evidence that estrogen exposure caused pathologies such as CVD and cancer (15). Sex hormones play a major factor in cardiovascular health, and must be used within the correct parameters to be an effective preventative measure for CVD.

Whether sex hormones affect vessels is no longer questioned, but their mode of action requires more elucidation. Research is now investigating the

exact mechanisms and pathways resulting from sex hormone initiation. Numerous studies have shown that the introduction of estrogen above "physiologic levels" causes vasodilation of vascular smooth muscle (VSM) cells. In contrast, other studies have shown that estrogen might possibly lead to vasoconstriction in older women (16) Like estrogen, testosterone is a vasodilatory hormone (17). The discovery of the effects of sex hormones on vascular tone and pressure has shed light on a possible complex pathway where sex hormones could induce vasodilation via a powerful molecular effector protein, the large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channel. **The purpose of this study will be to investigate molecular signaling mechanisms whereby sex hormones could produce vasodilation by opening BK_{Ca} channels.**

1.3 Hormones and Receptors

The suggested pathways by which sex hormones influence vascular tone are being established by a growing body of research. Most have singled out the production of nitric oxide as a major contributing factor to vasodilation, but sex hormones must prompt a series of biochemical signals before this happens (18). First, estrogen and testosterone bind to their respective receptors on the VSM cell. Estrogen can bind to at least two types of estrogen receptors (ER), ERα or ERβ. In addition, a novel G-protein-coupled ER (GPER) is also expressed in blood vessels. At present, there is only one known type of androgen receptor for testosterone and it has a highly conserved sequence (16, 19). Androgen and

estrogen receptors differ in their primary sequence by approximately 56 percent, but share a similar quaternary structure with both having a ligand-binding domain made of twelve helices. Sex hormone receptors are found on the membrane in VSM cells. They can also be located in the cytoplasm, which allows sex hormones to bind after permeating the cell membrane (19). The diversity in location of receptors points to the diversity of responses sex hormones are able to induce.

Estrogen and testosterone are able convert into other steroid derivatives and still elicit the same vasodilatory response. Aromatase is an enzyme present in the VSM that functions to aromatize testosterone into estrogen (20). This can then stimulate vascular signaling via the estrogen transduction pathway. There has been discussion as to whether the effects of testosterone on vascular tone and vasodilation are actually due to testosterone itself or to an aromatized form. Confirmation that testosterone, itself, affects vascular tonicity has been provided through the use of dihydrotestosterone (DHT). DHT is synthesized from testosterone via activity of 5a-reductase (21). DHT is incapable of aromatization, but can also initiate vasodilation (22). This demonstrates that androgen can cause vasodilation without aromatizing into estrogen. It also shows how sex hormone-induced vasodilation has multiple molecular effectors.

1.4 Production of Nitric Oxide: The First Messenger

The binding of the hormones to the receptors initiates the activity of several proteins that stimulate nitric oxide synthase (NOS) (23). ERs and ARs

activate phosphoinositide 3-kinase (PI3) and protein kinase B (Akt), which subsequently stimulate activity of NOS via phosphorylation (16, 20). NOS is a multifunctional enzyme exhibiting both oxygenase and reductase functions (24). Its oxidation of NADPH can be both coupled and uncoupled. A coupled reaction occurs when NOS uses all the electrons from NADPH to form monooxygenated products such as N^ω-hydroxy-L-arginine (NOHA) and NO. Uncoupled reactions of NOS fail to use all of the electrons from NADPH and the excess electrons bind to molecular oxygen reactive oxygen species (ROS) such as superoxide (25). Certain isoforms of the enzyme have a greater propensity towards a coupled or uncoupled reaction. NOS-1 or neuronal NOS (n-NOS) is found in vascular smooth muscle cells. It has the greatest tendency to produce superoxide under uncoupled conditions and nitric oxide under coupled conditions (16). In terms of vasodilation, a coupled condition is favorable because it yields a vasodilatory product, NO.

Although nNOS produces both NO and superoxide, its production of NO is important in the vasodilatory pathway as NO is the "first messenger" in the NO/cGMP/PKG sequence in coupled conditions. NO is produced by nNOS when L-arginine is converted into L-citruline and is considered to be an endothelium-derived relaxing factor (EDRF) (26, 27). The function of an EDRF is to maintain tone and resistance in vessels (27). It also activates a series of cascade phosphorylation steps that amplify the reactions of each enzyme involved (28). The ability of NO to influence vasodilation is largely dependent on its concentration. A varying basal concentration level of NO is constitutively

produced depending on the cell type. Changes in cell function occur when rapid increases or decreases in NO concentration deviate from the baseline level (24). A vasodilatory response occurs when the concentration of NO rapidly surpasses basal levels.

1.5. BK_{Ca} Channels and Hypertension

More specifically, the changes occurring in VSM cells are also due to the NO/cGMP/PKG pathway causing increased large conductance calcium-activated K^+ (BK_{Ca}) channel activity and hyperpolarization (29). BK_{Ca} channels are found in all cell types and at greater expression in VSM. A main regulatory mechanism for these channels occurs by phosphorylation or depolarization (30). Depolarization and calcium influx into the cell promotes BK_{Ca} channel openings. These openings allow for an efflux of K^+ ions and a resulting hyperpolarization of the cell (31). Hyperpolarization inhibits the cell from a stimulatory depolarization and lowers the concentration of cytosolic calcium levels. The decreased concentration of cytosolic calcium results in reduced VSM contraction (29). This series of reactions creates a standard negative-feedback loop. A muscle cell is stimulated by an increase in calcium concentration, and this increased calcium concentration activates BK_{Ca} channels which in turn cause a reverse response of vasodilation. This classic example of negative feedback is important because it is the body's aim to protect itself from a hypertensive state in vessels.

The use of electrophysiological methods has elucidated many of the understood roles of BK_{Ca} channels (32-34). Voltage-clamp and patch-clamp

experiments using VSM cells to determine current flow through the membrane have revealed a relationship between membrane potential (E_m) and vascular tone. An experiment comparing hypertensive animals to normotensive animals found that BK_{Ca} current was increased in hypertensive animals (35). The implication of this is that the increased current signified a hyperpolarized membrane and increased activity of BK_{Ca} channels. Another study validated the increased activity of BK_{Ca} by calculating higher levels of open channel probability (NP_o) in hypertensive animals (36). This use of electrophysiology has revealed that the increase of BK_{Ca} channels protects vessels from increased pressure/tension in a normotensive individual. It also serves as an indicator that an individual may be hypertensive if the activity is abnormally high. While it is a seemingly positive protective cellular function, increased level of BK_{Ca} channel activity over extended periods of time serve as an indicator of an individual suffering with hypertension or CVD (35).
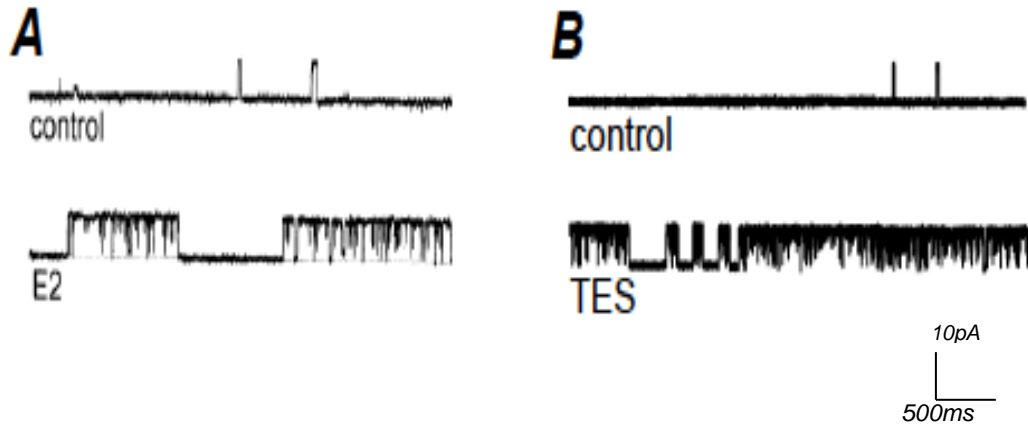
Figure 1: Gonadal steroids open up BK$_{Ca}$ channels in coronary artery smooth muscle cells (CASMC). Channel activity before (control) and 10 min after exposure to 100 nM 17β-estradiol (A) or testosterone (B). Openings are upward deflections from baseline (dashed line), +40 mV.

1.6 cGMP/cAMP Act As Second Messengers

NO production leads to enhanced cGMP synthesis in the VSM. NO-guanylyl cylcase acts as a catalyst in cGMP's production from GTP(24). Both cGMP and cAMP amplify the signal causing dilation of vessels. Cascading pathways using cGMP and cAMP signaling molecules increase the openings of BK_{Ca} channels and cause dilation (37). Experiments inhibiting NO, but not cGMP, showed that hyperpolarization still occurred in the cell (38). This isolates the importance of cGMP/cAMP on vasodilation independent of their precursors.

The direct influence of cGMP and cAMP on VSM still requires clarification because studies have revealed that they may activate multiple signaling pathways. cGMP and cAMP act as substrates for PKG and PKA, respectively. They typically elicit antagonistic responses in cells. These cyclic nucleotides have the capacity for cross-activation (PKA by cGMP and PKG by cAMP)(39). Cross-activation allows cAMP to play a role in the NO/cGMP/PKG pathway if cAMP cross-activates PKG. This leaves room for further study of a possible cAMP to PKG pathway and the resulting implications of such as well.

Establishing an accurate model for mechanisms by which the cyclic nucleotides interact with the each other and kinases could serve as the key to understanding the NO/cGMP/PKG pathway in the VSM. Cyclic nucleotides indirectly affect the concentration of each other by way of phosphodiesterase (PDE). cAMP increases the level of cGMP indirectly by inhibiting the PDE activity that hydrolyzes the molecule (37). Inhibiting PDE means an increased amount of

cGMP able to interact in the NO/cGMP/PKG pathway. cAMP concentration usually exceeds cGMP by marked amounts at basal levels in all cell types. However, PKG's strong affinity for cGMP offsets this disproportion to favor cGMP binding (24). The use of cross-activation becomes even more important in this regard, because high basal levels of cAMP add to the likelihood that cAMP cross-activates PKG.

Data has also shown that estrogen increases accumulation of cAMP in mouse granulosa cells or human coronary artery smooth muscle (40, 41). This shows a direct linkage of sex hormones to second messengers. Further, testosterone increases cGMP levels in coronary arteries and stimulates the activity of either PKG or PKA in these same vessels, although the effect on PKG was predominant (17). In addition, inhibition of PKG activity in coronary artery smooth muscle attenuates vasodilatory signaling mechanisms stimulated by either estrogen or testosterone(17, 42). Thus, these studies reveal a direct linkage of these cyclic nucleotides and their respective kinases to sex hormones and the possible role they play in this vasodilatory pathway. The increasing research in this area is showing that there are many ways in which this mechanism is carried out.

1.7 The role of PKA and PKG

The direct effect of activating or deactivating PKA or PKG is still uncertain. It has been shown that regulatory binding sites on both PKA and PKG are structurally similar making cross-activation possible due to similar consensus

sequences (43). Even upon this conclusion, there has been some difficulty in determining all of the specific substrates for these kinases because certain substrates are only phosphorylated *in vivo* or *in vitro* and not in both experimental conditions (39). There is also evidence showing that some PKG substrates phosphorylated *in vivo* are actually phosphorylated *in vitro* as PKA substrates and vice versa (44).

With much speculation still remaining on the exact pathway of VSM dilation, a focus should be placed on the role of PKA. The role of PKA in mediating relaxation of VSM is still controversial. Studies have shown that PKA promotes VSM hyperpolarization as a result of increased β-adrenoreceptors and adenylyl cyclase activity(45). Other experiments suggest that BK_{Ca} channels are not substrates for PKA phosphorylation, as BK_{Ca} channel activity appeared unaffected by PKA inhibition (37, 46). Another theory suggests a mechanism in which PKA phosphorylation subsequently leads to BK_{Ca} channel activation (47, 48). It is evident that the role of PKA is far from being understood. The use of phosphorylation and dephosphorylation to regulate BK_{Ca} channels necessitates the understanding of how these kinases modulate channel activity.
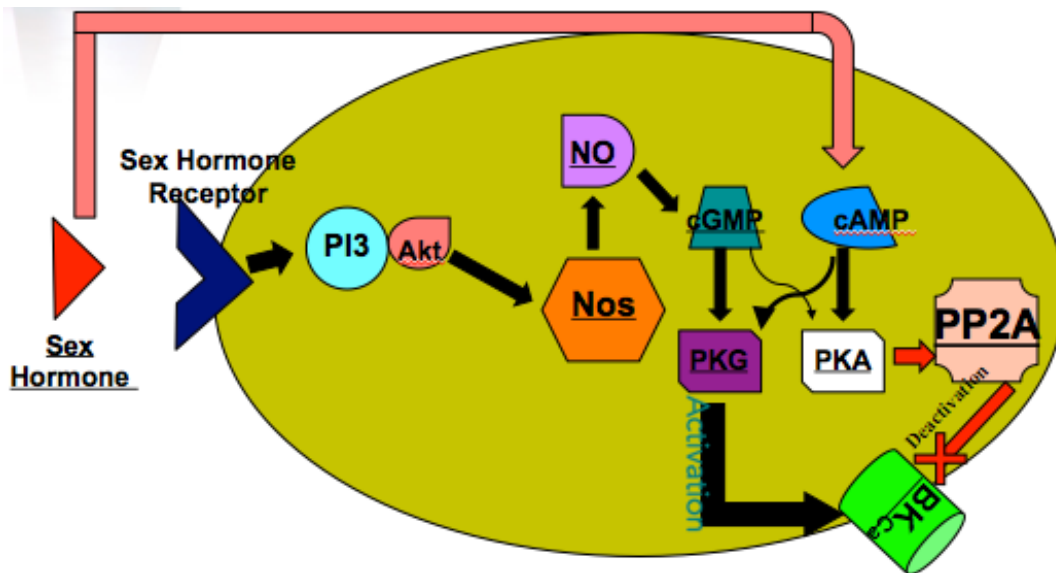
Figure 2: Proposed mechanism of kinase/phosphatase regulation of BK$_{Ca}$ channels- Binding of sex hormones to receptors elicits a cascade-signaling pathway with diverse components. Activation of NOS produces the first messenger, NO, and subsequent second messenger cGMP. Cyclic AMP may also be produced from adenylyl cyclase. Channels may be phosphorylated by PKA or PKG, and dephosphorylated by PP2A.

1.8 PP2A Mediates PKA Influence

As a number of studies have demonstrated that phosphorylation regulates BK_{Ca} channel activity, we hypothesize that PKA not only plays a role in BK_{Ca} channel activity, but that it may do so indirectly through protein phosphatase 2A (PP2A). PP2A is found ubiquitously in all cells (49). It is a serine/threonine phosphatase that has a regulatory site for phosphorylation and methylation. PP2A is a holoenzyme existing as a heterotrimer. This heterotrimer is composed of a scaffold, catalytic (C), and regulatory (B) subunit (50). More specifically, the B56δ regulatory subunit of PP2A has been phosphorylated by PKA in prior *in vitro* studies (51) validating a possible relationship between the two in the sex hormone cascade pathway.

Prior research has suggested that inhibition of PP2A leads to increased BK_{Ca} channel activity in mesangial cells (52), thus indicating PP2A as an overall inhibitory phosphatase. Nonetheless, the role of PP2A in regulating BK_{Ca} channel activity remains controversial, as other studies have suggested that PP2A activity may open BK_{Ca} channels (53, 54). We propose that PP2A dephosphorylates a stimulatory P_i that the BK_{Ca} channel has from PKG phosphorylation, leading to channel inactivation (48). It is possible that PKA activates PP2A by phosphorylation, and an activated PP2A removes a stimulatory P_i that PKG placed on the BK_{Ca} channel. With research on the specific role of PP2A and other intermediates in the mechanism growing yearly, the specific pathway that sex hormones have in influencing vessel dilation will

continue to be elucidated. This will ultimately yield more possibilities for effective drug targeting on specific proteins in this extensive signaling pathway.

1.9 Aims and Hypotheses

Aim # 1: BK_{Ca} channels are only one amongst the three types of voltage-sensitive calcium-activated potassium channels. Therefore, BK_{Ca} channel activity must be distinguished from that of the small-conductance calcium-activated potassium channels (SK_{Ca}) and the intermediate-conductance calcium-activated potassium channels (IK_{Ca}).

Hypothesis 1: If all three calcium-activated potassium channels are present, the primary channel stimulated by cAMP in HCASMC will be the large-amplitude BK_{Ca} channel with a single-channel conductance of approximately 150 pS(55, 56).

Approach #1:

1. Run Symmetrical and Physiological I/V Curve

Current –Voltage (I-V) relationship curves have been used to determine the conductance of the channels found in the patch clamp methods (57). Establishing that the channel inside the patch has a conductance of at least 150 pS will verify that any further experimentation is conducted with BK_{Ca} channels and not IK_{Ca} and SK_{Ca} channels that exhibit lower conductivity.

Aim #2: Evidence suggests cAMP-dependent vasodilators increase BK_{Ca} channel activity. The proposed experiments will employ the patch-clamp to establish whether or not cAMP-dependent vasodilators activate BK_{Ca} channels.

Hypothesis #2: The use of cAMP-dependent vasodilators above basal concentrations will cause an increase in BK_{Ca} channel openings.

Previous studies have suggested that forskolin activates the cAMP signaling pathway, leading to an increase in BK_{Ca} channel activity. The suggested mechanism is that cAMP cross-activates PKG, leading to BK_{Ca} activity increase(37). It is hypothesized that addition of cAMP vasodilators will increase BK_{Ca} channel activity.

Approach #2

1. Measuring the effect of cAMP-dependent vasodilators on VSM cells

Studies have shown that forskolin can increase cAMP concentrations by 6-fold in coronary arteries(37). In this proposed experiment. forskolin will be introduced into the cell suspension chamber to establish whether or not cAMP vasodilators can elicit an effect on BK_{Ca} channel activity.

2. Patch Clamp

BK_{Ca} channel activity will be recorded before and after addition of cAMP-dependent vasodilators via the patch clamp, and mean channel activity will be determined. This will be calculated by recording the product of the number of channels and the probability that these channels are open (NP_o) (# of channels x

open probability). It is anticipated that elevation of cAMP will increase BK_{Ca} channel NP_o.

Aim #3: Evidence has shown that cAMP works through the NO/cGMP/PKG pathway through cross-activation(58). Is there a mechanism by which cAMP activates PKA and causes some effect on BK_{Ca} channel activity? If PKA is not an agonist, is its function on BK_{Ca} channels inhibitory of activity or independent?

Hypothesis #3: cAMP does activate PKA similarly to their activation of PKG and will elicit a response in BK_{Ca} channels.

Measuring the response of the BK_{Ca} channels when only acted upon by PKA will provide a clear model for the exact effect PKA has on the BK_{Ca} channels. It is widely accepted that cAMP binds to the regulatory unit of PKA and induces a cascading effect from the resulting phosphorylation from PKA. However, the role that PKA has on BK_{Ca} channels is still controversial. The forerunning theory is that PKG acts to open BK_{Ca} channels (59). Due to the fact that PKG can be activated by cAMP and cGMP and induce an effect on BK_{Ca} channel activity, it is proposed that PKA will also affect activity as well. The evidence showing that only PKG elicits an agonistic response in BK_{Ca} channels leaves room for speculation on the purpose of PKA. PKA could serve a directly inverse function in BK_{Ca} channel activity by actually deactivating the channel.

1. Patch Clamp (Cell-Attached)

The effect of cAMP-dependent vasodilators (forskolin, or CPT-cAMP) on BK_{Ca} channel activity will be measured in intact (cell-attached) patches on coronary artery smooth muscle cells before and after pharmacological inhibition of PKA activity.

Aim #4: If PKA attenuates BK_{Ca} channel activity, does this occur indirectly through action on PP2A?

Hypothesis #4: PKA's inhibitory effect on BK_{Ca} channels occurs indirectly by its phosphorylation of PP2A and a subsequent dephosphorylation of the BK_{Ca} channels leading to inactivation.

Studies have shown that PKA does not open BK_{Ca} channels(37). Other studies have shown that PP2A functions to inhibit BK_{Ca} channels by dephosphorylation (52). PP2A may be the missing link connecting PKA to BK_{Ca} channels. PKA could act as a regulator of PP2A. PP2A would then function as a direct regulator of BK_{Ca} channels activity. The fact that the B56δ regulatory subunit of PP2A has been phosphorylated by PKA in prior *in vitro* studies (51) validates a possible relationship between the two in the sex hormone cascade pathway.

Approach #4:

1. Inhibition of PKG and Stimulation of cAMP

PKG will be inactivated by KT5823 to ensure that all BK_{Ca} channel activity is solely based on PKA 3. Forskolin will also be used to stimulate the production of cAMP. Since, PKG will be inhibited, all activity due to the presence of cAMP will be due to PKA and not PKG.

2. Inhibition of PP2A

Pharmacological inhibition of PP2A can be achieved in intact cells by using either okadaic acid or calyculin A.

3. Patch Clamp

Channel activity will be measured in cell-attached patches before and after PP2A inhibition. These findings will directly demonstrate either a positive or negative influence of PP2A on BK_{Ca} channel activity.

2. EXPERIMENTAL DESIGN

The purpose of this study was to elucidate a possible mechanism in which PKA interacted with BK_{Ca} channels. cAMP was first experimentally validated in influencing BK_{Ca} channel activity. Data had already shown cAMP acted as a known vasodilator and had stimulatory effects on BK_{Ca} channel activity (60). Other prior experimentation had shown that BK_{Ca} channel activity might work independently from PKA. Even if the channels were responsive to PKA activity, we had to ensure that PKA was sensitive to cAMP in our model first (37). BK_{Ca} channel activity was recorded by using voltage/patch clamp technique before and after addition of 10 μmol/L forskolin. Forskolin increases the accumulation of cAMP in the cell and increases activity (37). Recording activity before and after introduction of forskolin ensured that BK_{Ca} channels were responsive and more importantly; responsive to cAMP increase.

Evidence had shown that cAMP works through the NO/cGMP/PKG pathway through cross-activation. After establishing that cAMP does alter BK_{Ca} channel activity, cAMP's use of PKA to alter activity had to be singled out separately from NO/cGMP(cAMP)/PKG pathway. Is there a mechanism by which cAMP activates PKA and causes some effect on BK_{Ca} channel activity?

3. MATERIALS AND METHODOLOGY

3.1 Cell Culture Methods

Human vascular smooth muscle cells were obtained from Lonza/Clonetics. Gelatin was poured into 25 cm^2 cell culture flasks and 35mm cell culture dishes to cover the bottom surface. Each cell culture dish contained four 12 mm glass cover slips. Gelatin was left in the flasks and dishes for 30 min to allow it to solidify on the bottom. After 30 minutes, excess gelatin liquid was removed. A flask containing cells from the generation being split contained media. Media was decanted off, and cells were washed with 2.5 mL Dulbecco's Phosphate Buffer Saline (DPBS (1X)). The DPBS (1X) was decanted off and 1 mL of 0.25% Trypsin-EDTA (1X) was added and placed in the incubator for 2 min. After the incubation, cells were viewed under the microscope to ensure they had been freed from the flask's gelatinous bottom. Media was added to the solution containing cells and trypsin. The quantity of media added depended on the number of flasks and dishes receiving split cells. The total was found by adding 4 mL per flask and 1.5 mL per dish being generated. The solution containing cells, trypsin, and media was triturated to ensure a uniform concentration. This solution was split into the flasks and dishes. Each flask received 4.5 mL of split cells and each dish received 1.5 mL of split cells. Each flask of original cells was split into three new flasks for the next generation or two new flasks and three 35 mm cell culture dishes containing the cover slips. Cells were stored in an incubator at 37 ° C and 5% CO_2.

3.2 Thawing of Frozen Cultured Cells

HCASMC were frozen in liquid nitrogen for use at later dates in experimentation. Upon need, cells were removed from liquid nitrogen and placed in a water bath at 37 ° C for 2 minutes. 3 mL of media was added to vials containing 1.5 mL of cells and centrifuged for 5 min at 100 rpm. The supernatant was pipetted off and an additional 4 mL of media was added. This solution containing cells and media was placed in a 25 cm^2 cell culture flask already pre-coated with a 2% gelatin solution.

3.3 Primary Cell Isolation

Aortic tissue was excised from male Sprague-Dawley rats in order to harvest VSM cells. The tissue was placed in HEPES solution and stripped of its adventitial layer of cells under a dissecting microscope. The tissue was cut into fragments approximately 1 mm in size and placed in a high K$^+$ solution containing 140 mmol KCl, 10 mmol $MgCl_2$, .01 mmol $CaCl_2$, 10 mmol HEPES, and 30 mmol glucose. An enzymatic reaction to breakdown the media was created with the addition of 6 mg papain, 4 mg dithiothreitol (DTT), and 10 mg albumin dissolved into 5 mL DM solution. Excess high K+ solution was pipetted off the cells and the cells were added to the DM solution. The cellular DM solution was placed on a shaker at 70 rpm for 30 min to physically stimulate the enzymatic reactions. The cellular solution was then removed and triturated. Next, the solution was placed in a 15 mL centrifuge tube and spun in the centrifuge for 10 min at 1000 rpm. Cells in the solution formed a pellet at the bottom of the

centrifuge tube. Two or three drops of cellular solution were placed on microscope for electrophysiological patch clamping experiments. Remaining cellular solution was stored at 4°C and kept up to 24 hrs.

3.4 Patch Clamp Apparatus
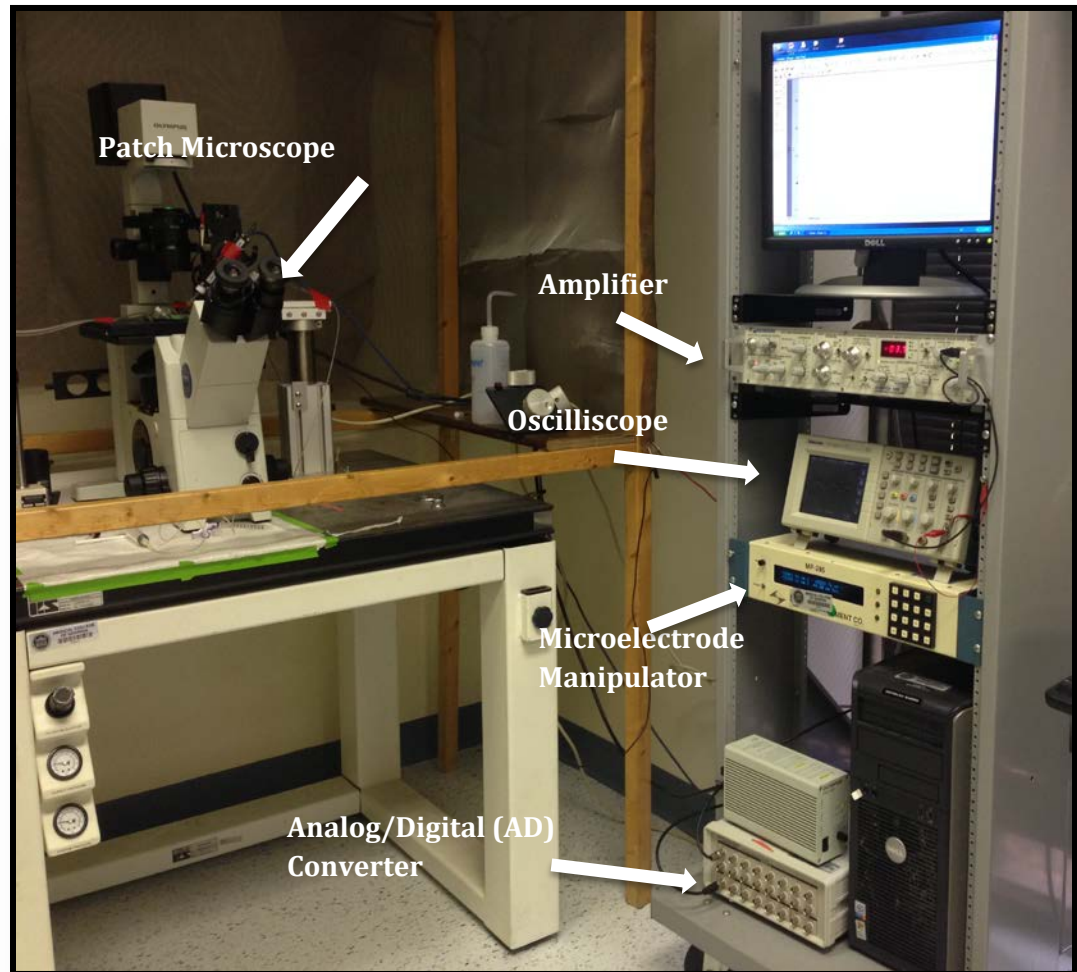
FIGURE 3: Patch Clamp Apparatus- The patch clamp microscope received voltage input from the amplifier via the microelectrode in a closed circuit system. This signal fed back to the A/D converter that converted the analog channel activity into a digital signal that the computer program could interpret. The microelectrode manipulator was used to position the microelectrode in proximity to the cells for patching. The oscilloscope was used to observe the generation of a seal before patching.
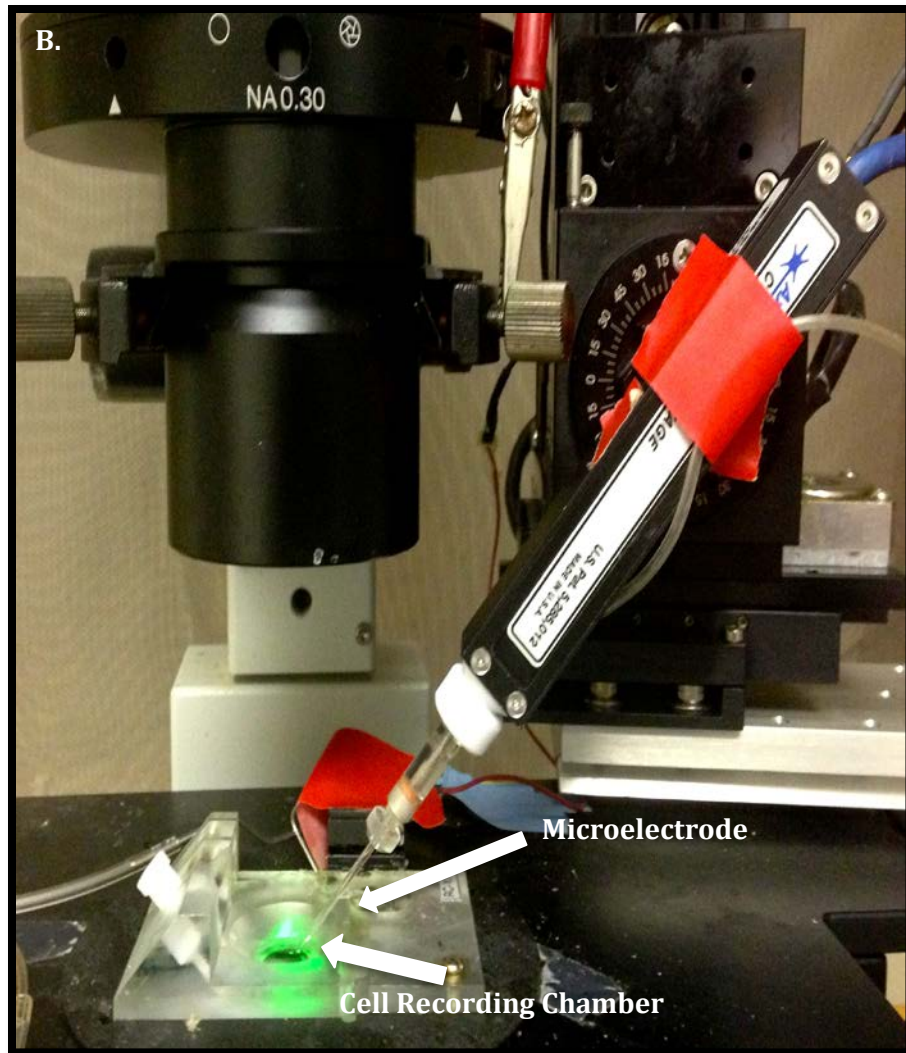
FIGURE 4: Cell Suspension Chamber- The microelectrode was positioned close to the cell inside of the cell suspension chamber using the microelectrode manipulator. The microelectrode contained a metal wire to allow conduction from the amplifier to the patch located inside its tip.
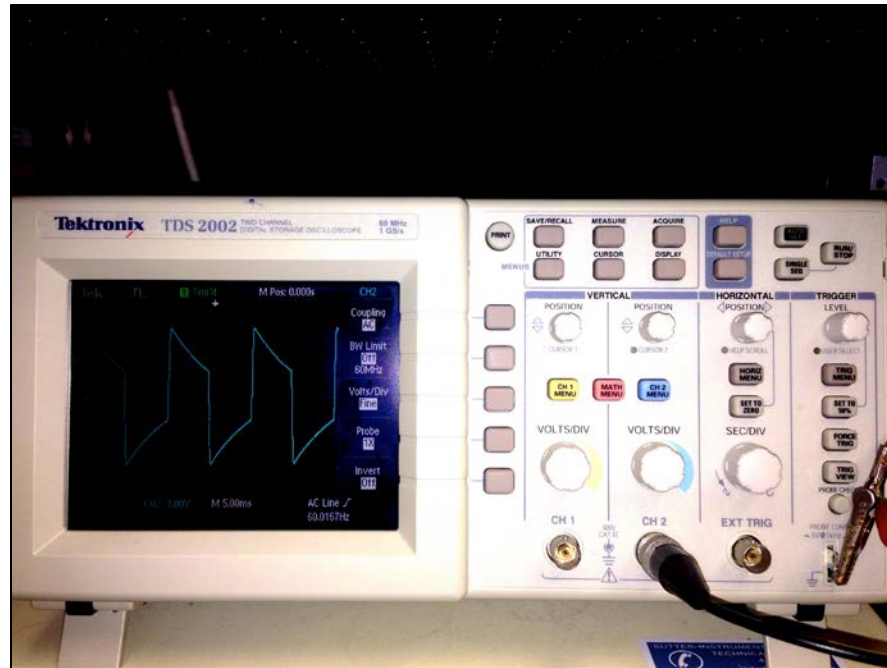
Figure 5: Generating a Mega-Ohm Seal- Microelectrodes were polished to have a 2-5 MΩ resistance prior to forming a giga-ohm seal. The figure shows a pipette with a calculated 2 MΩ resistance. As shown, the pipette has not formed a giga-ohm seal, therefore current is still flowing at a recordable value.

Figure 6: Seal Formation- A giga-ohm seal has been formed. The high level of resistance formed in the seal prevents current flow and is shown by a horizontal line on the oscilloscope.

3.5a Generating a Giga-Ohm Seal

Microelectrodes were generated by pulling 1.50 mm capillary glass tubes with the Sutter Instrument P-2000 microelectrode puller and polished by heat. A polished pipette was determined by having a 2-5 MΩ resistance calculated from the TDS 2002 Two-Channel Digital Storage Oscilloscope recording. Microelectrodes were filled with pipette solution and attached to the MP Sutter Instruments-285micro manipulator. The pipette solution varied depending on experimental conditions. In single channel-cell attached/inside-out conditions, the microelectrode was filled with a normal Ringer solution. In whole cell conditions, the microelectrode was filled with high K+ solution. The microelectrode was lowered into the recording chamber filled with cellular suspension using the micromanipulator. Once the microelectrode was in positioned in close proximity to the desired cell, suction was applied by mouth through a 1 mL syringe. To determine that a giga-ohm seal had been established, the AxoPatch 200B amplifier from Axon Instruments was set to 'seal test' and the oscilloscope reading showed a maximum resistance and no current flow. The prevention of current flow due to high resistance was represented by a horizontal line on the oscilloscope, and verified that a giga-ohm seal had been established.
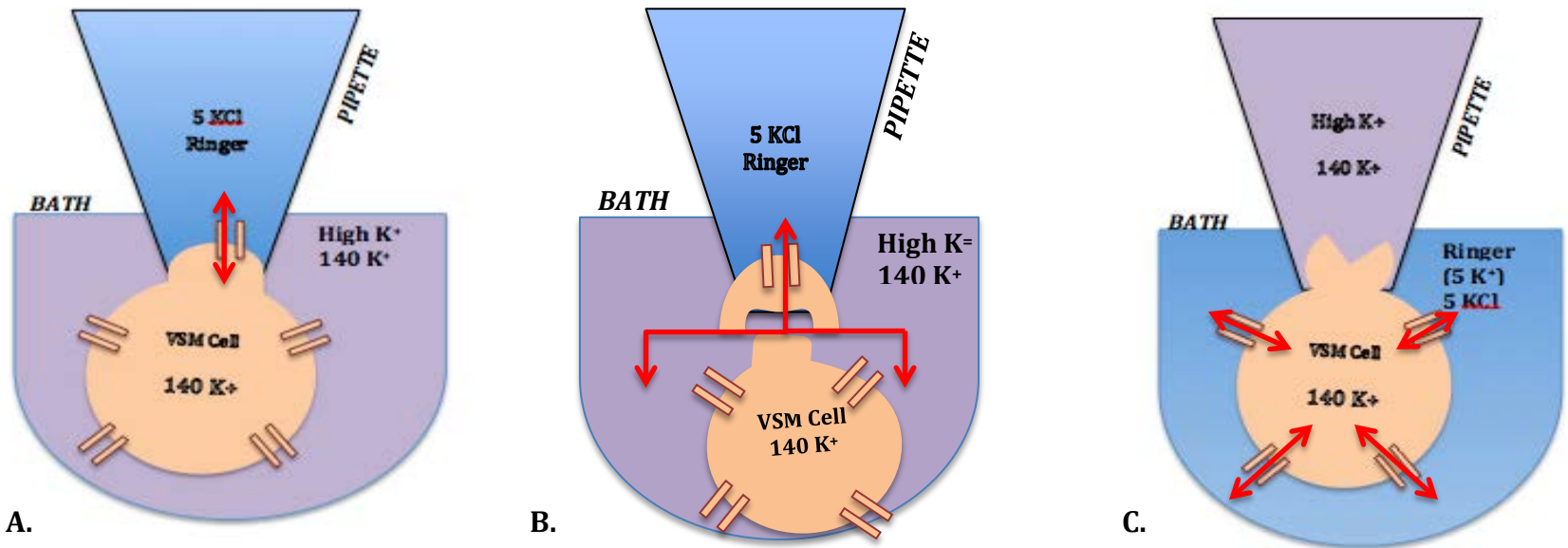
FIGURE 7: Patch Clamp Configurations

(A) Under single-channel cell-attached conditions, the pipette was filled with Ringer solution containing 5mM of K^+ in the form of KCl in order to ensure recording of physiologic cellular conditions for the flow of ions between the pipette and a single channel. (B) Under single-channel inside-out conditions, the pipette was dislodged from the rest of the cell with the seal still intact to a portion of the membrane. The current flow of ions was recorded solely between the channel in the patch and the High K+ bath in the recording chamber. (C) Whole cell recording occurred by rupturing the membrane inside of the patch while the rest of the cell was still attached. The flow of ions through the all channels outside of the patch was recorded. To ensure that physiologic conditions occurred here, Ringer pipette solution was added to the bath in the recording chamber.

3.5b Single-Channel Cell-Attached

For cell-attached patches, a few drops of cell suspension were placed in the recording chamber. Several drops of high K^+ solution were added to the cell suspension in the recording chamber as well. The microelectrode was filled with Ringer pipette solution and placed on the micromanipulator. Once a giga-ohm seal had been achieved, the 'seal test' parameters were removed. With the use of a high K+ bath in the recording chamber, the voltage across the patch was clamped at 0 mV. Currents were filtered at 2kHz and digitized at 10 kHz. Control and experimental groups were clamped at both 0 mV and 40 mV. Addition of forskolin, KT 5720, forskolin + KT 5720, okadaic acid, okadaic acid + KT 5720, chlorophenylthio-cAMP (CPT-cAMP), CPT-cAMP + KT 5720, sodium nitroprusside (SNP) or tetraethylammonimum (TEA) composed the experimental groups. The drugs were administered to the cell bath after a control recording of the channels was recorded at 0mV and 40 mV. After 10 minutes in the bath, the patch was clamped at 40 mV and activity was recorded. Administration of the second drug occurred after the recording of the first drug. Recording of the subsequent response of the second drug occurred after 10 min.

3.5c Single Channel Inside-Out

A few drops of cell suspension were placed in the recording chamber. Several drops of high K^+ solution were then added to the cell suspension in the recording chamber. The microelectrode was filled with Ringer pipette solution and placed on the micromanipulator. Once a giga-ohm seal had been achieved,

the 'seal test' parameters were removed. With the cell still attached to the microelectrode, the micromanipulator was used to raise the microelectrode out of the bath and immediately lowered back into the bath. The membrane still maintained a giga-ohm seal with the microelectrode, but the rest of the cell was now detached from the microelectrode. With the use of high K^+ bath in the recording chamber, the voltage across the patch was clamped at 0 mV. Currents were filtered at 2kHz and digitized at 10 kHz. Control and experimental groups were clamped at both 0 mV and 40 mV. In the case of the TEA experimental groups, the recordings occurred directly after drug administration.

3.5d Whole- Cell

A few drops of cell suspension were placed in the recording chamber. Several drops of normal Ringer solution were added to the cell suspension in the recording chamber as well. The microelectrode was filled with a high K^+ solution with added amphotericin-B and placed on the micromanipulator. Once a giga-ohm cell was produced, perforations in the cell membrane were produced from

amphotericin-B and visualized on the oscilloscope. After perforations were generated, the 'seal test' parameters were removed. Currents were filtered at 2kHz and digitized at 10 kHz. Control and experimental groups were clamped at and recorded at voltage ranging from -50 mV to 40 mV in 10 mV increments.

3.6 Measurement BK_{Ca} Channel Activity

BK_{Ca} channel activity was quantified by calculation of the mean open channel probability (NP_o). The NP_o values were generated by Clampfit 9.2 and Origin 2.0 computer software. The recordings of BK_{Ca} channel activity clamped at a range of voltages in 10 mV increments were also analyzed in Clampfit 9.2 to access the current amplitudes. The current amplitudes were then plotted in Microsoft Excel to calculate the current-voltage (I-V) curves tested in a physiological K^+ ionic condition.

Patch Clamp Solutions Table

Solution	mM / L
Dissociation Medium (DM)	110 NaCl 5 KCl 10 HEPES 10 $NHCO_3$ 0.5 KH_2PO_4 10 Glucose 2 $MgCl_2$ 0.16 $CaCl_2$
High Potassium Bath (High K^+)	140 KCl 10 $MgCl_2$ 0.1 $CaCl_2$ 10 HEPES 30 Glucose
Normal Ringer	140 NaCl 5 KCl 1 $MgCl_2$ 2 $CaCl_2$ 10 HEPES

4. RESULTS

4.1 Forskolin Increases Whole-Cell Current in HCASMC

Whole-cell patch-clamp recording was used to determine the effect of cAMP-
dependent vasodilators on HCASMC. Recordings were obtained at 10 mV
intervals in a range from -50 mV to +40 mV (Figure 8). Control group activity
was recorded before 10 μM forskolin was added to the cell suspension chamber
for experimental group recording. Generation of a current- voltage (I-V)
relationship curve quantitatively calculated the relationship of outward current
to voltage in the cell. As the clamped voltage increased, the current subsequently
increased revealing a positive correlation in both the control and experimental
groups. The addition of forskolin increased the outward current of the whole cell
in the experimental group compared to the control group when clamped at
voltages above -20 mV. At voltages under -20 mV, the control group had a
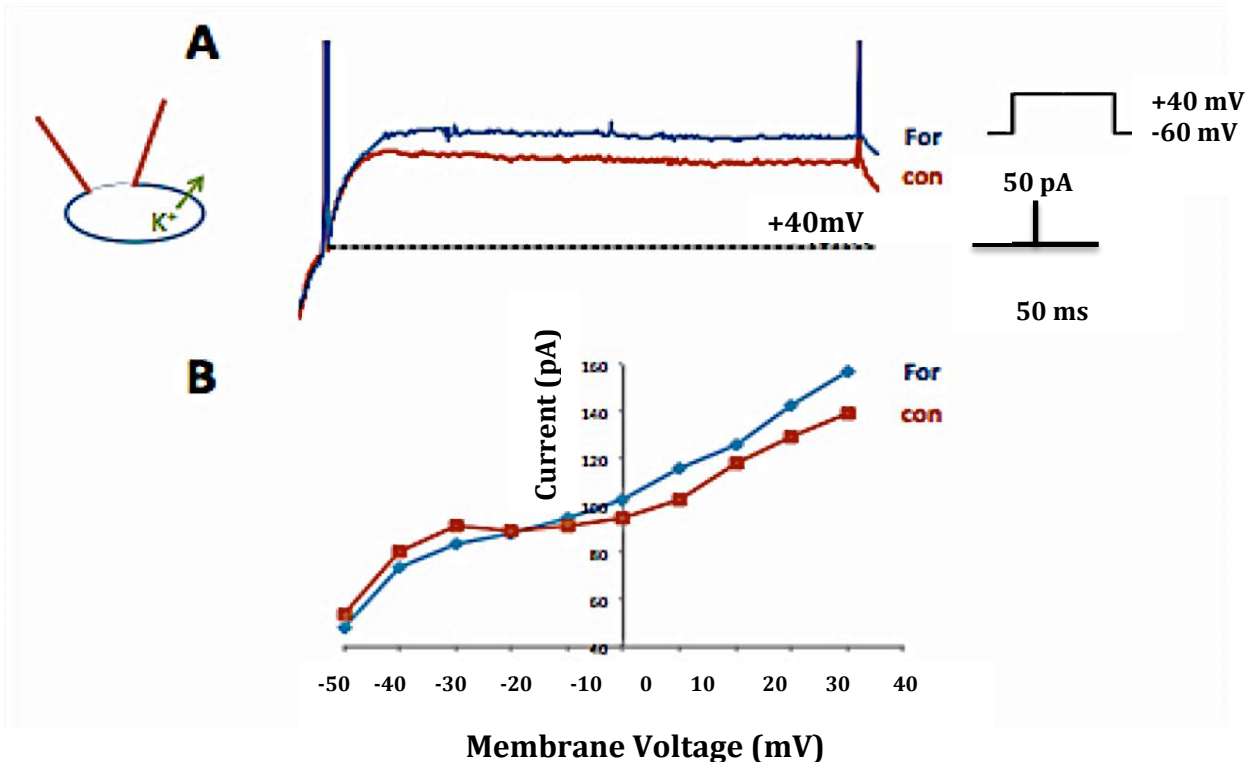greater outward current.

FIGURE 8: A cAMP-dependent vasodilator increases outward current in HCASMC. Forskolin (10 µM, 10 min) increased outward current in HCASMC. A) Current traces recorded before (red) and 10 min after (blue) addition of forskolin (+40mV). B) Complete current (I)-voltage (V) relationship before (red) and after (blue) forskolin.

4.2 BK_{Ca} Channels are expressed in HCASMC

Although cAMP generation increased outward currents, the identification

of the channel(s) involved cannot be determined from whole-cell studies.

Therefore we identified the specific channel stimulated by cAMP at the

molecular level by performing single-channel patch-clamp studies. Activity in

both cell-attached and inside-out patch configurations confirmed the presence of

BK_{Ca} channels in HCASMC when clamped at +40 mV. Recordings of the patch

clamp traces noted distinguishable channel openings and closings (FIGURE 9A,

9B). Channel openings were observable in physiological concentrations of

calcium (10^{-7} M) where channels were patched in cell-attached configurations.

Exposure to higher concentrations of calcium (10^{-4}M) in the high K^+ solution

showed a 1,000 fold (p< .001; n=3) increase in channel activity. This increased

exposure was accomplished by detaching the channel in the patch from the rest

of the cell so that the channel could now be exposed to the higher concentration

of calcium in the cell suspension chamber. The amplitude of the channel

openings was approximately 10 pA at +40mV.

Channel conductance was calculated to distinguish the presence of of BK_{Ca}

from IK_{Ca} and SK_{Ca} channels. The calculated channel conductance was 152.4 ± 7

pS (n= 4 patches) using the slope from an I-V relationship plot. The voltages for

this relationship were clamped with the cells being in physiological K^+

concentration (FIGURE 9C).

Addition of 1-5 mM of TEA to the cell suspension chamber in an inside-out

configuration clamped at 0 mV yielded a 100-fold decrease in BK_{Ca} channel

activity. The NP_O for the control and experimental recordings were 0.9130 and

0.0079, respectively. The patch clamp traces validated the data with an

observable decrease in BK_{Ca} channel activity after the addition of TEA (10A,

10B).

BK$_{Ca}$ Channels Are Expressed in HCASMC

A

Cell-attached patch, physiological Ca²⁺

Inside-out patch, high Ca²⁺ [100μM]

10pA

500ms

B

C

Slope Conductance= 152.4 ± 7

Membrane Voltage (mV)
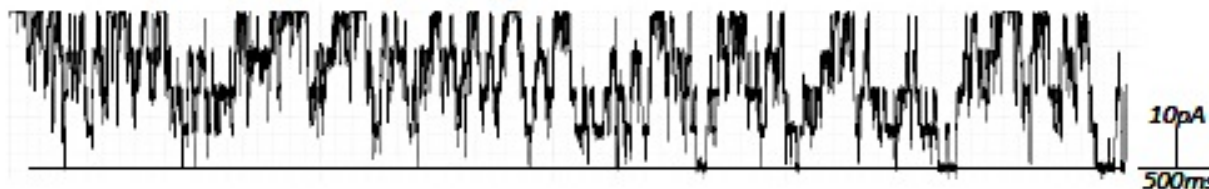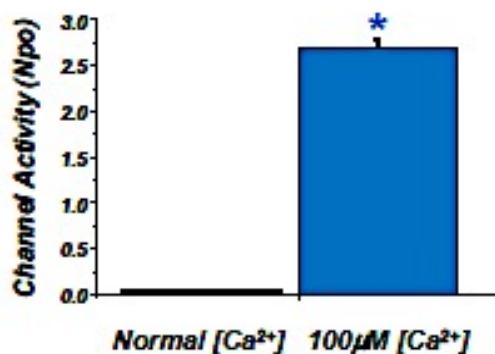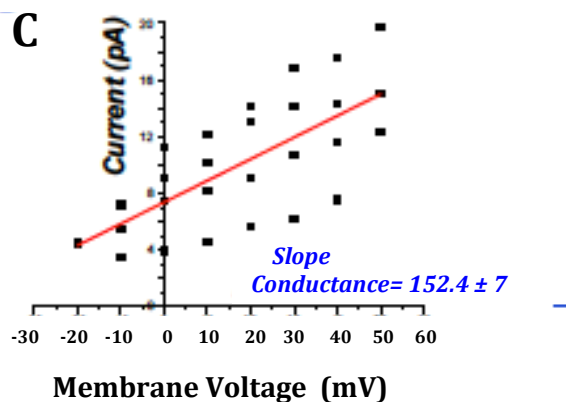
Figure 9: BKC$_a$ channel identification in HCASMC. (A) Channel activity recorded in the same HCASMC patch (+40mV). *Upper trace:* cell-attached patch (normal calcium; 10⁻⁷ M calcium). *Lower trace:* inside-out patch (100μM calcium). (B) On average, raising calcium increased channel activity by > 1000-fold (bar graph; *p<0.001; n=3). (C) Channel conductance is ~150 pS in physiological [K⁺] (n=4 patches).

TEA Inhibits BK$_{Ca}$ Channel Activity

A
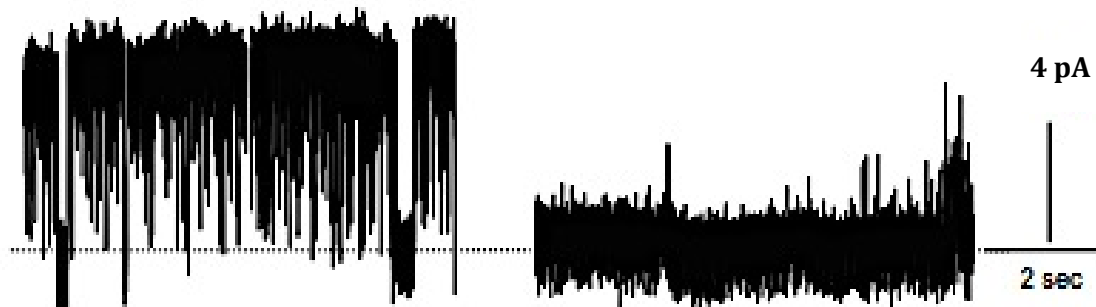
B
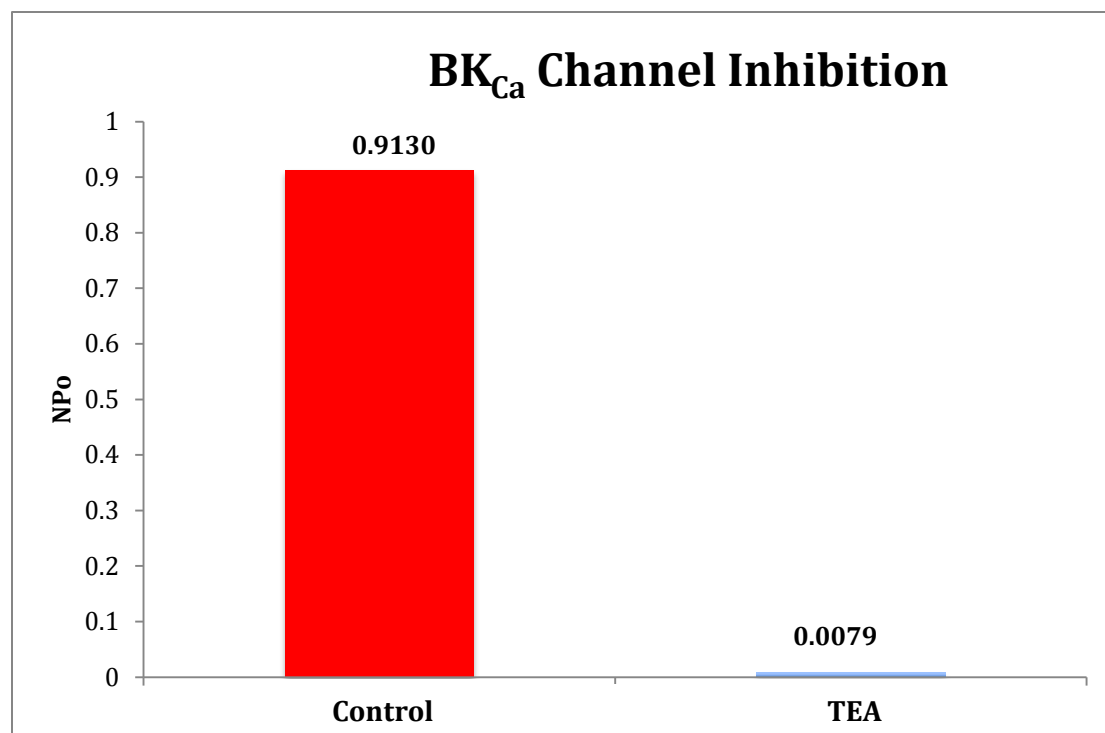
FIGURE 10: TEA inhibits BK$_{Ca}$ channel activity in inside-out patch. (A) *Left trace:* High level of BK$_{Ca}$ channel activity with distinguishable openings and closings (0 mV). *Right Trace*: Decreased level of BK$_{Ca}$ channel activity after addition of 10 μM TEA. (B) NP$_O$ decreased 100-fold after addition of TEA. (n=1)

4.3 Elevating cAMP Increases BK_{Ca} Channel Activity in Cell-Attached Patches

Increasing the level of cAMP in HCASMC increased the level of BK_{Ca} channel activity. Recordings of cell-attached patches at +40 mV before (control) and 10 min after addition of 10 μM forskolin were collected. The traces showed observable increase with forskolin(FIGURE 11). The NP_o of the control and forskolin group were 0.0525 and 0.0778, respectively (FIGURE 12) (n=1). Recordings after the addition of chlorophenythio-cAMP showed a similar trend. CPT-cAMP is a membrane-permeable derivative of cAMP that can influence channels still attached to the rest of the cell. Addition of either cAMP-stimulating drugs caused an NP_o of 0.0689, while the control NP_o was 0.0523 (FIGURE 12). The trace comparing channel activity before (control) and 30 min after the addition of 100μM CPT-cAMP showed an even more distinguishable difference in channel activity than when the control was compared to forskolin. This data suggests a positive correlation between stimulation of cAMP and BK_{Ca} channel activity.

Effect of cAMP on BK$_{Ca}$ Channel Activity
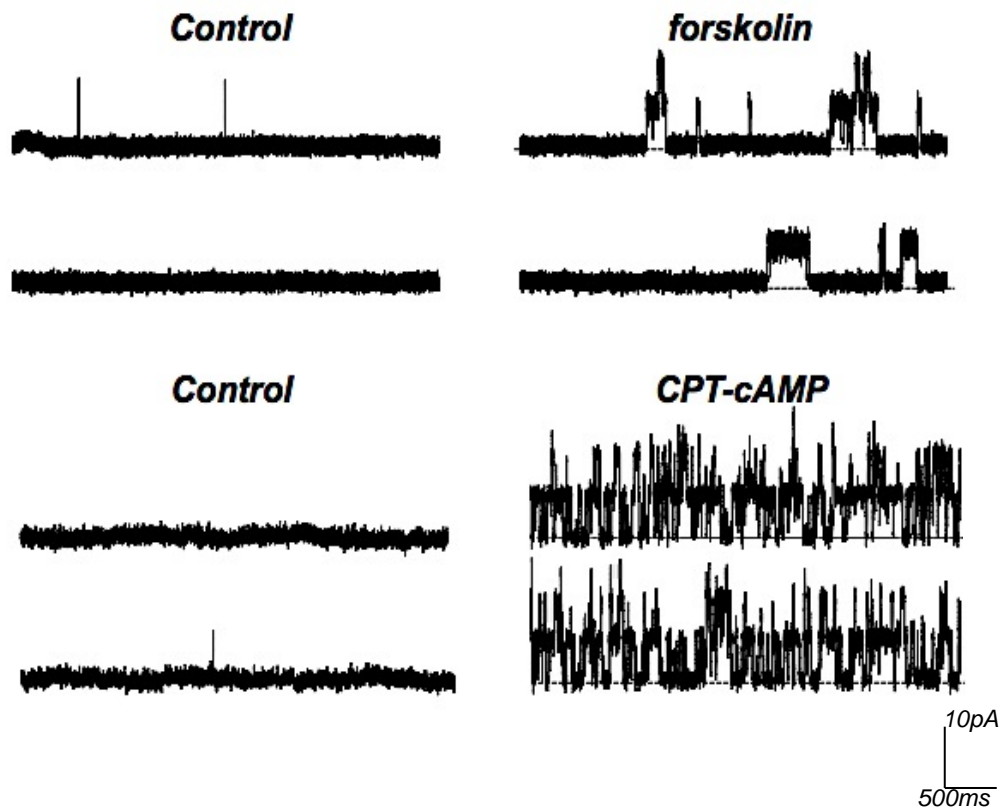
FIGURE 11: cAMP-dependent vasodilators increase BK$_{Ca}$ activity in HCASMC. *Upper traces:* Recordings from a cell-attached patch (+40mV) before and 10 min after addition of 10mM forskolin. *Lower traces:* Recordings from another cell-attached patch (+40mV) before and 30 min after addition of 100mM chlorophenylthio (CPT)-cAMP, a membrane-permeable derivative.Channel openings are upward deflections from baseline.
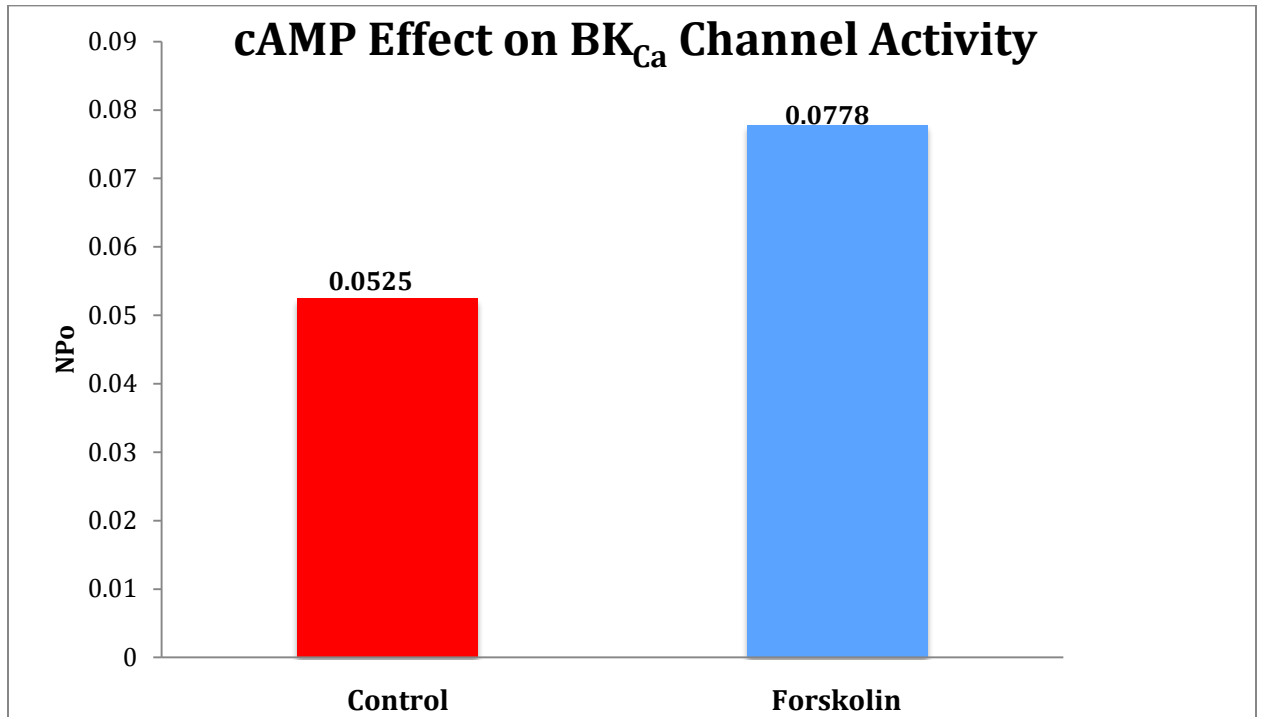
FIGURE 12: Addition of Forskolin Increases BK$_{Ca}$ Channel Activity.
Calculations of NP$_o$ from cells patched before (control) and 10 min after addition of 10 μM forskolin. Additions of forskolin increased NP$_o$ from 0.0525 to 0.0778

4.4 BK_{Ca} Channel Activation is Mediated Via PKG, But Independent of PKA

BK_{Ca} activity increased using 10 µM forskolin in cell-attached patches. Forskolin increased activity, so that any possible inhibition of activity would be observable on the trace. A trace was recorded 10 min after forskolin was added followed by the addition of the PKA inhibitor (300 nM KT 5720). The trace of the control showed observable channel openings for what appeared to be one channel present in the patch (FIGURE 13A). Forskolin addition increased the channel openings in the trace to a distinguishable level of second channel openings approximately 20 pA above baseline. The subsequent addition of KT 5720 showed no observable increase in channel activity from that of the forskolin addition suggesting BK_{Ca} channels may not be a target of PKA activity. The NP_O values for the control, forskolin addition, and subsequent KT 5720 addition were 0.0523, 0.0689, and 0.0675, respectively (FIGURE 14A). This validated the trace showing an increase in channel activity with the addition, but no change after the addition of KT 5720.

Comparing the traces before (control) and 10 min after the addition of a PKG stimulator sodium nitroprusside (SNP), an NO donor, showed an observable increase in BK_{Ca} channel activity suggesting that channel activity increases with activation of PKG. Stimulation of PKG activity with SNP showed an increase from control activity with NP_O values increasing from 0.0102 to 0.1310. Activation of PKG increased BK_{Ca} channel activity 10-fold (FIGURE 14B).
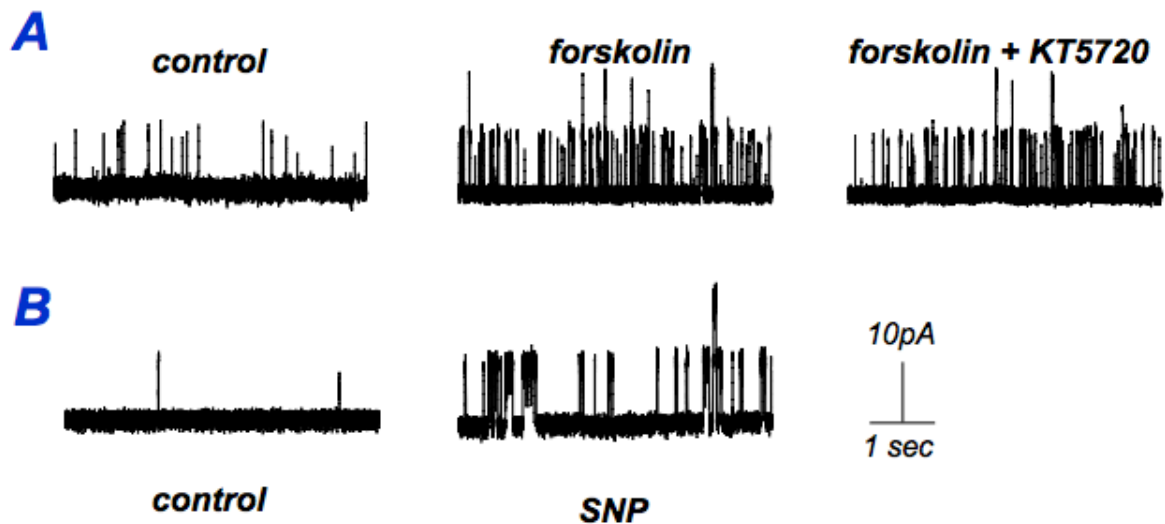
Figure 13: Inhibition of PKA does not affect BK$_{Ca}$ channel activity, but activation of PKG opens channels. (A) Channel activity recorded in the same HCASMC patch (+40mV) before (control), 10 min after 10 µM forskolin, and 20 min after addition of 300nM KT5720 (PKA inhibitor). Inhibition of PKA did not affect channel activity. (B) Channel activity recorded in the same HCASMC patch (+40mV) before (control) and 12 min after 10 µM sodium nitroprusside (SNP; PKG activator).

A
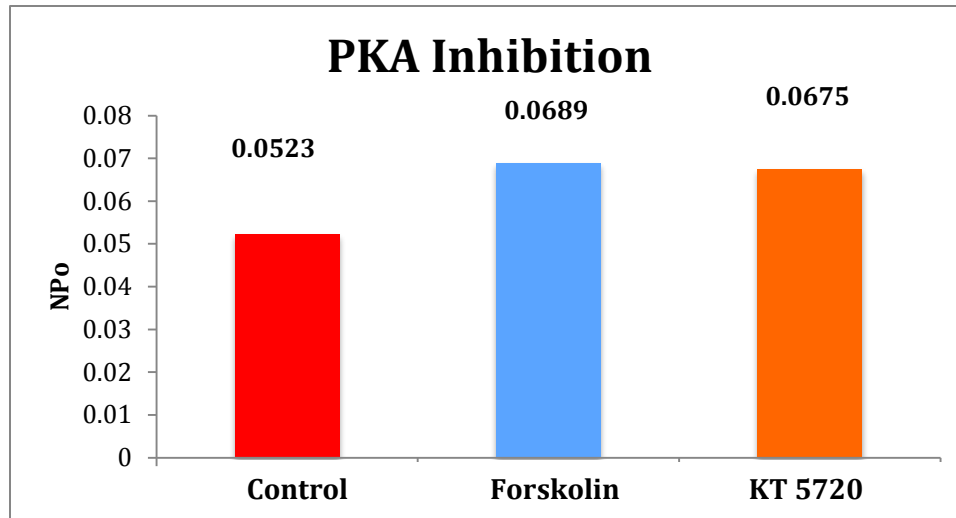
B
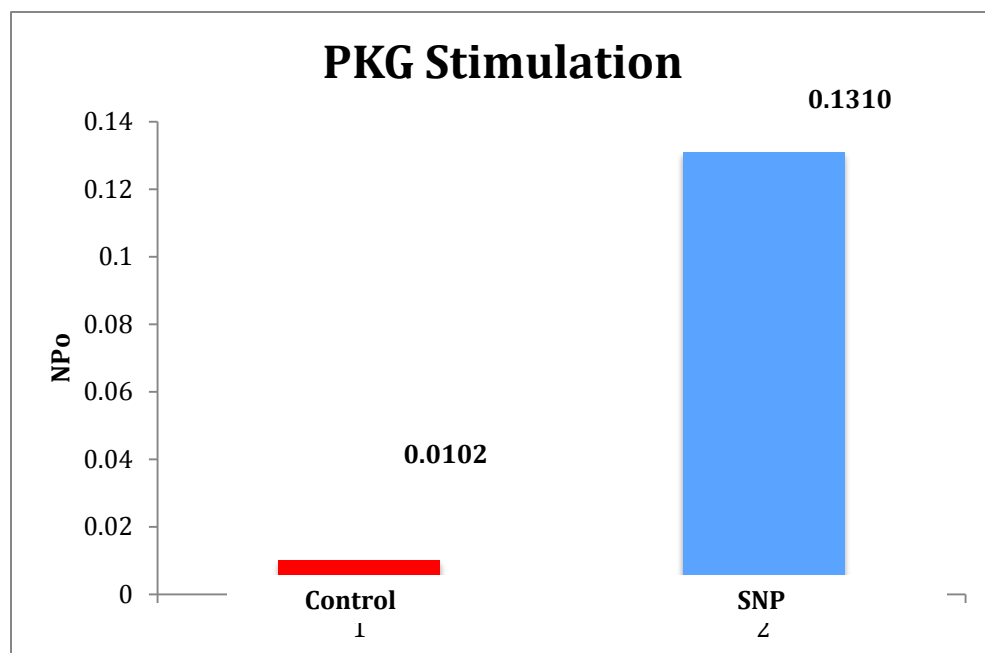
FIGURE 14: BK$_{Ca}$ Channel Activity increased with PKG activity and is independent of PKA activity. (A) After stimulating control activity with 10 μM forskolin, inhibition of PKA with KT 5720 showed now change in channel activity. (B) Stimulating PKG with 10 μM SNP increased channel activity 10 fold.

4.5 Inhibiting Channel Dephosphorylation Decreases BK$_{Ca}$ Channel Activity

Patch-clamp traces using cell-attached methods validated that BK_{Ca} channel activity was stimulated with the addition of 100 μM CPT-cAMP for 30 min when clamped at +40 mV. Addition of CPT-cAMP to the control increased the NP_O 100-fold from 0.0025 to 0.3176 (FIGURE 15B). In FIGURE 15A, two levels of BK_{Ca} channel activity can be distinguished by the openings at approximately 10 pA and 20 pA amplitudes. Subsequent addition of PP2A inhibitor (100 nM okadaic acid) for another 15 min showed a definitive decrease in channel activity now with only one channel being activated at an amplitude of approximately 10 pA. The subsequent addition of OA dropped the NP_O back down again 50-fold from 0.3176 to 0.0064. The graph showed significant increase in channel activity with CPT-cAMP and decrease with OA (*$p<0.05$) (n=3).
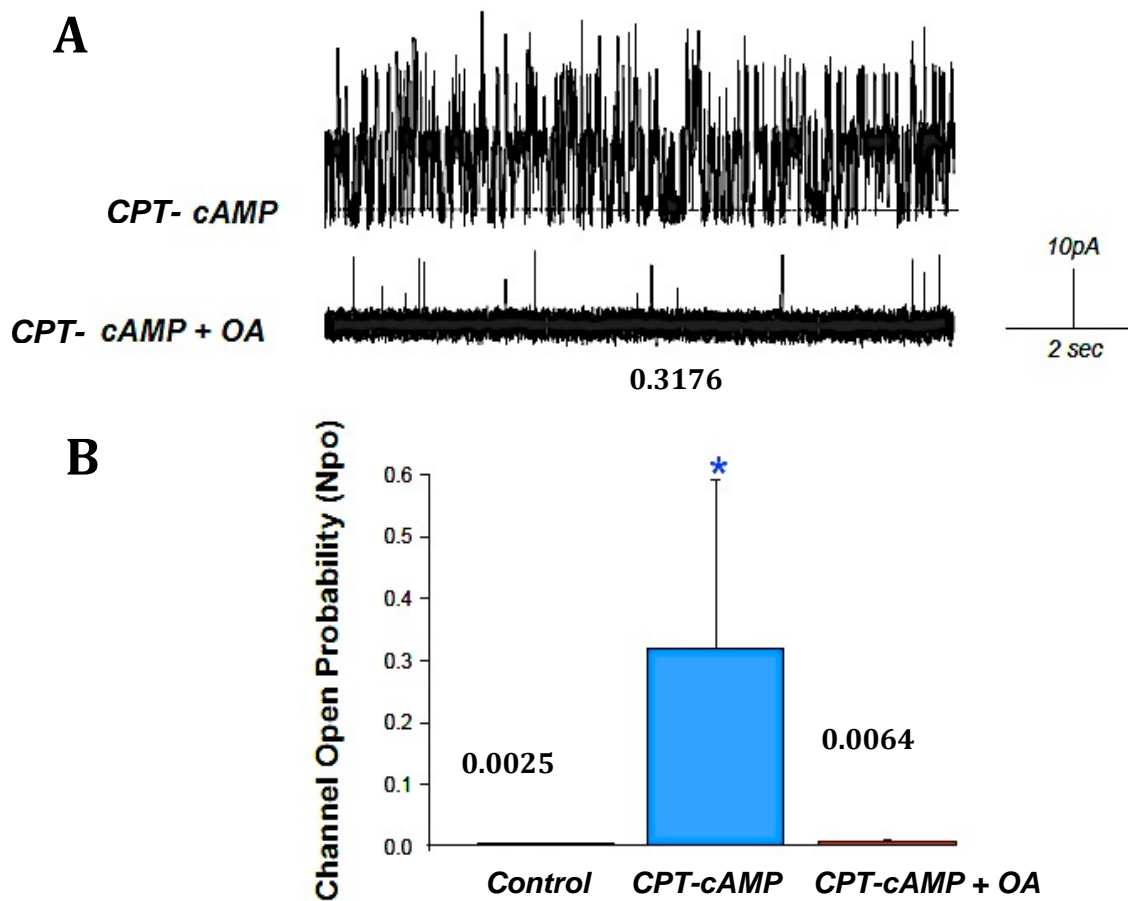
Figure 15: Okadaic acid reverses cAMP-stimulated BKCa channel activity in HCASMC.(A) Representative recordings from a cell-attached patch (+40mV) HCASMC treated with 100mM CPT-cAMP (30 min) and 45 min after 100nM okadaic acid (OA). Channel openings are upward deflections from the baseline (closed) state (dashed line). (B) Summary graph demonstrating significant (*p<0.05) channel stimulation by cAMP with subsequent inhibition by OA (n=3), which is a selective inhibitor for PP2A.

5. DISCUSSION

The importance of BK_{Ca} channels in vasodilation has been confirmed by many studies, however the mechanism behind this important regulatory effector still needs elucidation. It is apparent that after gonadal steriods bind to their receptors there is subsequent activation of PI3, Akt kinases, and nitric oxide synthase (61, 62). Nonetheless, we still do not understand the precise role of the cyclic nucleotide-dependent kinases activated subsequent to cyclic nucleotide generation. Working with the common precept that PKG activates BK_{Ca} channels, we constructed a mechanism in which PKG activates the channels via phosphorylation. Prior research shows that PKG and PKA exhibit antagonistic responses, leading to a proposal that PKA phosphorylation inhibited channel activity (39). The belief that PKA phosphorylation works with PP2A dephosphorylation stems from prior data reporting PP2A as a possible substrate for PKA (51) and data showing PP2A inhibition increasing BK_{Ca} activity. This evidence supported our initial hypothesis of indirect PKA and direct PP2A inhibition of BK_{Ca} via phosphorylation and dephosphorylation.

To examine the role of the cyclic nucleotide-dependent kinases on the channels, we first established that BK_{Ca} channels were actually expressed in HCASMC. The BK_{Ca} channel is known to be depolarization-activated and also calcium-dependent (31). The currents recorded in whole-cell experiments increased with voltage, so it verified a voltage-dependency of the channels in HCASMC. Subsequent experiments on inside-out patches revealed that

increasing calcium at the cytoplasmic surface of the channel stimulated activity. Stimulation of cAMP production increased whole-cell outward currents in HCASMC. Interestingly, however, we noted that cAMP did not appear to increase outward currents at negative (i.e., < -20mV) voltages. This finding could be attributed to the fact that BK_{Ca} channels are voltage-dependent and require a depolarized cell to activate. The lower the voltage, the less likely BK_{Ca} channels are to be active. In contrast, as the cell was increasingly depolarized (> -20mV) forskolin had more influence on BK_{Ca} activity. These findings are consistent with a model where BK_{Ca} channels carry the majority of outward current in HCASMC, are deplarization-activated, and highly sensitive to stimulation by cAMP. Thus, we propose that cAMP is an important signaling molecule in the signaling cascade governing BK_{Ca} channel activity.

However, these data alone could not verify that the increase of outward current on the cellular level was solely due to BK_{Ca} channel response. To verify this increase was due mainly to calcium-activated channels, we assessed the response of BK_{Ca} channels on a molecular level. Single channels in cell-attached patches are functioning at physiological levels of Ca^{2+} (10^{-7} M), and this basal level of BK_{Ca} activity was recorded. After excising the patch into an inside-out configuration to expose these channels to high Ca^{2+} concentration (10^{-4} M), the activity increased 1,000-fold. These findings verified the expression of calcium-activated potassium channels in HCASMC, and further suggested that the original increase in the outward current on the cellular level was due primarily to activity of calcium-activated channels.

Smooth muscle cells also express other calcium-activated K channels: IK_{Ca} and SK_{Ca} channels. Establishing that the channels were calcium-sensitive was important, but did not differentiate between the types of these K channels; i.e., BK_{Ca}, IK_{Ca} and/or SK_{Ca}. Generation of a single-channel I-V relationship in physiological potassium gradients allowed us to calculate the conductance of the calcium-activated channel to be an average of 152.4 ± 7 pS. Experimentation has shown that the conductance of BK_{Ca}, IK_{Ca}, and SK_{Ca} channels under these conditions is $\sim$150 pS, $\sim$39 pS, and $\sim$10 pS, respectively. (57). Our calculated conductance value agreed with the accepted value and validated that the activity observed in the traces was due primarily to BK_{Ca} channels.

Measuring the open-channel amplitude and exposing an inside-out patch to TEA also confirmed the presence of BK_{Ca} channels in HCASMC. The recorded amplitude of the channel openings was approximately 10 pA at +40mV. This was in the characteristic range for BK_{Ca} channels found in other studies involving myocytes from both porcine and human tissues when clamped at this voltage (63, 64). Channels were clamped at +40 mV because prior experimentation from our lab and other studies have shown that BK_{Ca} channels are clearly distinguishable at this voltage (37, 65). Lastly, when the channel was exposed to TEA in an inside-out patch, the channel activity decreased 100-fold. TEA is a known BK_{Ca} channel blocker with higher selectivity for BK_{Ca} channels at lower concentrations, i.e., $\leq$1 mM (66). Thus, our experiments have defined that the channel responsible for the majority of outward current in HCASMC is depolarization-activated, highly calcium-dependent, sensitive to inhibition by

TEA, and has a large molecular conductance. Therefore, we have identified this protein as the BK_{Ca} channel known to be highly expressed in coronary artery smooth muscle cells.

We had observed that forskolin increased whole-cell outward currents in HCASMC. However, the cell-attached patch revealed the effect of this cAMP-dependent vasodilator on BK_{Ca} channels. The NP_O increased after the addition of forskolin, which confirmed BK_{Ca} channel sensitivity to cAMP. The NP_O increased even greater with the addition of CPT-cAMP. The variation in the BK_{Ca} response between forskolin and CPT-cAMP could be attributed to the fact that forskolin directly activates adenylyl cyclase and indirectly increases cAMP, while CPT-cAMP is a membrane-permeable derivative of cAMP and serves to directly increase the cyclic nucleotide. These experiments clearly demonstrated that increasing intracellular cAMP levels stimulates BK_{Ca} channel activity in HCASMC.

Testing for the role of cyclic nucleotide-dependent kinases suggested that PKG stimulates BK_{Ca} channel activity while PKA activity may not affect channel activity. Addition of a PKG stimulator (SNP) increased NP_O 10-fold, while addition of a PKA inhibitor (KT 5720) showed no observable change in activity. The data validated the common belief that PKG stimulates BK_{Ca} channels, but still leaves many things to question about the role of PKA. The decreased channel activity following addition of the PP2A inhibitor (okadaic acid) suggests that PP2A may play a stimulatory role rather than inhibitory. The data showed that PKG and PP2A both play a role in activating the channel, and perhaps they do

this in conjunction. Instead of PP2A working with PKA to dephosphorylate a stimulatory phosphate off of the channel, PP2A may function following activation by PKG to dephosphorylate an inhibitory phosphate from the channel. In other words, PP2A activity may mediate a more indirect stimulatory effect of PKG on BK_{Ca} channel activity, instead of requiring direct PKG-dependent phosphorylation of the channel protein. To our knowledge, this is the first time this mechanism has been demonstrated in coronary arteries (particularly in human coronary artery smooth muscle). However, support for this mechanism is derived from similar studies done in mesenteric microvascular smooth muscle cells (53). Nonetheless, the role of PP2A may be more complicated, as researchers found that PP2A activation could inhibit BK_{Ca} channel activity in mesangial smooth muscle cells (67). Although these data differ from our original proposed function of PP2A, it now sheds light on possible variability in PP2A function depending on cell type and localization.

Our study adds to an increasing effort to identify the signaling pathways regulating BK_{Ca} channel activity. The activity of BK_{Ca} channels is regulated by their phosphorylation by cyclic nucleotide-dependent kinases and dephosphorylation by protein phosphatases. We concluded that PKG stimulates the channels. Other studies also support this finding. Fukao et al. (1999) reported similar findings as SNP also increased BK_{Ca} channel activity in cloned BK_{Ca} channels from canine colonic smooth muscle cells (68). More importantly, the addition of PKG and ATP to the cytosolic surface of BK_{Ca} channels increased the NP_o 3.2-fold in cloned colonic smooth muscle cells (37, 69). Another study

found that BK_{Ca} channels have a strong consensus site for PKG phosphorylation near the COOH terminal end. The channels also have 11 possible sites for PKA phosphorylation, however, these are all weaker consensus sites (70). The presence of a PKG consensus site in this study confirms that the BK_{Ca} channel is a likely substrate for PKG-dependent phosphorylation. The evidence of weak consensus PKA phosphorylation sites gives cause to further investigate its role as our data suggests that channels are actually unaffected by PKA activity. The role of PP2A also needs further investigation. Imig et al. (54) inhibited PP2A in renal artery smooth muscle cells and observed decreased BK_{Ca} channel activity. This was congruent to results from our experiments in coronary artery smooth muscle cells. Further, White et al. (46) reported that okadaic acid inhibited cGMP-stimulated BK_{Ca} channel activity in neuroendocrine cells. Thus, there is evidence from both vascular and non-vascular cells that PP2A mediates a stimulatory effect on BK_{Ca} channels. In contrast, other studies report an inhibitory role for PP2A. For example, Sansom et al. (1997) reported that the inhibition of PP2A increased BK_{Ca} channel activity in mesangial cells (52). Taken together, the contradictory results with these kinases and phosphatase confirm the need for increased investigation into defining the signaling mechanism whereby phosphorylation / dephosphorylation regulates BK_{Ca} channel activity.

This study investigated the molecular signaling mechanisms of BK_{Ca} channels that mediate coronary artery relaxation. The BK_{Ca} channel is a highly-expressed protein in HCASMC, and we found activity of this channel dominates membrane electrical activity in these cells. These channels provide a powerful

repolarizing influence that closes voltage-dependent calcium channels, thereby leading to HCASMC relaxation and coronary artery vasodilation. Given the important role played by BK_{Ca} channels, integration of our data for potential clinical use is most important. It is critical to determine the exact vasodilatory mechanism involving BK_{Ca} channel activation because this will provide strong implications for these mechanisms serving as novel pharmacological targets to relieving myocardial ischemia or hypertension. Hypertension accompanies multiple disease states, such as diabetes, hypoxia, obesity, and CVD (71-73) As these diseases are spread amongst all demographics, finding a drug therapy to target hypertension, one of the major symptoms, could greatly increase the health of an entire population.

There is increasing evidence that hypertension-related diseases exhibit alterations in molecular signaling. One study showed that increased levels of phosphorylated-Akt (p-Akt) were linked to pulmonary hypoxia, and that the use of an anti-hypoxic drug, baicalin, increased BK_{Ca} channel activity (71). Research showed that NOS activity was increased in obese rats in comparison to control (74) Another study linked higher BK_{Ca} channel NP_0 and lower channel conductance to rats with streptozotocin-induced diabetes in their thoracic aortic smooth muscle cells (75). A wide variety of physiological and disease states are influenced by activity of BK_{Ca} channels and their regulatory molecules (e.g., Akt, NOS). Understanding the cascading pathways that determine their function will help clinicians and pharmacuetical companies more effectively target the many diseases that exist due to BK_{Ca} channel function and dysfunction.
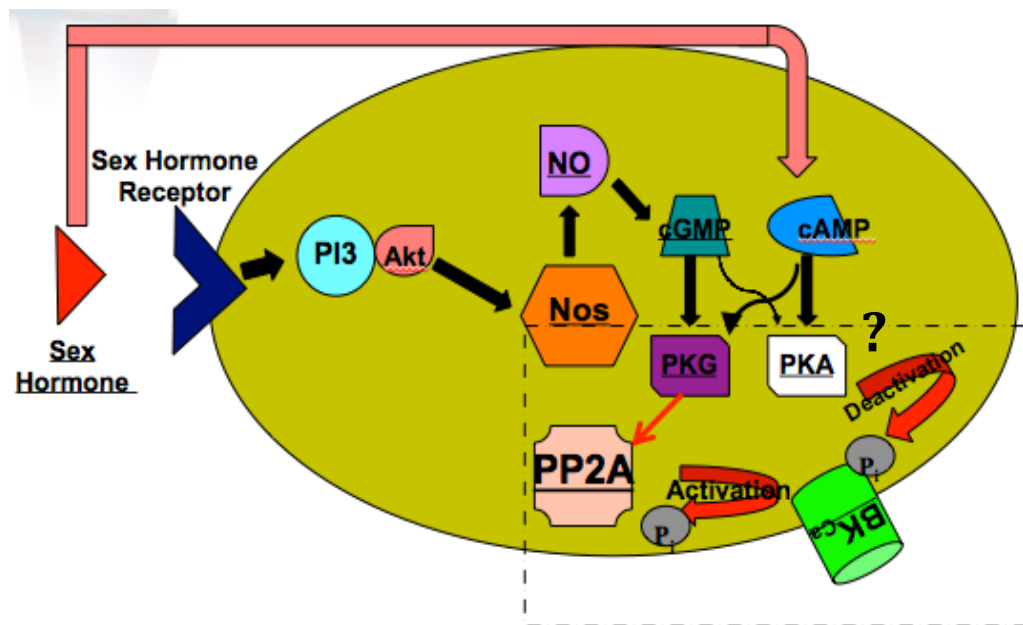
FIGURE 16: Modified BK$_{Ca}$ mechanism with stimulatory PP2A. Both PKG and PP2A elicited increased BK$_{Ca}$ response. In this modified mechanism, PKG phosphorylates PP2A. This activates PP2A to remove an inhibitory P$_i$. The source of the P$_i$ still needs to be determined. PKA could be the kinase responsible for the addition of the P$_i$.

6. CONCLUSION AND FURTHER STUDIES

In conclusion, these findings are the first to provide evidence suggesting that dephosphorylation by PP2A opens BK_{Ca} channels in human coronary artery smooth muscle cells. The data also links gonadal steroids, cyclic nucleotides, and BK_{Ca} channel activity. We propose PP2A to be an exciting novel therapeutic target to promote coronary artery vasodilation and thereby reduce ischemic injury. Given the increased use of gonadal steroids, particularly in older women and men, it would be of subtantial health benefit to understand how these powerful hormones can modify cardiovascular function. An important goal would be to isolate the signaling mechanisms of gonadal steroids in blood vessels from the endocrine effects of these hormones, thus helping to potentially maximize their therapeutic use while minimizing unwanted or dangerous side effects.

Further objectives for this research would call for running more trials to establish the potential clinical significance of the data. I also propose testing for the influence of PP2A working in conjunction with PKG on BK_{Ca} channel activity. He (2000) showed that PKG may not directly activate channels leading to the belief that there is an intermediate molecule involved in activation (76). Experimentation on whether or not PP2A is actually a substrate for PKG would clearly support their relationship. Finally determining the level of PP2A in various types smooth muscle tissues would shed light on the why its function has shown varying effects depending on the cell type.

7. BIBLIOGRAPHY

1. **Go, A. S., D. Mozaffarian, V. L. Roger, E. J. Benjamin, J. D. Berry, W. B. Borden, D. M. Bravata, S. Dai, E. S. Ford, C. S. Fox, S. Franco, H. J. Fullerton, C. Gillespie, S. M. Hailpern, J. A. Heit, V. J. Howard, M. D. Huffman, B. M. Kissela, S. J. Kittner, D. T. Lackland, J. H. Lichtman, L. D. Lisabeth, D. Magid, G. M. Marcus, A. Marelli, D. B. Matchar, D. K. McGuire, E. R. Mohler, C. S. Moy, M. E. Mussolino, G. Nichol, N. P. Paynter, P. J. Schreiner, P. D. Sorlie, J. Stein, T. N. Turan, S. S. Virani, N. D. Wong, D. Woo, and M. B. Turner.** 2013. Heart Disease and Stroke Statistics--2013 Update: A Report From the American Heart Association. Circulation. **127:**e6 <last_page> e245. doi: 10.1161/CIR.0b013e31828124ad.

2. **Finegold, J. A., P. Asaria, and D. P. Francis.** 2013. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organisation and United Nations. Int. J. Cardiol. **168:**934 <last_page> 945. doi: 10.1016/j.ijcard.2012.10.046.

3. **World Health Organization.** 2009. ed. *Global health risks: mortality and burden of disease attributable to selected major risks*. World Health Organization. **2014:**.

4. **American Heart Association.** 2011. What is Cardiovascular Disease (Heart Disease)? AHA. **2014:**.

5. **Urbina, E., B. Alpert, J. Flynn, L. Hayman, G. A. Harshfield, M. Jacobson, L. Mahoney, B. McCrindle, M. Mietus-Snyder, J. Steinberger, and S. Daniels.** 2008. Ambulatory Blood Pressure Monitoring in Children and Adolescents: Recommendations for Standard Assessment: A Scientific Statement From the American Heart Association Atherosclerosis, Hypertension, and Obesity in Youth Committee of the Council on

Cardiovascular Disease in the Young and the Council for High Blood Pressure Research. Hypertension. **52:**433 <last_page> 451. doi: 10.1161/HYPERTENSIONAHA.108.190329.

6. **Wentland, A. L., T. M. Grist, and O. Wieben.** 2014. Review of MRI-based measurements of pulse wave velocity: a biomarker of arterial stiffness. Cardiovasc. Diagn. Ther. **4:**193-206. doi: 10.3978/j.issn.2223-3652.2014.03.04 [doi].

7. **Kanber, B., T. C. Hartshorne, M. A. Horsfield, A. R. Naylor, T. G. Robinson, and K. V. Ramnarine.** 2013. Wall motion in the stenotic carotid artery: association with greyscale plaque characteristics, the degree of stenosis and cerebrovascular symptoms. Cardiovasc. Ultrasound. **11:**37-7120-11-37. doi: 10.1186/1476-7120-11-37 [doi].

8. **National Institute for Health and Care Excellence.** 2014. Hypertension Guidance CG127. NICE. **2014:**. http://publications.nice.org.uk/hypertension-cg127/guidance.

9. **Yeap, B. B.** 2013. Sex steroids and cardiovascular disease. Asian J. Androl. . doi: 10.4103/1008-682X.122357.

10. **K. Nathan, L., W. Shi, H. Dinh, T. K. Mukherjee, X. Wang, A. J. Lusis, and G. Chaudhuri.** 2001. Testosterone inhibits early atherogenesis by conversion to estradiol: Critical role of aromatase. Proceedings of the National Academy of Sciences. **98:**3589 <last_page> 3593. doi: 10.1073/pnas.051003698.

11. **Hak, A. E., J. C. M. Witteman, F. H. de Jong, M. I. Geerlings, A. Hofman, and H. A. P. Pols.** 2002. Low Levels of Endogenous Androgens Increase the Risk of Atherosclerosis in Elderly Men: The Rotterdam Study. The Journal of Clinical Endocrinology & Metabolism. **87:**3632 <last_page> 3639. doi: 10.1210/jcem.87.8.8762.

12. **R Haas, E., M. R. Meyer, U. Schurr, I. Bhattacharya, R. Minotti, H. H. Nguyen, A. Heigl, M. Lachat, M. Genoni, and M. Barton.** 2007. Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis. Hypertension. **49:**1358-1363. doi: 10.1161/HYPERTENSIONAHA.107.089995.

13. **Kannel, W. B., M. C. Hjortland, P. M. McNamara, and T. Gordon.** 1976. Menopause and risk of cardiovascular disease: the Framingham study. Ann. Intern. Med. **85:**447-452.

14. **Q Lisabeth, L. D., A. S. Beiser, D. L. Brown, J. M. Murabito, M. Kelly-Hayes, and P. A. Wolf.** 2009. Age at Natural Menopause and Risk of Ischemic Stroke: The Framingham Heart Study. Stroke. **40:**1044 <last_page> 1049. doi: 10.1161/STROKEAHA.108.542993.

15. **T Rossouw, J. E., G. L. Anderson, R. L. Prentice, A. Z. LaCroix, C. Kooperberg, M. L. Stefanick, R. D. Jackson, S. A. Beresford, B. V. Howard, K. C. Johnson, J. M. Kotchen, J. Ockene, and Writing Group for the Women's Health Initiative Investigators.** 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA. **288:**321-333.

16. **White, R., R. Gerrity, S. Barman, and G. Han.** - Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. - Steroids.2010 Nov;75(11):788-93.Doi: 10.1016/J.Steroids.2009.12.007.Epub 2010 Jan 7. .

17. **Deenadayalu, V., Y. Puttabyatappa, A. Liu, J. Stallone, and R. White.** Testosterone-induced relaxation of coronary arteries: activation of BKCa channels via the cGMP-dependent protein kinase. - Am J Physiol Heart Circ Physiol.2012 Jan 1;302(1):H115-23.Doi: 10.1152/Ajpheart.00046.2011.Epub 2011 Nov 11. .

18. **Dhanakoti, S., Y. Gao, M. Nguyen, and J. Raj.** Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP. J Appl Physiol (1985).2000 may;88(5):1637-42. .

19. **S Gelmann, E. P.** 2002. Molecular biology of the androgen receptor. J. Clin. Oncol. **20:**3001-3015.

20. **White, R., G. Han, C. Dimitropoulou, S. Zhu, K. Miyake, D. Fulton, S. Dave, and S. Barman.** Estrogen-induced contraction of coronary arteries is mediated by superoxide generated in vascular smooth muscle. - Am J Physiol Heart Circ Physiol.2005 Oct;289(4):H1468-75. .

21. **Kaufman, J. M., and A. Vermeulen.** 2005. The decline of androgen levels in elderly men and its clinical and therapeutic implications. Endocr. Rev. **26:**833-876. doi: 10.1210/er.2004-0013.

22. **Jones, R. D., P. J. Pugh, T. H. Jones, and K. S. Channer.** 2003. The vasodilatory action of testosterone: a potassium-channel opening or a calcium antagonistic action? Br. J. Pharmacol. **138:**733 <last_page> 744. doi: 10.1038/sj.bjp.0705141.

23. **Hodgin, J. B., J. W. Knowles, H. S. Kim, O. Smithies, and N. Maeda.** 2002. Interactions between endothelial nitric oxide synthase and sex hormones in vascular protection in mice. J. Clin. Invest. **109:**541-548. doi: 10.1172/JCI14066 [doi].

24. **Francis, S., J. Busch, J. Corbin, and D. Sibley.** cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol Rev.2010 Sep;62(3):525-63.Doi: 10.1124/Pr.110.002907. .

25. **ZA Gao, Y. T., L. J. Roman, P. Martasek, S. P. Panda, Y. Ishimura, and B. S. S. Masters.** 2007. Oxygen Metabolism by Endothelial Nitric-oxide Synthase. J. Biol. Chem. **282:**28557 <last_page> 28565. doi: 10.1074/jbc.M704890200.

26. **Burnett, A.** Role of nitric oxide in the physiology of erection. Biol Reprod.1995 Mar;52(3):485-9. .

27. **U Bauer, V., and R. Sotnikova.** 2010. Nitric oxide--the endothelium-derived relaxing factor and its role in endothelial functions. Gen. Physiol. Biophys. **29:**319-340.

28. **Kvietys, P., and D. Granger.** Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. - Free Radic Biol Med.2012 Feb 1;52(3):556-92.Doi: 10.1016/J.Freeradbiomed.2011.11.002.Epub 2011 Nov 12. .

29. **Feletou, M.** Calcium-activated potassium channels and endothelial dysfunction: therapeutic options? Br J Pharmacol.2009 Feb;156(4):545-62.Doi: 10.1111/J.1476-5381.2009.00052.X.Epub 2009 Jan 29. .

30. **Alioua, A., Y. Tanaka, M. Wallner, F. Hofmann, P. Ruth, P. Meera, and L. Toro.** The large conductance, voltage-dependent, and calcium-sensitive K+ channel, Hslo, is a target of cGMP-dependent protein kinase phosphorylation in vivo. J Biol Chem.1998 Dec 4;273(49):32950-6. .

31. **V Sobey, C. G.** 2001. Potassium Channel Function in Vascular Disease. Arterioscler. Thromb. Vasc. Biol. **21:**28 <last_page> 38. doi: 10.1161/01.ATV.21.1.28.

32. **Wu, X., Y. Yang, P. Gui, Y. Sohma, G. A. Meininger, G. E. Davis, A. P. Braun, and M. J. Davis.** 2008. Potentiation of large conductance, Ca2+-activated K+ (BK) channels by

alpha5beta1 integrin activation in arteriolar smooth muscle. J. Physiol. **586:**1699-1713. doi: 10.1113/jphysiol.2007.149500 [doi].

33. **Obukhov, A. G., J. J. Ji, L. Chen, X. Duan, X. Song, W. Su, P. Zhang, L. Li, S. Bai, Y. Sun, and N. Inagaki.** 2012. BK Channels Reveal Novel Phosphate Sensitivity in SNr Neurons. PLoS ONE. **7:**e52148. doi: 10.1371/journal.pone.0052148.

34. **Ahring, P. K., D. Strøbæk, P. Christophersen, S. Olesen, and T. E. Johansen.** 1997. Stable expression of the human large-conductance Ca2+-activated K+ channel a- and ß-subunits in HEK293 cells. FEBS Lett. **415:**67 <last_page> 70. doi: 10.1016/S0014-5793(97)01096-X.

35. **X Rusch, N. J., R. G. De Lucena, T. A. Wooldridge, S. K. England, and A. W. Cowley Jr.** 1992. A Ca(2+)-dependent K+ current is enhanced in arterial membranes of hypertensive rats. Hypertension. **19:**301-307.

36. **W Martens, J. R., and C. H. Gelband.** 1996. Alterations in Rat Interlobar Artery Membrane Potential and K+ Channels in Genetic and Nongenetic Hypertension. Circ. Res. **79:**295 <last_page> 301. doi: 10.1161/01.RES.79.2.295.

37. **White, R. E., J. P. Kryman, A. M. El-Mowafy, G. Han, and G. O. Carrier.** 2000. cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK(Ca) channel activity in coronary artery smooth muscle cells. Circ. Res. **86:**897-905.

38. **Y Archer, S. L., J. M. Huang, V. Hampl, D. P. Nelson, P. J. Shultz, and E. K. Weir.** 1994. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A. **91:**7583-7587.

39. **Butt, E., C. Nolte, S. Schulz, J. Beltman, J. Beavo, B. Jastorff, and U. Walter.**
Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets
using a specific activator 8-para-chlorophenylthio-cGMP. Biochem Pharmacol.1992 Jun
23;43(12):2591-600. .

40. **Deroo, B., K. Rodriguez, J. Couse, K. Hamilton, J. Collins, S. Grissom, and K.
Korach.** Estrogen receptor beta is required for optimal cAMP production in mouse granulosa
cells. Mol Endocrinol.2009 Jul;23(7):955-65.Doi: 10.1210/Me.2008-0213.Epub 2009 Mar
26. .

41. **Mugge, A., M. Riedel, M. Barton, M. Kuhn, and P. Lichtlen.** Endothelium
independent relaxation of human coronary arteries by 17 beta-oestradiol in vitro. Cardiovasc
Res.1993 Nov;27(11):1939-42. .

42. **White, R., D. Darkow, and J. Lang.** Estrogen relaxes coronary arteries by opening
BKCa channels through a cGMP-dependent mechanism. Circ Res.1995 Nov;77(5):936-42. .

43. **Francis, S., and J. Corbin.** vi. Phosphodiesterase-5 inhibition: the molecular biology of
erectile function and dysfunction. Urol Clin North Am. **2005:**419-429.

44. **Butt, E., K. Abel, M. Krieger, D. Palm, V. Hoppe, J. Hoppe, and U. Walter.** cAMP-
and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-
stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem.1994
may 20;269(20):14509-17. .

45. **Nelson, C. P., R. D. Rainbow, J. L. Brignell, M. D. Perry, J. M. Willets, N. W.
Davies, N. B. Standen, and R. A. J. Challiss.** 2011. Principal role of adenylyl cyclase 6 in

K+ channel regulation and vasodilator signalling in vascular smooth muscle cells. Cardiovasc. Res. **91:**694 <last_page> 702. doi: 10.1093/cvr/cvr137.

46. **White, R., R. White, A. El-Mowafy, G. Han, and G. Carrier.** cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK(Ca) channel activity in coronary artery smooth muscle cells. - Circ Res.2000 Apr 28;86(8):897-905. .

47. **Tanano, I., T. Nagaoka, T. Omae, A. Ishibazawa, T. nKamiya, S. Ono, and A. Yoshida.** Dilation of porcine retinal arterioles to cilostazol: roles of eNOS phosphorylation via cAMP/protein kinase A and AMP-activated protein kinase and potassium channels. - Invest Ophthalmol Vis Sci.2013 Feb 19;54(2):1443-9.Doi: 10.1167/Iovs.12-10115. .

48. **Zhou, X., P. Ruth, J. Schlossmann, F. Hofmann, and M. Korth.** Protein phosphatase 2A is essential for the activation of Ca2+-activated K+ currents by cGMP-dependent protein kinase in tracheal smooth muscle and Chinese hamster ovary cells. J Biol Chem.1996 Aug 16;271(33):19760-7. .

49. **Ahn, J. H., T. McAvoy, S. V. Rakhilin, A. Nishi, P. Greengard, and A. C. Nairn.** 2007. Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit. Proc. Natl. Acad. Sci. U. S. A. **104:**2979-2984. doi: 10.1073/pnas.0611532104.

50. **Letourneux, C., G. Rocher, and F. Porteu.** 2006. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. EMBO J. **25:**727-738. doi: 10.1038/sj.emboj.7600980.

51. **Agarwal, E., A. Chaudhuri, P. D. Leiphrakpam, K. L. Haferbier, M. G. Brattain, and S. Chowdhury.** 2014. Akt inhibitor MK-2206 promotes anti-tumor activity and cell

death by modulation of AIF and Ezrin in colorectal cancer. BMC Cancer. **14:**145. doi: 10.1186/1471-2407-14-145.

52. **Sansom, S., J. Stockand, D. Hall, and B. Williams.** Regulation of large calcium-activated potassium channels by protein phosphatase 2A. J Biol Chem.1997 Apr 11;272(15):9902-6. .

53. **Dimitropoulou, C., L. West, M. Field, R. White, L. Reddy, J. Falck, and J. Imig.** Protein phosphatase 2A and Ca2+-activated K+ channels contribute to 11,12-epoxyeicosatrienoic acid analog mediated mesenteric arterial relaxation. Prostaglandins Other Lipid Mediat.2007 Feb;83(1-2):50-61.Epub 2006 Nov 7. .

54. **Imig, J., C. Dimitropoulou, D. Reddy, R. White, and J. Falck.** Afferent arteriolar dilation to 11, 12-EET analogs involves PP2A activity and Ca2+-activated K+ Channels. Microcirculation.2008 Feb;15(2):137-50.Doi: 10.1080/10739680701456960. .

55. **Dalaklioglu, S., and G. Ozbey.** 2014. Role of different types of potassium channels in the relaxation of corpus cavernosum induced by resveratrol. Pharmacogn Mag. **10:**47-52. doi: 10.4103/0973-1296.126658 [doi].

56. **Wu, S. N.** 2003. Large-conductance Ca2+- activated K+ channels:physiological role and pharmacology. Curr. Med. Chem. **10:**649-661.

57. **Vogalis, F., and R. K. Goyal.** 1997. Activation of small conductance Ca2+-dependent K+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. J. Physiol. (Lond.). **502:**497 <last_page> 508. doi: 10.1111/j.1469-7793.1997.497bj.x.

58. **Nugent, F. S., J. L. Niehaus, and J. A. Kauer.** 2009. PKG and PKA signaling in LTP at GABAergic synapses. Neuropsychopharmacology. **34:**1829-1842. doi: 10.1038/npp.2009.5 [doi].

59. **Puttabyatappa, Y., J. Stallone, A. Ergul, A. El-Remessy, S. Kumar, S. Black, M. Johnson, M. Owen, and R. White.** - Peroxynitrite mediates testosterone-induced vasodilation of microvascular resistance vessels. - J Pharmacol Exp Ther.2013 Apr;345(1):7-14.Doi: 10.1124/Jpet.112.201947.Epub 2013 Jan 14. .

60. **Z Lincoln, T. M., T. L. Cornwell, and A. E. Taylor.** 1990. cGMP-dependent protein kinase mediates the reduction of Ca2+ by cAMP in vascular smooth muscle cells. Am. J. Physiol. **258:**C399-407.

61. **Hurt, K. J., B. Musicki, M. A. Palese, J. K. Crone, R. E. Becker, J. L. Moriarity, S. H. Snyder, and A. L. Burnett.** 2002. Akt-dependent phosphorylation of endothelial nitric-oxide synthase mediates penile erection. Proc. Natl. Acad. Sci. U. S. A. **99:**4061-4066. doi: 10.1073/pnas.052712499 [doi].

62. **Yoshitomi, H., Q. Xu, M. Gao, and Y. Yamori.** 2011. Phosphorylated endothelial NOS Ser1177 via the PI3K/Akt pathway is depressed in the brain of stroke-prone spontaneously hypertensive rat. J. Stroke Cerebrovasc Dis. **20:**406-412. doi: 10.1016/j.jstrokecerebrovasdis.2010.01.014 [doi].

63. **Jacob, M. K., and R. E. White.** 2000. Diazepam, gamma-aminobutyric acid, and progesterone open K(+) channels in myocytes from coronary arteries. Eur. J. Pharmacol. **403:**209-219. doi: S0014299900005987 [pii].

64. **Doheny, H. C., D. D. Houlihan, N. Ravikumar, T. J. Smith, and J. J. Morrison.** 2003. Human chorionic gonadotrophin relaxation of human pregnant myometrium and activation of the BKCa channel. J. Clin. Endocrinol. Metab. **88:**4310-4315. doi: 10.1210/jc.2003-030221 [doi].

65. **Wang, Y., and D. A. Mathers.** 1993. Ca(2+)-dependent K+ channels of high conductance in smooth muscle cells isolated from rat cerebral arteries. J. Physiol. **462:**529-545.

66. **Ransom, C. B., X. Liu, and H. Sontheimer.** 2002. BK Channels in Human Glioma Cells | Journal of Neurophysiology. Glia. **2014:**. doi: 10.1002/glia.10064.

67. **Stockand, J. D., M. Silverman, D. Hall, T. Derr, B. Kubacak, and S. C. Sansom.** 1998. Arachidonic acid potentiates the feedback response of mesangial BKCa channels to angiotensin II | Renal Physiology. Am. J. Physiol. **274:**F658-64.

68. **Fukao, M., H. S. Mason, F. C. Britton, J. L. Kenyon, B. Horowitz, and K. D. Keef.** 1999. Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. J. Biol. Chem. **274:**10927-10935.

69. **Fukao, M., H. S. Mason, F. C. Britton, J. L. Kenyon, B. Horowitz, and K. D. Keef.** 1999. Cyclic GMP-dependent Protein Kinase Activates Cloned BKCa Channels Expressed in Mammalian Cells by Direct Phosphorylation at Serine 1072. J. Biol. Chem. **2014:**10927-10935.

70. **Toro, L., M. Wallner, P. Meera, and Y. Tanaka.** 1998. Maxi-KCa, a Unique Member of the Voltage-Gated K Channel Superfamily | Physiology. News Physiol. Sci. **2014:**13.

71. **Zhang, L., Z. Pu, J. Wang, Z. Zhang, D. Hu, and J. Wang.** 2014. Baicalin Inhibits Hypoxia-Induced Pulmonary Ar... [Int J Mol Sci. 2014] - PubMed - NCBI. Int. J. Mol. Sci. **2014:**15. doi: 10.3390/ijms15058153 [doi].

72. **Liu, W., Y. Wei, P. Sun, W. H. Wang, T. R. Kleyman, and L. M. Satlin.** 2009. Mechanoregulation of BK channel activity in the mammalian cortical collecting duct: role of protein kinases A and C. Am. J. Physiol. Renal Physiol. **297:**F904-15. doi: 10.1152/ajprenal.90685.2008 [doi].

73. **Rohani, F., N. Hooman, S. Moradi, M. Mobarra, M. Najafizadeh, and P. Tatarpoor.** 2014. The Prevalence of Pre-hypertension in Children with Type 1 Diabetes Mellitus. Int. J. Prev. Med. **5:**S44-9.

74. **Howitt, L., T. H. Grayson, M. J. Morris, S. L. Sandow, and T. V. Murphy.** 2012. Dietary obesity increases NO and inhibits BKCa-mediated, endothelium-dependent dilation in rat cremaster muscle artery: association with caveolins and caveolae. AJP: Heart and Circulatory Physiology. **302:**H2464 <last_page> H2476. doi: 10.1152/ajpheart.00965.2011.

75. **Ye, C. L., B. Shen, X. D. Ren, R. J. Luo, S. Y. Ding, F. M. Yan, and J. H. Jiang.** 2004. An increase in opening of BK(Ca) channels in smooth muscle cells in streptozotocin-induced diabetic mice. Acta Pharmacol. Sin. **25:**744-750.

76. **Anonymous** Cellular mechanisms involved in carotid body inhibition produced by atrial natriuretic peptide | Cell Physiology. **2014:**.