Exchange Protein directly Activated by cAMP (Epac) in the vasculature

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy (or other degree as appropriate) by

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Abstract

Background -Vascular smooth muscle forms the media layer of blood vessels and is responsible for maintaining total peripheral resistance, an integral factor in blood pressure. Relaxation of vascular smooth muscle, which increases vessel lumen diameter and decreases resistance, is brought about by a decrease in cytoplasmic Ca^{2+} and/or a decrease in the Ca^{2+} sensitivity of contractile proteins. Relaxation can be initiated by vasoactive transmitters working via the activation of G-protein coupled receptors; with the downstream effects being attributed to the action of numerous signalling mediators. Exchange protein directly activated by cAMP (Epac), a major effector for the second messenger 3'-5'-cyclic adenosine monophosphate (cAMP), has recently been shown to be involved in a novel vascular relaxation pathway that increases the frequency of localised Ca^{2+} release from intracellular stores in vascular smooth muscle.

Methods – Multiple biochemical techniques such as immunoblotting and immunoprecipitation were used to assess cellular signalling pathways involved in Epac activation. Patch clamping was used in the whole cell format to record the electrical currents regulated by Epac activation.

Results - In immunoblots of rat mesenteric artery lysates, pretreatment with 8-(4-chloro-phenylthio)-2´-O-methyladenosine-3´, 5'-cyclic monophosphate-AM (8-pCPT-AM), a selective Epac activating cAMP analogue, induced phosphorylation of calcium/calmodulin-dependent kinase 2 (CaMKII) at position Thr286/7, an autophosphorylation site that indicates CaMKII activation. Epac activation preferentially induced auto-phosphorylation of specific CaMKII gamma (γ) isoform/s, as shown by CamKIIγ and CamKII

delta (δ) isoform knock out mice. In whole-cell recordings from isolated rat mesenteric artery myocytes, inhibition of CaMKII reversed the increase in STOC frequency and amplitude induced by 8-pCPT-AM. Epac-induced CaMKII activation is probably initiated by inositol 1, 4, 5-trisphosphate (IP_3)-mobilized Ca^{2+}: 8-pCPT-AM failed to induce CaMKII activation following intracellular Ca^{2+} store depletion and inhibition of IP_3 receptors blocked both 8-pCPT-AM-mediated CaMKII phosphorylation and STOC activity.

Conclusion - Epac-mediated STOC activity occurs via activation of specific isoforms of CaMKII activated by IP_3-mediated Ca^{2+} release and is driven by changes in the underlying kinetic behaviour of RyR channels. To our knowledge, this is the first report of CaMKII initiating cellular activity linked to vasorelaxation and suggests novel roles for this Ca^{2+} and redox-sensing enzyme in the regulation of vascular tone and blood flow.

Abbreviations

2-APB	2-aminoethoxydiphenylborate
8-AHA-2´-O-Me-cAMP	8- (6-Aminohexylamino) adenosine- 3', 5'- cyclic monophosphate
8-pCPT-AM	8-(4-chloro-phenylyyhio)-2'-O-methyladenosine-3',5-cyclic monophosphate-AM
AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
Akt	Protein Kinase B
BIM IX	Bisindolylmaleimide IX
Bk_{Ca}	Big-conductance, calcium activated potassium channel
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CaMKII	Calcium/calmodulin-dependent kinase
cAMP	3'-5'-cyclic-adenosine-monophosphate
CFP	Cyan fluorescent protein
cGMP	Cyclic guanosine monophosphtase
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary
CNB	Cyclic nucleotide binding domain
CNG	Cyclic nucleotide gated
CREB	cAMP response element binding partner
CsCl	Caesium chloride
DAG	Diacylglycerol
DEP	Dishevelled, Egl-10, Pleckstrin domain
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
EDHF	Endothelial derived hyperpolarising factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

eNOS	Endothelial nitric oxide synthase
EPAC	Exchange Protein directly Activated by cAMP
FKBP	FK-506 binding protein
FRET	Fluorescence resonance energy transfer
FSK	Forskolin
GAP	GTPase activation protein
GC	Guanylyl Cylcase
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GTP	Guanosine triphosphate
GTPase	GTP hydrolzying enzyme
GST-RalGDS-RBD	Glutathione S-transferase RalGDS Rap binding domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP27	Heat shock protein 27
HSP20	Heat shock protein 20
IC_{50}	Half maximal inhibition concentration
IP	Immunoprecipitation
IP_3	Inositol 1,4,5-triphosphate
IP3R	Inositol 1,4,5-triphosphate Receptor
IPG	Immobilised pH gradient
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K_{ATP}	ATP sensitive potassium channel
KCl	Potassium Chloride
L-NAME	L-N^6-Nitroarginine methyl ester
LTCC	L-type calcium channel
$MgCl_2$	Magnesium chloride
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
Na_2ATP	Disodium adenosine tri-phosphate
Na_2HPO_4	Disodium phosphate
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate

NaH_2PO_4	Sodium diphosphate
NCX	Sodium/Calcium exchanger
NO	Nitric oxide
PBC	Phosphate binding cassette
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PKI	PKA inhibitor
PLB	Phospholamban
PLC	Phospholipase C
PVDF	Polyvinylidene Fluoride
RA	Ras-association domain
REM	Ras-exchange motif
RIPA	Radioimmunoprecipitation assay buffer
RMASMC	Rat mesenteric artery smooth muscle cell
ROCC	Receptor operated calcium channel
ROCK	RhoA associated protein kinase
ROS	Reactive Oxygen Species
RyR	Ryandoine Receptor
SAC	Stretch activated channel
SACC	Stretch associated calcium channel
SB	Sample Buffer
SCF	Skp, Culin, F-box containing complex
SDS	Sodium dodecyl sulfate
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SKP2	S-phase kinase-associated protein 2
SOCC	Store-operated calcium channel
STOC	Spotaneous transient outward current
TRIS	Tris (hydroxymethyl) aminomethane

TRPC	Transient Receptor Potential Classical channel
TRPV4	Transient Receptor Potential Vanilloid 4 channel
VDCC	Voltage dependent calcium channel
VASP	Vasodilator-stimulated phosphoprotein
VSMC	Vascular smooth muscle cell
YFP	Yellow fluorescent protein

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Preface

Publications

Work presented in this thesis was used for the following publications:

Humphries, E. S. A. & Dart, C. Neuronal and Cardiovascular Potassium Channels as Therapeutic Drug Targets. J. Biomol. Screen. 20, 1055–1073 (2015).

Humphries, E. S. A., Kamishima, T., Quayle, J. M. & Dart, C. Calcium/calmodulin-dependent kinase 2 (CaMKII) mediates Epac-induced spontaneous transient outward currents (STOCs) in rat vascular smooth muscle. J. Physiol. (2017).

Contributions

Caffeine induced Ca^{2+} transient experiments (Chapter 5) were undertaken by Dr. Tomoko Kamishima using cells isolated by myself.

1 Introduction

1.1 The Vasculature

The vasculature in mammals is organised into three main types of blood vessel; arteries, capillaries and veins. The function of arteries is to deliver blood to organs in the body, and they fall into two general categories based on the size of their relaxed lumen. Conduit arteries possess a relaxed luminal diameter of >400μm and are responsible for absorption of pressure pulses produced by the pumping of the heart muscle (O'Rourke 1995). These arteries have a low ratio of wall thickness:diameter with large amounts of elastin and collagen and moderate amounts of vascular smooth muscle (Shadwick 1999). Resistance arteries possess a resting diameter ranging between 400μm and ~100μm (Intengan & Schiffrin 2000; Schiffrin 1992). These arteries have a higher ratio of wall thickness:diameter and contain large amounts of muscle with lower levels of elastin and collagen compared to conduit arteries. Resistance arteries are the primary site of vascular resistance since contraction and relaxation of vascular smooth muscle within these vessels regulates lumen diameter and controls blood flow and distribution between and within tissues. As blood pressure is proportional to cardiac output and peripheral vascular resistance, the resistance produced by vascular smooth muscle contraction controls blood pressure (Intengan & Schiffrin 2000). Resistance is determined using Poiseuille's law, including parameters such as vessel length, vessel radius and blood viscosity (Sutera & Skalak 1993).

Blood Pressure ∝ Cardiac Output x Total Peripheral Resistance

After blood passes through large resistance arteries responsible for delivering blood to organs it drains into arterioles which, together with venules, control intracapillary pressure to keep capillaries open or closed (Gardner & Fox 2001). Capillaries are endothelial tubes with no nerve innervations and are key in allowing the movement of oxygen, nutrients and hormones to tissues via the extracellular fluid. After flowing through capillaries and then venules, blood enters veins which possess little muscle and are thought of as blood reservoirs. Veins, unlike arteries and capillaries, possess valves which inhibit retrograde flow of blood.

1.1.1 Smooth Muscle Tone

Vascular smooth muscle is categorised as tonic smooth muscle, in that it has properties of slow and sustained contraction that may be maintained with minimal energy (Khalil 2010; Baker et al. 2003). This is compared to phasic smooth muscle, such as gastrointestinal and uterine smooth muscle, which produce phasic contractions and relaxations (Fisher 2010). The level of contraction experienced by an artery relative to its fully dilated state (referred to as vascular tone) is determined by the balance of vasodilating and vasoconstricting influences.

As with other muscle, the contractile apparatus is made up of myosin motor proteins that induce contraction by moving along a framework of actin filaments. Unlike skeletal and cardiac muscle, there is no regular, striated arrangement of actin and myosin (hence the name ‘smooth’ muscle), and actin and myosin bundles are found throughout the sarcoplasm. These bundles are fastened to the cell membrane by adhesion densities and anchored within the cytoplasm by focal densities (Young 2006). Smooth muscle cells also do not

possess the Ca^{2+}-binding protein troponin that regulates contraction in striated muscle. Contraction is still triggered by elevation of intracellular Ca^{2+} (see **Section 1.2**), but Ca^{2+} instead binds to calmodulin. The Ca^{2+}-calmodulin complex then activates myosin light chain kinase (MLCK), which in turn phosphorylates the myosin heads, initiating interaction with actin.

1.2 Mechanism of Contraction

A rise in intracellular Ca^{2+} is one of the two methods of contraction in vascular smooth muscle, the other being **Ca^{2+} sensitization** (discussed below). The Ca^{2+} required for activation of MLCK is produced either through intracellular store Ca^{2+} release or through influx of extracellular Ca^{2+} via membrane Ca^{2+} channels, such as voltage-dependent Ca^{2+} channels (VDCCs), store-operated Ca^{2+} channels (SOCCs), receptor-operated Ca^{2+} channels (ROCCs) and stretch-activated Ca^{2+} channels (SACCs).

This rise in intracellular Ca^{2+} concentration, either via influx or store release, is predominately mediated by activation of cell-surface receptors coupled to G_q-type, heterotrimeric guanine nucleotide binding proteins (G proteins) (Harnett & Biancani 2003). The α subunit of G_q is responsible for activation of phospholipase C (PLC); it hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These two signalling molecules are responsible for protein kinase C (PKC) mediated Ca^{2+} entry and IP_3-mediated store Ca^{2+} release, respectively (Khalil 2010).

Once activated by the binding of Ca^{2+}-calmodulin, MLCK phosphorylates myosin heads at position 19, producing a conformational change. This allows myosin heads and actin to cycle through crossbridging, due to an increase in

actin-activated myosin MgATPase activity (Somlyo & Somlyo 1994; Gallagher et al. 1991) . Removal of this position 19 phosphorylation by myosin light chain phosphatase (MLCP) inhibits contraction (**Figure 1.1**).

Alternative mechanisms of contraction exist that do not rely on an increase in intracellular Ca^{2+} and one of these is termed 'Ca^{2+} sensitization'. Ca^{2+} sensitization involves the modulation of MLCP through the downstream kinase of Rho-A, Rho-A-associated protein kinase (ROCK). MLCP contains three distinct subunits, MYPT1, PP1c and M20 (Grassie et al. 2012). ROCK phosphorylates the MYPT1 subunit of MLCP at positions, T696 and T853, which inhibits MLCP thereby reducing the amount of dephosphorylation of myosin (i.e. inhibits MLCP leading to contraction). ROCK is not the only kinase to mediate Ca^{2+} sensitization. PKC phosphorylates PKC potentiated inhibitor 17 kDa protein (CPI-17), which also decreases the activity of MLCP and is therefore procontractile (Somlyo & Somlyo 2003; Khalil 2010).

A third mechanism of contraction has also been explored in the vasculature, involving the protein family of heat shock proteins. HSP27 has been shown to interact with tropomyosin and caldesmon (part of the thin filament) by addition of a vasoconstrictive agonist (Somara & Bitar 2004). In colonic smooth muscle, tropomyosin involvement in contraction has been shown. Phospho-HSP27 (induced by acetylcholine) causes a sliding of tropomyosin, allowing myosin to bind actin (Somara & Bitar 2004). This dissociation of tropomyosin with actin is dependent on phosphorylated caldesmon (Somara & Bitar 2004; Bitar et al. 1991; Somara & Bitar 2010).

Therefore, in essence, vascular smooth muscle contraction is produced through two main mechanism: 1) increase in Ca^{2+} and 2) increase in Ca^{2+}

sensitivity. A number of endogenous hormones and signalling molecules which produce contraction are discussed below.

1.2.1 Endogenous vasoconstrictors

Table 1.1- Summary of common endogenous vasoconstrictors including category/structure, target receptors, release site and mechanism of action.

Vasoconstrictor	Receptor	Structure	Release Site	Mechanism of Action	References
Adrenaline	$\alpha 1$	Catecholamine	Nerves	PLC/IP_3/DAG	(Wu et al. 1992)
Angiotensin II	AT_1	Peptide	Blood Stream	PLC/IP_3/DAG Intracellular alkalisation Increased $[Na]_I$ and decreased $[Mg^{2+}]$ inside the cell	(Berk et al. 1987; Griendling et al. 1994; Griendling et al. 1997; Touyz & Berry 2002; Wynne et al. 2009) In depth-review (Touyz & Schiffrin 2000)
ATP	P2X/Y	Nucleotide	Paracrine/ Blood Stream	Increase Ca^{2+} via P2X receptor VDCC/Ca^{2+}-activated Cl^- channel via P2Y	(Mitchell et al. 2012; Liu et al. 1989)
Endothelin	ET_A	Peptide	Endothelium	PLC/IP_3/DAG ROCK	(Schneider et al. 2007; Kedzierski & Yanagisawa 2001; Inoue et al. 1989; Ivey et al. 2008; Klöckner & Isenberg 1991)
Neuropeptide Y	NYP Y_1	Peptide	Nerves	Potentiate adrenaline contraction (Na^+ influx/Ca^{2+} mobilization/PLC activity) Adrenaline independent contraction via Ca^{2+} channels	(Franco-Cereceda & Liska 1998; Prieto et al. 2000; Wahlestedt et al. 1985)
Thromboxane A2		Hormone	Platelets/ Endothelium	G_q/Ca^{2+} influx/Ca^{2+} sensitivity (caudal; not PKC) PKC/ROCK/Ca^{2+} mobilization (coronary)	(Wilson et al. 2005; Grann et al. 2016; Ding & Murray 2005; Nobe & Paul 2001; Leung et al. 2010)
Vasopressin	V1	Hormone	Blood Stream (produced in posterior pituitary gland)	>μM PLC/IP_3/DAG <μM PKC/VDCC	(Henderson & Byron 2007; Nemenoff 1998)

Summarised in **Figure 1.**

1.2.2 Stretch/Pressure

Stretch activated channels (SACs) exist in vascular smooth muscle cells. They are activated by elongation of the vascular smooth muscle i.e. stretching the membrane. Stretch is associated with increases in an inward cation current which produces depolarization of cells increasing VDCC mediated Ca^{2+} entry (Zou et al. 2002). SACs themselves conduct Ca^{2+} with these ions activating RyR causing Ca^{2+} induced Ca^{2+} release (Kirber et al. 2000; Gilbert et al. 2014). Also activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels hyperpolarize the cell membrane and amplify the Ca^{2+} entry through SACs due to an increased electrochemical driving force for Ca^{2+} (Zou et al. 2002; Gilbert et al. 2014).

Myogenic tone, produced by arterial pressure within an artery causing elongation of smooth muscle cells is pivotal to maintaining and controlling blood flow to organs. Specifically, it allows a baseline level of contraction which can be increased or decreased. Elegant studies by Osol *et al.* (2002) showed the development of myogenic tone at 60 mmHg in cerebral arteries (Osol et al. 2002). In this study, stepwise increments (10mmHg) from a starting pressure of 20mmHg showed an initial increase in diameter, before an increase in Ca^{2+} and depolarization took place to produce contraction and development of tone at around 60 mmHg. Increasing this pressure up to ~160mmHg does not have any further effect on blood vessel diameter i.e. contraction counters the pressure and the vessel lumen diameter does not widen (Osol et al. 2002).

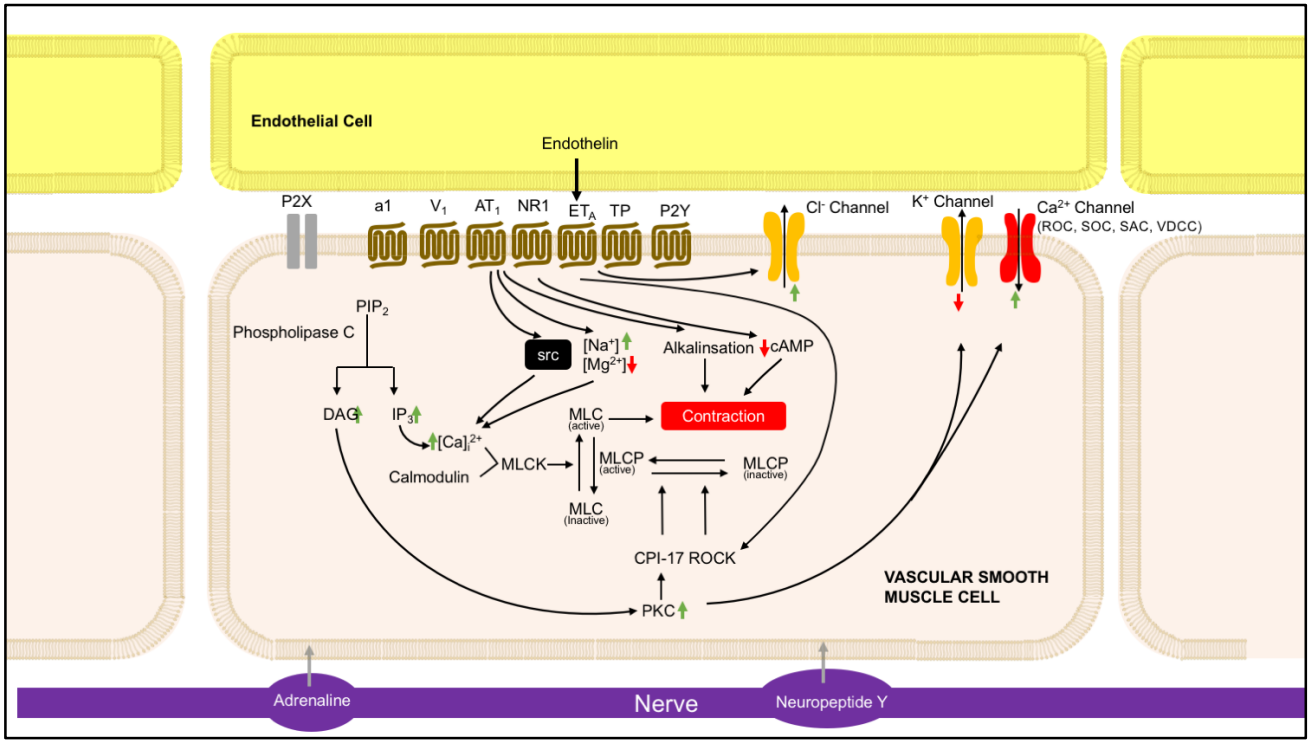


Figure 1.1- Endogenous vasoconstrictors in the vasculature. Many vasoconstrictors use GPCR and Nicotinic receptors to produce contraction in vascular smooth muscle cells. These pathways aim to increase Ca^{2+} or increase Ca^{2+} sensitivity of the contractile proteins. Many endogenous vasoconstrictors such as adrenaline, released from nerves, work by activating $G_{\alpha q}$ coupled receptors which activate phospholipase C to produce IP_3 and DAG, both capable of producing vasoconstriction. The endothelium also produces procontractile peptides such as Endothelin which works via GCPRs on smooth muscle cells. More complex vasocontractile pathways are present such as alkalinisation, decreasing cAMP and Mg^{2+} regulation.

1.3 Mechanisms of Vasodilation

Relaxation of vascular smooth muscle i.e. decreasing vascular tone, is the opposite to contraction in that pathways aim to reduce available cytoplasmic [Ca^{2+}] and decrease Ca^{2+} sensitivity of the contractile apparatus. Reduction of available cytoplasmic [Ca^{2+}] can be through sequestration of Ca^{2+} to intracellular stores, such as the sarcoplasmic reticulum, Ca^{2+} extrusion via plasma membrane Ca^{2+} ATPase or decreasing voltage-dependent Ca^{2+} entry (VDCC) (Khalil 2010). Inhibition of VDCC can be brought about by hyperpolarisation of the membrane, through the activation of outward K^+ currents (Somlyo & Somlyo 1994). Receptor operated channels (ROCs) and store operated channels (SOCs) can be inhibited by removal of the stimulus i.e. receptor activation or recovery of Ca^{2+} load, respectively. Reduction of available Ca^{2+} removes the activation of MLCK produced through Ca^{2+}/CaM and therefore limits contraction (Somlyo & Somlyo 2003). The other route of relaxation is that of decreasing Ca^{2+} sensitivity. Vasodilating hormones can produce an increase in activity of MLCP, therefore increasing the dephosphorylation of myosin light chain and limiting contraction (Somlyo & Somlyo 2003). Most of these mechanisms, which aim at reducing Ca^{2+} availability or Ca^{2+} sensitivity, are initiated by phosphorylation events mediated by cAMP-dependent protein kinase (protein kinase A, PKA). PKA is activated by 3'-5'-cyclic adenosine monophosphate (cAMP), a ubiquitous second messenger produced by the enzyme adenylyl cyclase following activation of G_s-coupled cell-surface receptors (Canaves & Taylor 2002; Taylor et al. 2004; Kim et al. 2005; Walsh et al. 1968; Rall et al. 1956). Phosphorylation targets of PKA in vasodilation, include ion channels, MLCP and heat shock proteins.

Indeed, many endogenous vasodilators responsible for maintaining the vascular tone of arteries work through PKA mediated signalling pathways and are summarised in **Section 1.3.1 and Table 1.2.** A number of endogenous vasodilators also utilise cGMP pathways, activating PKG and producing vasodilation through downstream phosphorylation of PKG targets. This is a common pathway by which nitric oxide produces relaxation, through activation of guanylyl cyclase increasing cGMP in smooth muscle cells.

1.3.1 Endogenous Vasodilator

Table 1.2- Endogenous vasodilators summarising target receptor, type of structure, release site and mechanism of action.

Vasorelaxant	Receptor	Structure	Release Site	Mechanism of Action	References
Adenosine	$A_{1/2}$	Hormone	Cells	cAMP/PKA (AT_2) Decrease cAMP (AT_1) cAMP/Ca^{2+}/NO	(Kleppisch & Nelson 1995; Dart & Standen 1993; K. T. Cheng et al. 2008; Karczewski et al. 1998)
Adrenaline	β_2	Catecholamine	Nerves	cAMP/PKA in VSMC cAMP independent signalling cAMP/PKA/eNOS/NO in endothelium	(Butt et al. 2000; Ferro et al. 2004; Queen & Ferro 2006; Tanaka et al. 2005)
ATP	Endothelial P2Y	Nucleotide	Platelets/ Cells	Ca^{2+}/NO cAMP?	(Mitchell et al. 2012; Shah et al. 2001; Kaiser et al. 2002)
Calcitonin-gene related peptide	$CGRP_1$	Peptide	Peptide	cAMP/PKA in VSMC cAMP/PKA/eNOS/NO in endothelium	(Y Hayabuchi, Dart, et al. 2001; Brain & Grant 2004)
Endothelin	Endothelial ET_B	Peptide	Endotheliu m	NO production	(Schneider et al. 2007; Kedzierski & Yanagisawa 2001; Filep et al. 1993; Pollock 2001; Kisanuki et al. 2010)
Vasoactive intestinal polypeptide	VDAC1/2	Peptide	Nerves	cAMP/PKA NO	(Yang et al. 2008; Ergün & Oğülener 2001; Henning & Sawmiller 2001; Severi et al. 2006; D'Amato et al. 1992)
Endothelial Derived Relaxant Factors (Reviewed *Edwards 2010* (Edwards et al. 2010))					
Nitric Oxide (EDRF)	Guanylyl Cyclase (GC)	N/A	Endotheli um	NO released by endothelial cell acts on GC activating PKG	(Murad et al. 1978; Palmer et al. 1987; Furchgott & Zawadzki 1980)
Endothelial derived hyperpolarisng factor (EDHF)	N/A	N/A		Extracellular K^+ increase leads to hyperpolarization via Na^+/K^+ exchanger and K_{ir} family. Spread of hyperpolarization from endothelial cells to VSMC via gap junctions.	(Edwards et al. 1998; Richards et al. 2001) (Garland et al. 2011; Yamamoto et al. 1999; Sonkusare et al. 2012)
11,12 epoxyeico-satrienoic acid (EET)	TRPV4?	Acid		Activate TRPV4 increase Ca^{2+} sparks	(Filosa et al. 2013; Earley et al. 2005; Earley et al. 2009)
Prostacyclin	IP receptors			cAMP/PKA	(Karczewski et al. 1998; Parkington et al. 2004; Edwards et al. 2010)

1.3.2 Ca^{2+} Sparks

Within smooth muscle cells an important caveat exists in regards to increases in Ca^{2+} producing contraction, whereby local releases of Ca^{2+} counter intuitively elicit relaxation (Nelson et al. 1995). These local releases of Ca^{2+} are termed "Ca^{2+} sparks" and can be up to 10μM in concentration (Jaggar et al. 2000; Nelson et al. 1995; Zhuge et al. 2002). In smooth muscle Ca^{2+} influx through VDCCs leads to an increase in Ca^{2+} store load, increasing the frequency of Ca^{2+} sparks (Essin et al. 2007; Collier et al. 2000; ZhuGe et al. 1999). These Ca^{2+} sparks are produced by Ca^{2+} release from ryanodine-sensitive Ca^{2+} release channels (RyRs) located on the sarcoplasmic reticulum (Vaithianathan et al. 2010). Ryanodine receptors are named due to their inhibition by the plant alkaloid ryanodine. Ca^{2+} sparks produce both contractile and relaxant effects by activating Ca^{2+} activated Cl^- channels or Ca^{2+} activated K^+ channels (BK_{Ca}) , respectively (ZhuGe et al. 1998). These activations of BK_{Ca} channels are termed spontaneous transient outward currents, or STOCs (Jaggar et al. 1998). Importantly, these STOCs carrying a large outward current of K^+, which leads to a hyperpolarisation of the cell membrane and a decrease in Ca^{2+} entry via VDCCs, lowering global [Ca^{2+}], leading to vasorelaxation. Indeed inhibition of RyRs, with ryanodine, to pressurised arteries causes vasoconstriction showing the importance of STOCs in vascular tone regulation (Nelson et al. 1995). Ca^{2+} sparks and with them STOCs can be modulated by cAMP and cAMP-raising endogenous agonists, such as adenosine (Porter et al. 1998). STOC frequency is also increased by depolarisation, induced by elevations in pressure (Jaggar 2001).

1.3.3 Shear stress induced vasorelaxation

Under shear stress, produced by the dragging force generated by blood flow, numerous pathways are involved in flow-induced vasodilation including the integrin pathway, which produces NO by PKA mediated or Akt mediated phosphorylation of eNOS (Boo et al. 2002; Dimmeler et al. 1999), prostacyclin release via cyclooxygenase (COX) or inhibition of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Lu & Kassab 2011). The ion channel TRPV4 has also been shown to be flow sensitive; activation increases both the classical NO dependent vasodilation and the novel endothelial derived-hyperpolarising factor (EDHF) pathway (Filosa et al. 2013; Sonkusare et al. 2012; Mendoza et al. 2010).

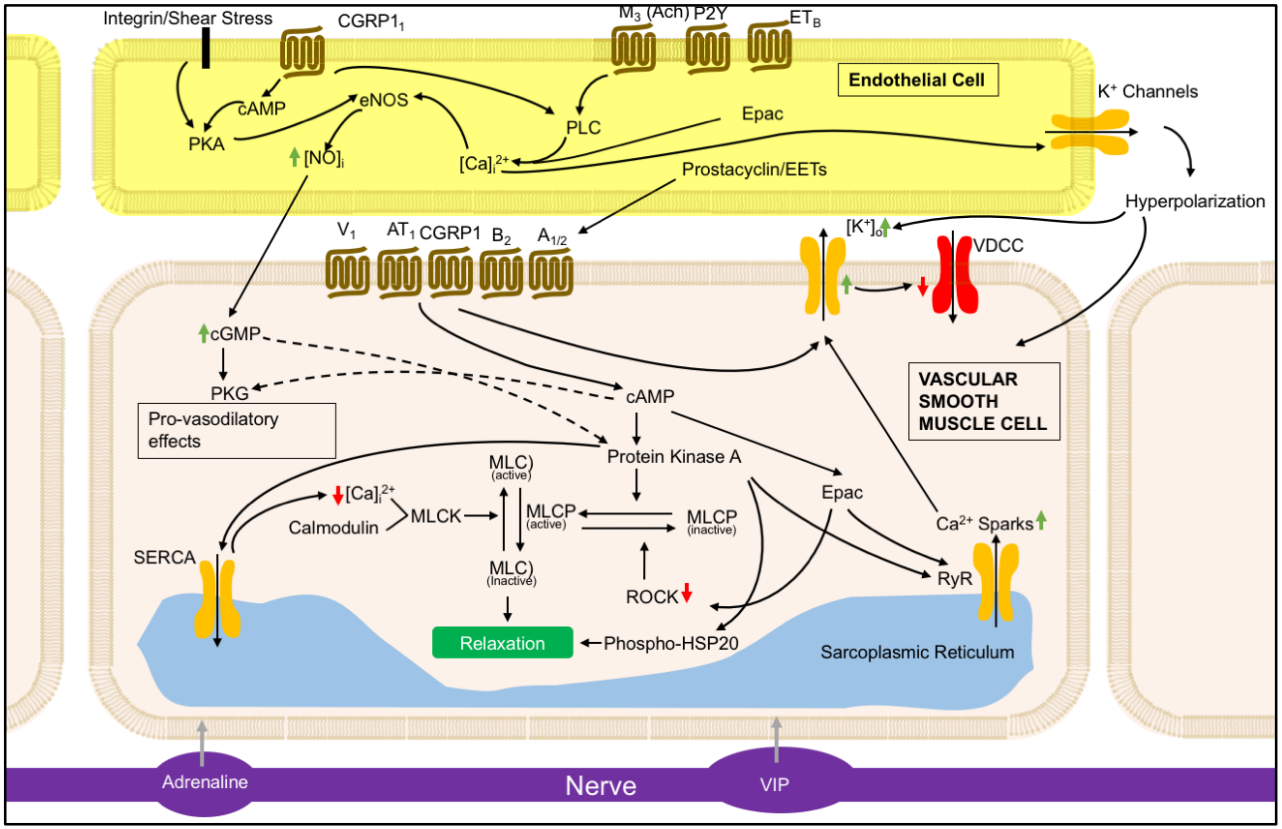


Figure 1.2- Endogenous Vasodilators. Numerous vasodilators use cAMP to activate PKA and cause relaxation, including Gs coupled GPCRs such as β adrenoceptors and calcitonin gene-related peptide receptor. cAMP can also activate PKG as depicted by the dotted line, as can cGMP activate PKA. Many vasodilators use the endothelium as a source of Nitric Oxide to produce relaxation, such as muscarinic 3 receptors which increase Ca^{2+} in the endothelial cell and activates eNOS to produce NO. This NO can then increase cGMP and activate PKG or PKA (cross over annotated using dotted line). The rise in $[Ca^{2+}]$ in endothelial cells also activates Ca^{2+} activated K^+ channels causing a hyperpolarisation of the cell which spreads to VSMC through gap junctions. The K^+ "cloud" from such channels also causes hyperpolarisation through increasing the outward current K^+ current of Inward-rectifying K^+ channels such as $K_{ir}2$

1.4 cAMP Signalling Overview

A common and generally conserved feature of vasorelaxant pathways is the use of the second messenger cAMP to convey the relaxant signal from cell-surface receptors to downstream effectors **(Table 1.2 and Figure 1.2).**

1.4.1 History

cAMP is a small, diffusible nucleotide first identified as an intracellular second messenger by Sutherland and Rall (Rall et al. 1956) in 1956, who suggested that a membrane-bound receptor must mediate the activation of liver phosphorylase in the cytoplasm by a heat stable factor. This was demonstrated by separating membrane and cytoplasmic fractions of the liver and treating the membrane fraction with adrenaline. Membranes were then removed by centrifugation and the soluble supernatant was added to the original cytoplasmic fraction. Addition of this supernatant increased phosphorylation of liver phosphorylase and gave the first indication that a soluble heat resistant messenger molecule was present (Rall et al. 1956). Later, research showed that this second messenger was cAMP and was produced from heart, liver and brain particulate fractions (Sutherland & Rall 1958). Further research by Sutherland and Rall identified an enzyme (phosphodiesterase) that, particularly in brain homogenates, converted cAMP to 5'-AMP. These phosphodiesterases were inhibited by caffeine and dependent on Mg^{2+} (Sutherland & Rall 1958). Since its original discovery, the production (by adenylyl cyclases) and destruction (by phosphodiesterases) of cAMP has been extensively explored.

1.4.2 Adenylyl Cyclases

Activation of G_s coupled receptors present in the membrane causes GTP recruitment and dissociation of the heterotrimeric G protein (α,β and γ subunits). $G_{\alpha s}$ produces an increase in cAMP through activation of adenylyl cyclase, which produces cAMP by converting ATP in the presence of Mg^{2+} into cAMP and pyrophosphate (Garrett & Grisham 2012). Ten isoenzymes of adenylyl cyclase are known, 9 membrane bound and a single soluble isoform (sAC) (Hanoune & Defer 2001). Adenylyl cyclase isoenzymes are each regulated differently, for instance certain isoenzymes are inhibited by Ca^{2+} (AC5/6), whereas AC3 is inhibited by calcium/calmodulin-dependent protein kinase II (CaMKII) (Hayn et al. 2010; Hanoune & Defer 2001) (**Table 1.3)**.

Isoform	Activators	Inhibitors
1	$G_{\alpha s,}$ Fsk, Ca^{2+}, PKC	$G_{\alpha i}$
2	$G_{\alpha s}$, Fsk, PKC	Ca^{2+}, CaMKIV
3	$G_{\alpha s}$, Fsk, Ca^{2+}(in vitro), PKC	$G_{\alpha i}$, CaMKII
4	$G_{\alpha s}$, Fsk, PKC	
5	$G_{\alpha s}$, Fsk, PKC	$G_{\alpha i}$, Ca^{2+}, PKA
6	$G_{\alpha s}$, Fsk	$G_{\alpha i}$, Ca^{2+}, PKC, PKA
7	$G_{\alpha s}$, Fsk, PKC	
8	$G_{\alpha s}$, Fsk, Ca^{2+}	$G_{\alpha i}$
9	$G_{\alpha s}$, Fsk (weak)	$G_{\alpha l}$, Calcineruin,
Soluble	Mn^{2+} , Ca^{2+}, HCO_3^- (Tresguerres et al. 2011)	

Table 1.3- Adenylyl Cyclase regulation. Unless stated, data was taken from *Hanoune and Defer (2001)* (Hanoune & Defer 2001)*. Fsk denotes Forskolin a commonly used adenylyl cyclase activator.*

Vascular smooth muscle expresses isoforms 3,5 and 6 as assessed by mRNA analysis and immunoblotting (Ostrom et al. 2002; Nelson et al. 2011); AC7 expression has only been shown by mRNA analysis from freshly isolated aortic smooth muscle, with no confirmed protein expression available (Nelson et al. 2011). However, in the same study knockdown of AC3/5/6 inhibited cAMP accumulation by 90% suggesting only a minor role for AC7. Importantly, AC expression changes in culture, with AC2/8 mRNA detected in cells passaged 3-6 (Nelson et al. 2011; Ostrom et al. 2002). Overexpression of AC6 in rat aortic smooth muscle cells enhances both β_1 and β_2 adrenoceptor-mediated cAMP production, but did not affect basal cAMP (Ostrom et al. 2002). Furthermore, AC6 proved predominately responsible for the increase in K_{ATP} current induced by isoprenaline in freshly isolated aortic smooth muscle cells, with AC6 knockdown attenuating the evoked current (Nelson et al. 2011). AC3 knockdown also produced a modest reduction in K_{ATP} currents. Surprisingly, knockdown of AC5, which shares a high homology to AC6, produced no effect on the K_{ATP} current.

In pulmonary arterial smooth muscle cells AC 2/5/6 are the predominant isoenzymes, with AC3 expressed at lower levels (Jourdan et al. 2001).

1.4.3 Phosphodiesterases

Cyclic nucleotide phosphodiesterases are a family of enzymes that degrade the phosphodiester bond in cAMP and cGMP. Twenty-one genes encode the 11 known mammalian PDE families, with alternative mRNA splicing producing multiple variants (Maurice et al. 2014). This produces a highly complex situation with almost 100 different PDE isozymes possible. These isoforms collectively produce a high level of control on cAMP, with small areas

containing a high concentration of cAMP, known as a cAMP “hotspots” (Maurice et al. 2014) **(Figure 1.3)**.

PDEs fall into three general groups based on their substrate specificity: PDEs 4, 7 and 8 selectively hydrolyse cAMP; PDEs 5, 6 and 9 are cGMP-specific; and PDEs1-3,10 and 11 degrade both cAMP and cGMP (Maurice et al. 2014). PDE 1, 3, 4 and 5 are expressed in vascular myocytes (Komas, Lugnier, Andriantsitohaina, et al. 1991; Polson & Strada 1996) . PDE 2, 3 ,4 and 5 have been shown to be expressed in endothelium cells (Netherton & Maurice 2005).

PDE 1 has been shown to be involved in vascular remodelling, where they have a role in the proliferative phenotypic smooth muscle cells i.e. dividing and growing. PDE 1 is also involved in vascular tone regulation in the contractile phenotype i.e. no growth, force generating phenotype. PDE1’s localisation is different in contractile and proliferative VSMCs, cytoplasmic in contractile and nuclear in proliferative cells. The role of PDE1A in contractile smooth muscle is to regulate myosin light chain kinase phosphorylation (Nagel et al. 2006). However, in proliferative VSMCs, PDE1A is involved in the cell cycle, whereby inhibition of PDE1 (total family inhibition) or knock down (PDE1A specific) decreases cell growth (Nagel et al. 2006). PDE1C expression levels increase after the phenotypic change from contractile to proliferative and this limits cAMP-mediated inhibition of proliferation (Rybalkin et al. 2002). Disease involvement of PDE1 in pulmonary hypertension has been shown whereby there is over 250-fold increase in PDE1C mRNA from idiopathic pulmonary artery hypertension patients pulmonary myocytes compared to control. Upon knockdown the disease associated increase in proliferation of vascular smooth muscle is decreased (Murray et al. 2007). PDE1A and 1C do not produce their

increase in cell proliferation via the same pathways, further showing compartmentalisation and the complexity of cAMP signalling (Nagel et al. 2006; Growth et al. 2015; Chan & Yan 2011).

PDE3, also known as cGMP-inhibited phosphodiesterase, is a low K_m (i.e. high affinity) cAMP hydrolysing PDE shown to have an involvement in vascular tone. Inhibition of PDE3 in a number of vascular beds relaxes arteries by inducing leftward shift in the dose response curve to β-adrenergic agonists, such as isoprenaline (Lindgren et al. 1990). These responses are not dependent on the presence of a working endothelium due to PDE3 expression in vascular myocytes (Komas, Lugnier & Stoclet 1991). PDE3 inhibition was not the same in different vascular beds, with milrinone (PDE3 inhibitor) producing a more potent relaxation in coronary than cerebral or renal arteries (Polson & Strada 1996). PDE3 inhibition has been assessed as a therapeutic target in heart failure, however, chronic administration of milrinone (Primacor) and amrinone (imamrinone, Inocor) leads to increased mortality through an increase in the incidence of arrhythmias and sudden cardiac death (Maurice et al. 2014). A PDE3 inhibitor, cilostazol (Pletal; Otsuka Pharmaceutical), is licensed for the treatment of intermittent claudication, an arterial disease characterized by ischaemia-induced leg pain, and works by decreasing platelet aggregation and directly stimulating arterial vasodilation (De Backer et al. 2010).

PDE4s are expressed in arteries, however inhibition of PDE4 in arteries denuded of endothelial cells produces modest relaxation, whereas the presence of an intact endothelium significantly increases this response. PDE4 inhibition caused leftward shifts in relaxation curves to known cAMP

dependent vasodilators e.g. Isoprenaline (Maurice et al. 1991; Schoeffter et al. 1987). Precontraction with different contractile agonists affected the response to PDE inhibition, isoproterenol relaxation sensitive to PDE inhibition in Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) pre-contracted preparations, but not sensitive to guanfacine pre-contracted preparations (Lindgren et al. 1990). The need for a working endothelium in PDE4 inhibition induced relaxation poses the questions to its involvement in healthy vessels; However, increased cGMP produced by NO releasing endothelium may inhibit PDE3, "pushing" PDE4 into the major cAMP hydrolysing role. This was supported by experiments where, after PDE3 inhibition, non-denuded preparations were significantly more sensitive to PDE4 inhibition (Lugnier & Komas 1993) (**Figure 1.3)**.

PDE5 is a cGMP sensitive PDE, and the therapeutic target of sildenafil (Viagra, Pfizer) a drug used for erectile dysfunction. PDE5 inhibition in aorta preparations initially produced contradictory reports, however this was probably due to poor selectivity (Polson & Strada 1996). Saeki *et al.* (1995) produced a highly specific potent inhibitor of PDE5 and showed it produced relaxation of porcine coronary artery, which was dependent on endothelium and nitric oxide (Saeki et al. 1995). PDE5 inhibition potentiated nitroglycerin (NO donor) mediated relaxations in porcine arteries, while L-NAME inhibited PDE5 inhibition mediated relaxations. This relaxation was as a result of a rise in cGMP not cAMP (Saeki et al. 1995). Targeting PDE5 inhibition and the NO-cGMP pathway may be a possible treatment in stroke, Raynaud's phenomenon, ventilation/perfusion mismatch and heart failure (Ghofrani et al. 2006).

Phillips *et al.* (2005) reported the presence of PDE 8/9 mRNA in aortic and renal artery smooth muscle cells but not in coronary artery smooth muscle cells. PDE7 was also present in all vascular smooth muscle cells studied. Importantly, these vascular smooth muscle cells in Phillips *et al.* were in culture (Passage 2) and therefore the transition of contractile to synthetic may produce a different PDE isoform expression (Phillips et al. 2005).

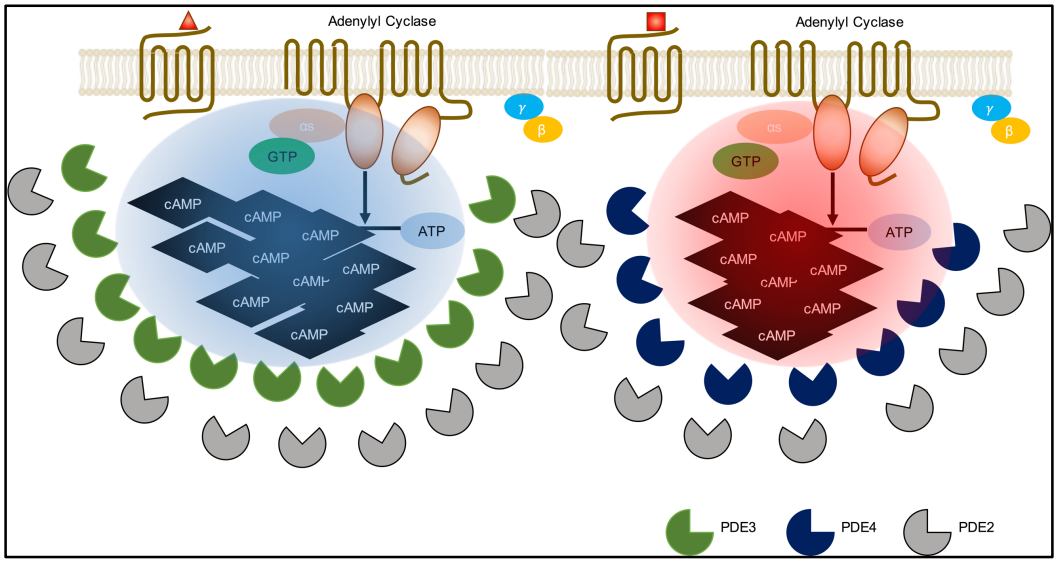


Figure 1.3- Phosphodiesterase control of cAMP hotspots in the vasculature. Within certain cells high concentrations of localised cAMP (hotspots) exist. These hotspots are maintained by the presence of localised phosphodiesterases, along with the production of cAMP from adenylyl cyclases. PDE3 and PDE4 are the main PDE's present in the vasculature responsible for maintaining cAMP hotspots.

1.5 cAMP Effectors

cAMP has four known effector proteins: protein kinase A (PKA); cyclic nucleotide-gated (CNG) channels, exchange protein directly activated by cAMP (Epac) and PDE10, the cAMP-inhibited phosphodiesterase **(Figure 1.4)**. Below three of the effectors are discussed including their structural properties. PDE10 is not explored due to the lack of expression in vascular preparations (Phillips et al. 2005; de Rooij et al. 1998; Kawasaki et al. 1998; Walsh et al. 1968).

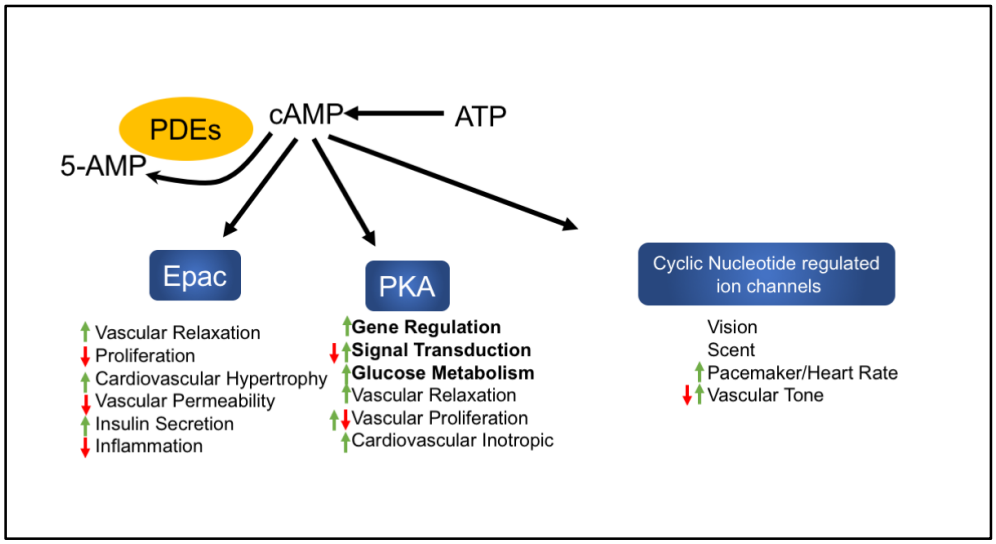


Figure 1.4- cAMP Effectors and their downstream responses. cAMP has 3 main effectors in the vasculature, Epac, PKA and CNGCs. Epac mediates a number of signalling pathways including vascular relaxation, insulin secretion and inflammation. PKA mediates pathways including gene regulation, glucose metabolism and vascular tone. CNGCs have a role within the heart and vascular tone.

1.5.1 Protein Kinase A

PKA was first discovered in 1968 by Walsh, Perkins and Krebs (Walsh et al. 1968). Their discovery that a cAMP-dependent kinase phosphorylated casein and protamine has led the way for kinase research over 50 years with PKA still thought of as the original and simplest kinase (Walsh et al. 1968).

1.5.1.1 *Structure and Activation of Protein Kinase A*

Protein Kinase A is a tetramer in its inactive state, with two regulatory subunits (RI or RII, alpha or beta) forming a complex with two PKA catalytic subunits (Kim et al. 2005; Taylor et al. 2012; Zhang et al. 2012). PKA regulatory subunit, as the name states, regulates the catalytic region by an inhibitory domain towards the N-terminus (Taylor et al. 2012).

The regulatory subunits of PKA were originally named I (1) and II (2) after the order in which they elute from ion exchange chromatography. Further isoforms, utilising the names α and β, were discovered due to their different

mobility on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In total there are four genes encoding PKA regulatory subunit isoforms (Canaves & Taylor 2002; Taylor et al. 2012). At the N-terminus of the regulatory subunits is the dimerization domain responsible for the interaction of the two regulatory subunits with one another. This dimerization is produced by the anti-parallel arrangement of the two regulatory subunits. This regulatory subunit dimer binds two catalytic subunits to form the tetramer. Each regulatory subunit possesses two cAMP binding domains named A and B in the carboxyl-terminus. In the inactive state only the cAMP binding domain B is accessible to cAMP, and it is after this initial binding of cAMP that the cAMP binding domain A is accessible for cAMP binding. cAMP binding to PKA RI at only site B *in vitro* has a reported K_d 0.9 nM. However, Dao *et al.* (2006) showed that cAMP affinity is dramatically affected when PKA is in its holoenzyme state (K_d 2.9μM) (Dao et al. 2006). PKA Type II holoenzymes (RIIα_2:C_2 and RIIβ_2:C_2) have an activation constant of 200-400 nM cAMP, compared to 50-100 nM cAMP for PKA Type I holoenzymes (RIα_2:C_2 and RIβ_2:C_2) (Taylor et al. 2012; Zhang et al. 2012). The original text book understanding of PKA regulation was that, upon binding of 4 cAMP molecules to the regulatory dimer, a conformation change occurs that results in the release of the two catalytic domains held in an inactive conformation with the regulatory subunits under basal conditions. These catalytic subunits then phosphorylate serine and threonine residues on target proteins (Guo et al. 2012; Rust & Thompson 2011) **(Figure 1.5)**. However, in recent studies it has been shown both *in vivo* and *in vitro* that at physiological cAMP concentrations, regulatory and catalytic subunits remain intact (Smith et al. 2017).

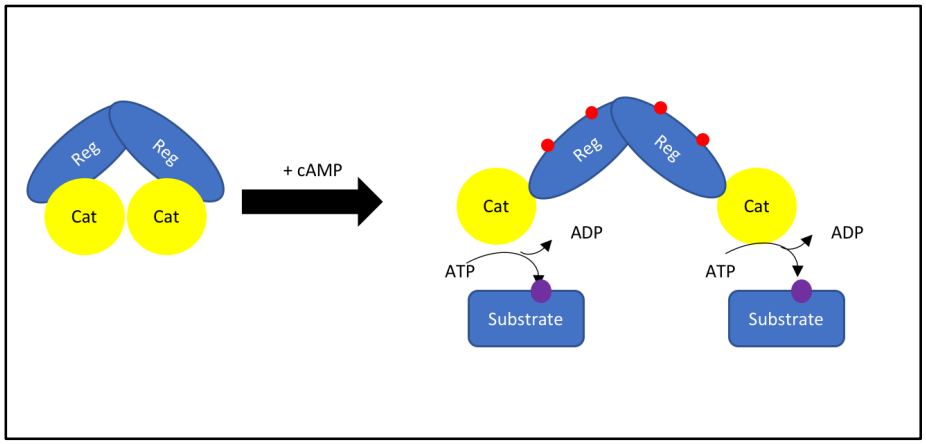


Figure 1.5- Regulation and Activation of Protein Kinase A. PKA RII subunits inhibit PKA catalytic subunits under basal conditions. Upon cAMP binding the PKA RII subunit, inhibition is removed, with PKA catalytic subunits able to phosphorylate downstream substrates.

1.5.2 Cyclic Nucleotide gated ion channels

Cyclic nucleotide gated channels (CNGCs) are Ca^{2+}-permeable, non-selective cation channels expressed in various tissues, including smooth muscle cells and vascular endothelial cells (Cheng et al. 2003; Shen et al. 2008; Yao et al. 1999). CNGCs have six isoforms, CNGA1 to 4 and CNGB1 and 3; CNGA1/2 being shown to possess functional effects in the vasculature (Yao et al. 1999; K.-T. Cheng et al. 2008; Biel & Michalakis 2002; Cheng et al. 2003; Shen et al. 2008). CNGA1 expression is predominately in endothelial cells in guinea pig arteries (Yao et al. 1999). CNGA2 is expressed in both smooth muscle and endothelial cells, with higher expression in the vascular smooth muscle layer (Cheng et al. 2003). CNGCs are activated by direct binding of cAMP or cGMP, with different sensitivities depending on the isoform. They do not desensitize to ligand activation (Biel & Michalakis 2002). Contrary to the thought that cyclic nucleotides cause relaxation, it has been shown that CNGs are involved in thromboxane induced contraction in rat mesenteric arteries (Leung et al. 2010). Endothelium denuded arteries were susceptible to relaxation upon

blockade with selective CNGC blockers such as *L-cis*-diltiazem in U46619 pre-contracted arteries (Leung et al. 2010).

1.5.3 Exchange Protein directly Activated by cAMP

1.5.3.1 *History of Epac*

Exchange protein directly activated by cAMP (Epac) was discovered simultaneously by two laboratories in 1998. The laboratory of Johannas Bos, discovered Epac based on the PKA-independent Rap1 activation (de Rooij et al. 1998). Activation in response to forskolin showed no difference between normal Chinese hamster ovary (CHO) cell lines and CHO cell lines expressing a mutant regulatory PKA subunit that rendered PKA inactive. Furthermore, Rap1 was activated in the rat fibroblasts (Rat1 cell line) by forskolin, but not inhibited by the PKA inhibitor, H89. Epac1 mediated Rap 1 activation was not dependent on PKA activation (de Rooij et al. 1998). Epac bound cAMP to a similar level as PKA RI subunit, as shown by cAMP-binding assays. This is expected as the original search for Epac utilised a gene encompassing a cyclic nucleotide binding region and guanine exchange factors for Rap1 and Ras (de Rooij et al. 1998). Within the same month an independent laboratory presented two proteins, cAMP-GEFI and cAMP-GEFII, which directly activated Rap1 *in vitro* and bound cAMP, shown through cAMP-agarose pull downs (Kawasaki et al. 1998). Kawasaki *et al.* (1998) further explored the expression of both cAMP-GEFI and cAMP-GEFII, now known as Epac1 and Epac2, in various tissues (Kawasaki et al. 1998). Epac1 was almost ubiquitously expressed with predominant heart and kidney expression. Epac2 was shown to be predominately expressed in the brain and adrenal glands. Interestingly, they showed a difference in mRNA expression between adult and foetal human

tissues, with Epac1 and Epac2 expressed in foetal lung tissue but undetectable in adult tissue (Kawasaki 1998).

1.5.3.2 *Structure and cAMP binding*

Epac1 (99 kDa) and Epac2 (109 kDa) both possess cyclic nucleotide binding domains (CNB), a guanine exchange factor domain (CDC25-HD), Ras-exchange motif (REM) and a Ras-association domain (RA) along with a dishevelled, Egl-10, and Pleckstrin domain (DEP) (Bos 2006; Gloerich & Bos 2010). Epac1 contains a single cyclic nucleotide binding domain (CNB) termed B, with Epac2 possessing an extra CNB, termed A. The CNB-B domain is responsible for the auto-inhibition (self inhibition in the absence of cAMP) that takes place with both Epac proteins. This domain possesses a common alanine amino acid, which is crucial for the selectivity of cAMP over cGMP, as in PKA and CNGCs (Kawasaki 1998; de Rooij et al. 1998). The CNB-A domain has a lower cAMP affinity and binding does not relieve auto-inhibition i.e. cAMP binding does not produce GEF activity. In fact, binding of cAMP to CNB-A does not affect Epac2 activity, leading to the idea that this domain is redundant (de Rooij et al. 2000). However, recently an Epac2 splice variant, Epac2B, which is without CNB-A was shown to have an increased cytoplasmic distribution compared to Epac2 suggesting a possible role of CNB-A in localisation of Epac2A (Niimura et al. 2009) **(Figure 1.6)**.

The DEP domain present in Epac1 and Epac2 is responsible for the translocation of Epac to the plasma membrane upon activation. De Rooij *et al.* (1998) and Ponsioen *et al.* (2004) showed that deletion of the DEP domain caused either an increase in the amount of Epac1 in the cytoplasmic fraction after activation, assessed by immunoblotting, or a more diffuse cytoplasmic

distribution, assessed using Epac-based FRET biosensors (Ponsioen et al. 2004; de Rooij et al. 2000). There appears to be a slight difference in what effect deletion of the DEP domain has on Rap1 activation, with *de Rooij* (1998) showing a clear Rap1-GTP activation and Ponsioen *et al.* showing a clear lack of Rap1-GTP activation upon DEP deletion. The CDC25 homology domain (CDC25-HD) domain present in Epac1, Epac2 and other guanine exchange factors (GEFs) is responsible for the exchange of GDP for GTP in small Ras superfamily G-proteins (Bos et al. 2007). This CDC25-HD domain works by causing a dissociation of the nucleotide from a small GTPase such as Rap1, followed by the reuptake of a new nucleotide; with a 50x higher concentration of cellular GTP than GDP, there is a higher probability of the small G-protein (Rap1/2 in the example of Epac) binding GTP than GDP (Bos et al. 2007). The REM is almost always seen adjacent to the CDC25-HD domain in GEFs where it stabilises the GEF, allowing CDC25-HD to catalyse the exchange of nucleotides (Bos et al. 2007; Gloerich & Bos 2010). The function of the RA in Epac1 is still unclear, however Epac2 has been shown to interact with Ras-GTP through the RA domain, leading to nuclear location (Li et al. 2006).

cAMP binding to Epac1 through its CNB-B domain has an *in vitro* K_d of 2.8 μM for cAMP and an AC_{50}, the concentration needed for 50% activation, of 50 μM (Dao et al. 2006; Poppe et al. 2008). It was initially thought that Epac is a standby/back up cAMP effector to PKA due to PKA R1 subunit have an *in vitro* K_d of 1.3 nM. However, due to the R1 subunit within a holoenzyme having a K_d of 2.9 μM for cAMP, some have questioned this secondary role (Dao et al. 2006). Importantly, in terms of the AC_{50}, Epac1 has a value of 50μM whereas the PKA holoenzyme is shown to be below 1μM and therefore would be

expected to activate prior to Epac (Dao et al. 2006; Zhang et al. 2012). Different *in-vitro* protocols such as buffer composition may give rise to varying K_d/Ac_{50} values and therefore *in-vivo* data will be key to establish the order of activation and under what stimulus PKA and Epac are activated.

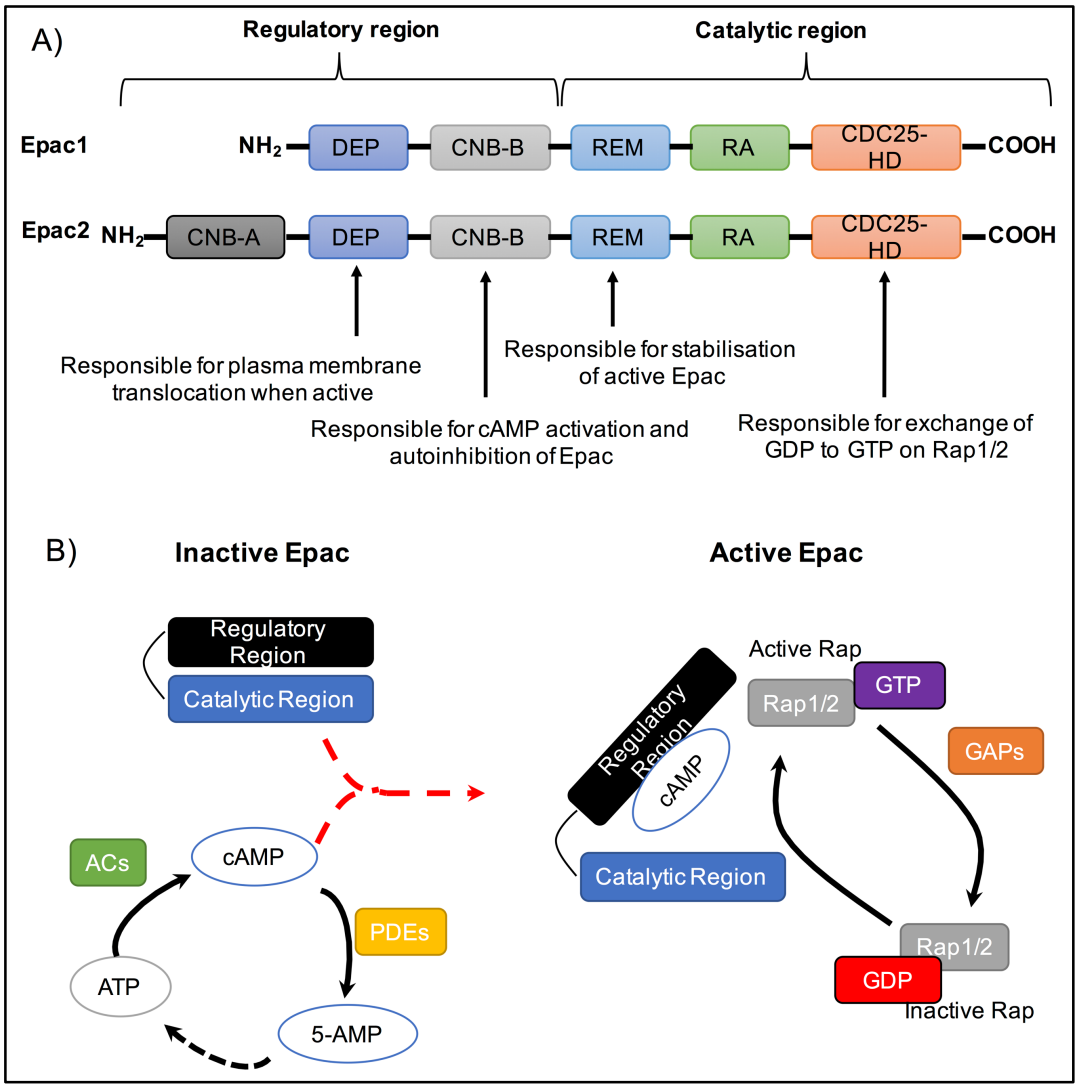


Figure 1.6- Epac1 and Epac 2 Structure and Activation: A) Epac1 and Epac2 have similar domains. Epac2 contains a CNB-A domain that binds cAMP with a low affinity. DEP domain is responsible for the translocation of Epac to the plasma membrane under activation. cAMP binds to CNB-B with a higher affinity than CNB-A of Epac2 and is responsible for the auto inhibition of Epac proteins. REM are present in all GEF's and is responsible for stabilisation of the protein to assist the exchange of GDP for GTP which is undertaken by the CDC25-HD domain. Epac activation by cAMP produces a conformational which allows Rap1 to access the CDC25-HD domain and exchange GDP for GTP. **B)** Inactive Epac in an auto-inhibited state is activated by cAMP formed from ATP by adenylyl cyclases, predominately positioned at the membrane. Upon cAMP binding, Epac "unhinges" allowing the small Ras-related G-protein Rap1 to exchange GDP to GTP and become activated. Rap1 can be deactivated by GTPase-activating proteins (GAPs), which exchange GTP for GDP and inactive the small G-protein.

1.5.3.3 *Structural basis of Epac activation*

Upon binding of cAMP to the CNB-B domain a cascade of conformational changes takes place. The first contact of cAMP is with the phosphate-binding cassette (PBC) present in the CNB domain. This interaction causes some residues to undergo a conformational change to adapt an optimal docking pose. This destabilises the C-terminal hinge-helix and allows the CNB to reach the docking position at the REM domain. The cAMP not only activates this Epac structure but stabilises the 'lid and hinge' to maintain the active conformation (Rehmann et al. 2008).

1.5.3.4 *Tool used to study Epac*

Appreciation of the potential functional importance of Epac has come with the development of cell-permeant, Epac-specific cAMP analogues. Most significant is the Epac "super agonist" 8-pCPT-2'-O-Me-cAMP (8-pCPT), which utilises a single difference between the cyclic nucleotide binding domains of Epac, compared with CNGCs and PKA **(Figure 1.7)**. A single glutamate residue found only in PKA and CNGCs interacts with the 2-OH group of cAMP and is needed for activation of these proteins (Yagura & Miller 1981; Diller et al. 2001; Enserink et al. 2002). Replacement of this 2-OH group in cAMP with 2-OCH_3 resulted in selective activation of Epac proteins, as indicated by an increased maximal activity (K_{max}) (de Rooij et al. 2000) (Epac1 and Epac2 are both activated by 8-pCPT). This analogue was modified to further improve Epac affinity, with the addition of pCPT (4-chlorophenythio) at the 8 position. Addition of an AM ester increased permeability in tissue and cells (Gloerich & Bos 2010). The AC_{50} of Epac1 by 8-pCPT *in vitro* is 1.8μM, over 25x lower than cAMP (50 μM) (Rehmann et al. 2003). 8-pCPT-AM not

only increases the half maximal concentration, but also increases the K_{max} (x3.6 compared to cAMP) (Rehmann et al. 2003). One area of caution is the possible inhibition of certain phosphodiesterases by 8-pCPT. Specifically, it inhibits PDE1B and 6 with IC_{50} below 10μM, while it inhibits PDE3 with an IC_{50} of ~20 μM. As discussed above, the major PDEs in vascular smooth muscle are 1 (A and C), 3, 4 and 5, of which none are inhibited by 8-pCPT at the 5μM concentration used by our group (Poppe et al. 2008).

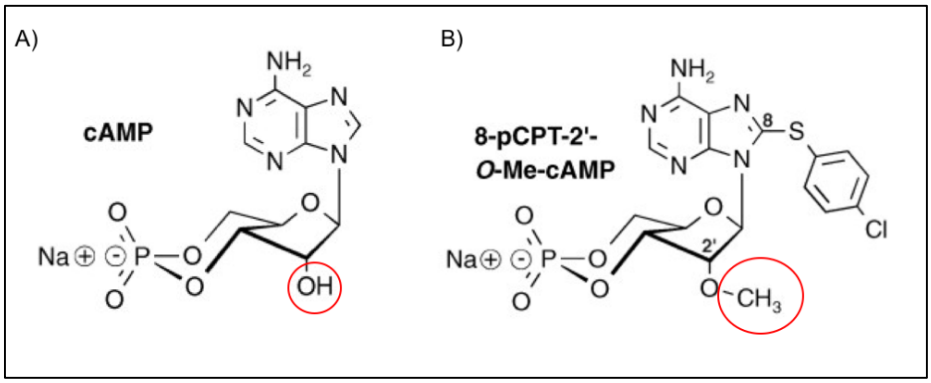


Figure 1.7- Various Tools used to Explore Epac-mediated effects: A) Cyclic adenosine monophosphate (cAMP) produced by adenylyl cyclase from ATP is a major second messenger within cells. **B)** 8-pCPT-2′-O-Me-cAMP is a cAMP analogue designed to activate Epac without activation PKA or CNGs. Red circle indicates the position of the substitution (–OH group to an $–OCH_3$) making 8-pCPT-AM a selective Epac agonist. cAMP analogue diagrams were from Holz *et al.* (Holz et al. 2006).

1.6 cAMP signalling in vascular smooth muscle

1.6.1 cAMP causes quiescence in vascular smooth muscle

In many cell types including vascular smooth muscle cells cAMP causes an inhibition of proliferation *in vitro* and *in vivo* (Assender et al. 1992). Since discovering the role of cAMP in proliferation, cAMP response element binding protein (CREB), a transcription factor, has been shown to be a molecular determinant for smooth muscle cell proliferation (Bito et al. 1996). cAMP analogues or cAMP increasing compounds increase CREB phosphorylation, thereby decreasing proliferation-associated genes that encode endothelin

receptors, platelet-derived growth factor receptors and cyclins (Ichiki 2006; Klemm et al. 2001). As with numerous cAMP-mediated effects, this inhibition of proliferation was thought to be exclusively mediated through PKA. More recently skp2, an F-box protein component of SCF^{skp2} ubiquitin ligase, has been shown to be increased in proliferative vascular smooth muscle cells, both in culture and *in vivo* following balloon injury of arteries (Y. J. Wu et al. 2006). Skp2 is important in cell cycle regulation, by degrading $p27^{kip1}$, itself responsible for halting the progression of quiescent cells into S phase. cAMP analogues decrease skp2 expression, increase $p27^{kip1}$ expression and inhibit focal adhesion kinase activation, which is responsible for skp2 expression (Y. J. Wu et al. 2006; Bond et al. 2004). This effect of cAMP is reversed in the presence of PKI, an inhibitor peptide of PKA (Cheng et al. 1986). Not mentioned specifically in the study showing PKI effects on skp2 expression was the level of reversal, with only ~20% (approximate due to estimation of the reversal effect from figures) of the original skp2 expression being reversed. This is surprising as PKI was used at 40 μM, 1000 fold higher than the IC_{50} (Y. J. Wu et al. 2006). After the discovery of Epac this same group reassessed the involvement of Epac in this cAMP-mediated event involving skp2. Using PKA-specific and Epac-specific activators it demonstrated that PKA and Epac1 work synergistically to cause both vascular smooth muscle cell quiescence though proliferation studies, and with it a decrease in skp2 expression as shown by immunoblots (Hewer et al. 2011). Activation of a single cAMP effector (PKA or Epac) did not produce any significant effect (Hewer et al. 2011). Other studies have shown a single cAMP effector had an effect, with 8-pCPT increasing the level of the transcription factor NR4A1, along with decreasing cell proliferation

(Mayer et al. 2011). The involvement of Epac in proliferation and migration was shown by Epac1 $^{-/-}$ mice, where aortic smooth muscle cells showed increased proliferation in response to serum compared to control mice (Kato, Yokoyama, Yanai, Ishige, Kurotaki, Umemura, Fujita, Kubota, Okumura, Sata, et al. 2015). Epac1$^{-/-}$ mice (from the same animal strain as above) also had reduced neointima formation after injury, primarily due to a decrease in cell invasion and migration in response to PDGF-BB (Kato, Yokoyama, Yanai, Ishige, Kurotaki, Umemura, Fujita, Kubota, Okumura & Sata 2015). Interestingly, beraprost (a prostacyclin analogue) activated Epac and inhibited migration (the opposite to the Epac1 $^{-/-}$ study), but had no effect on proliferation (McKean et al. 2015). The difference in results is yet unexplained but may be due to species differences or the role of other receptors present such as EP receptors (Orie & Clapp 2011).

1.6.2 cAMP improves vascular permeability

cAMP-elevating hormones have long been associated with attenuation of both *in vitro* endothelial cell permeability and *ex vivo* artery permeability (Langeler & van Hinsbergh 1991; Stelzner et al. 1989; Kobayashi et al. 1987). This is not the case in all arterial beds, for example the coronary circulation, where cAMP increases permeability (Bindewald et al. 2004). Originally, PKA was shown to be the cAMP effector responsible for decreasing endothelial permeability, after over-expression of the PKA inhibitor gene (PKI) abolished cAMP-mediated decreases in permeability (Lum et al. 1999). The potential substrates of PKA in endothelial cells are commonly thought of as MLCK and RhoA, both of which are involved in the control of MLC phosphorylation, however other substrates have been investigated, such as IP_3 receptors, filamin and protein

phosphatases (Lum et al. 1999). MLC phosphorylation is important in endothelial permeability, as contraction of endothelial cells changes endothelial cell shape, and with it opens intracellular gaps (Wysolmerski & Lagunoff 1990; Yuan et al. 2002). In coronary artery endothelial cells MLC phosphorylation and cAMP production are similar to aortic endothelial cells, however forskolin treatment produces opposing effects on permeability i.e. increases membrane permeability (Bindewald et al. 2004). This opposing effect is due to rapid disintegration of cellular adhesion complexes containing VE (vascular-endothelial) -cadherin and paxillin (Bindewald et al. 2004). Epac has been shown to be involved in both MLC phosphorylation and gap junction modulation in the endothelium of arteries. Cullere *et al*. (2005) showed that Epac activation in human pulmonary artery endothelial cells causes an increase in cortical versus stress fibre actin, as well as a continuation of VE-cadherin staining between cells (Cullere et al. 2005). This continuous VE cadherin improves vascular integrity and decreases permeability (Giannotta et al. 2013). Thrombin treatment increases RhoA activation and decreases continuality of VE-cadherin staining, both of which were reversed by incubation with 8-pCPT-AM or by increasing cAMP through forskolin and rolipram (PDE4 inhibitor) application (Cullere et al. 2005). Epac's role has been further elucidated in endothelial vascular permeability, with a plethora of signaling pathways potentially involved. Epac/Rap1 activates Rac1, which strengthens adheren junctions (Baumer et al. 2008). Rac1-independent, but Rap1-dependent, signaling may exist with RhoA activity decreased through a multimeric protein complex (Rho GTPase activating protein 29, Radil1 and Rasip1) (Post et al. 2013). Rap1-independent Epac-mediated signaling has

been shown, with AKAP9 being required for increasing microtubule growth, but not for basal vascular permeability (Sehrawat et al. 2011). Further confirmation of Epac1 involvement in vascular permeability has been shown where Epac1$^{-/-}$ mice have an increase in microvascular permeability (Kopperud et al. 2017).

1.6.3 Cyclic nucleotide induced relaxation

Vascular smooth muscle cells in the normal, non-pathophysiological state are in a contractile phenotype being quiescent (no cell division) with the role of maintaining and controlling blood vessel diameter. cAMP was first explored in relation to relaxation, when researchers acknowledged that the increase in cAMP seen by β-adrenoceptor activation was accompanied by smooth muscle relaxation. For a thorough and complete review on early cAMP studies in the vascular see *Kramer and Hardman* (1980) (Kramer & Hardman 1980).

1.6.3.1 *PKA effect on contractility*

Vascular smooth muscle cell relaxation dependent on cAMP is vascular bed specific and difficult to explore due to the potential for cAMP to not only activate PKA, but also at high concentrations activate PKG (Morgado et al. 2012). However, in general cAMP, produces relaxation through four major routes involving the regulation of Ca^{2+} homeostasis, signaling and cytoplasmic contraction apparatus (Murthy 2006; Webb 2003; Beavo & Brunton 2002; Hardman 1984; Kramer & Hardman 1980; Roberts & Dart 2014; Morgado et al. 2012; Akata 2007a; Akata 2007b):

1. **Decrease in Ca^{2+} store release:** cAMP decreases Ca^{2+} store release through phosphorylation of IP_3 receptors, commonly associated with Ca^{2+}

store release. *In vitro* PKA has been shown to phosphorylate IP_3R and attenuate Ca^{2+} release. However in intact rat aorta, PKG was shown to mediate IP_3 phosphorylation by cAMP increases, presenting it as the major kinase for this role through possible (i.e. cAMP activating PKG not PKA) (Komalavilas & Lincoln 1996; Narayanan et al. 2012; Murthy & Zhou 2003). The difference in effect of PKA and PKG may be due to the presence of IP_3R associated cGMP kinase substrate (IRAG) (Taylor 2017).

2. **Increase in Ca^{2+} store reuptake:** cAMP working via PKA causes an increase in Ca^{2+} reuptake to the intracellular stores in the sarcoplasmic reticulum (Webb & Bhalla 1976; Wegener et al. 1989). The addition of PKA catalytic subunit and cAMP to vascular microsomes, showed an increase in the level of phosphorylation for a 22kDa protein, as assessed by radioactive ^{32}P incorporating experiments (James et al. 1989; Bidlack et al. 1982). This protein was later shown to be phospholamban, which when phosphorylated disinhibits the sarco/endoplasmic reticulum Ca^{2+} ATPase (Simmerman & Jones 1998).
3. **Increased efflux of Ca^{2+} to the extracellular space:** The two main "escape" routes for Ca^{2+} from the cytoplasm of smooth muscle cells are the plasma membrane Ca^{2+} ATPase (PMCA) or the Na^+/Ca^{2+} exchanger (NCX) (Morgado et al. 2012; Kamishima & McCarron 1998; Oloizia & Paul 2008). PMCA exchanges 2 H^+ ions into the cell in exchange for 1 Ca^{2+} ion out, with ATP needed for this exchange. This ATPase is highly regulated by numerous kinases, including Ca^{2+}/calmodulin dependent kinase (CaMKII) along with PKA and PKG (Kamishima & McCarron 1998; Oloizia

& Paul 2008; Brini & Carafoli 2011). The role of NCX in vascular smooth muscle has not fully been elucidated, due to contrasting results, but certain groups have shown potentiation of Ca^{2+} extrusion in response to PKG (Brini & Carafoli 2011; Morgado et al. 2012; Kamishima & McCarron 1998; Murray 1990).

4. **Decrease in extracellular Ca^{2+} entry, both directly and through membrane potential modulation:**
    a. **Direct**: PKA and its role in direct regulation of Ca^{2+} entry is complex and still not completely understood. For example, T-type Ca^{2+} channel activity has been shown to be inhibited by PKA in cerebral arterial myocytes. This effect of decreasing Ca^{2+} entry is fitting for a relaxant mechanism, but is paradoxical to the potentiation of L-type Ca^{2+} channels (LTCC) in murine arterial myocytes by PKA, which causes vasoconstriction (Nystoriak et al. 2017). Enhanced LTCC currents may also produce relaxation through activating BK_{Ca} channels (Guia et al. 1999). However, LTCC currents have also commonly been associated with attenuation by PKA (Keef et al. 2001). The varying effects seen on LTCCs may be due to “crossover” of cAMP activating PKG and *vice versa*. Due to excessive rundown of LTCC in experiments that utilize cell rupture, the majority of experiments utilize the perforated patch technique and use specific PKA or PKG activators to assess kinase involvement. PKG has been shown to reliably inhibit LTCC currents (Ruiz-Velasco et al. 1998; Ishikawa et al. 1993; Keef et al. 2001). Inhibition of store-

operated calcium channels (SOCCs) by PKA and CaMKII takes place in rat mesenteric arterial myocytes through inhibition of TRPC1 (Transient receptor potential canonical 1) channel and TRPC5 channel (Albert et al. 2007). SOCCs were also inhibited by PKA and PKG in pulmonary artery myocytes (Chen et al. 2011). Receptor operated calcium channels (ROCC) have been subject to a number of experiments to determine their regulation by kinases. The consensus is that PKA/PKG inhibit ROCC with the TRP channel TRPC6 being inhibited (Nishioka et al. 2011).

b. **Membrane potential modulation:** PKA also decreases Ca^{2+} entry by phosphorylating target proteins that induce hyperpolarization of the cell membrane. Positive modulation of K^+ channels at membrane potentials positive to E_k (K^+ reversal potential) produce an outward current of K^+, which causes a hyperpolarization of the cell membrane. This hyperpolarisation of the cell membrane attenuates Ca^{2+} entry via LTCC by decreasing the opening probability of LTCC's and lowering the driving for Ca^{2+}.

    i. **Voltage-activated K^+ channels:** K_v channels are activated by PKA phosphorylation including endogenous K_v currents in smooth muscle cells (Brignell et al. 2015; Y Hayabuchi, Standen, et al. 2001).

    ii. **ATP-sensitive K^+ channels:** PKA-mediated phosphorylation increases K_{ATP} current (Yang et al. 2008; Kleppisch & Nelson 1995).

iii. **Inward-rectifying K^+ channels:** PKA mediates an increase in K_{ir} current induced by hypoxia (Park et al. 2005).

iv. **Large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}):** cAMP via PKA or PKG increases open-probability (White et al. 2001; Minami et al. 1993; Porter et al. 1998; Scornik et al. 1993).

See *Humphries & Dart* (2015) for ion channel structure and classifications (Humphries & Dart 2015).

PKA regulation of vascular tone is not only produced by altering available cytoplasmic Ca^{2+}, but by regulating the Ca^{2+} sensitivity of the contractile proteins in vascular smooth muscle cells. Phosphorylation at site S695 in the MYPT1 subunit of MLCP increases myosin dephosphorylation by inhibiting ROCK phosphorylation (Somlyo & Somlyo 2003; Wooldridge et al. 2004). Another site, S852, is also phosphorylated, but its effect has not been examined (Wooldridge et al. 2004). PKA-mediated inhibition of MLCK has been shown *in-vitro* (Conti & Adelstein 1981), however *in-vivo,* CaMKII appears to play a more physiological role in inhibitory phosphorylation of MLCK (Stull et al. 1993).

A third relaxation pathway dependent on PKA has been shown. In this phosphorylation of heat shock protein 20 (HSP20) produces a decrease in the interaction between myosin and actin. The exact mechanism is still to be elucidated **(Figure 1.8)** (Beall 1999; Salinthone et al. 2008).

The above mechanisms of relaxation are through PKA-mediated events in vascular smooth muscle cells, however PKA also modulates events in endothelial cells. G_s-coupled receptors on endothelial cells have been shown to increase NO synthase activity by PKA dependent phosphorylation, leading to an increase in nitric oxide (NO) a prominent vasodilatory through PKG (Ferro et al. 2004; Butt et al. 2000).

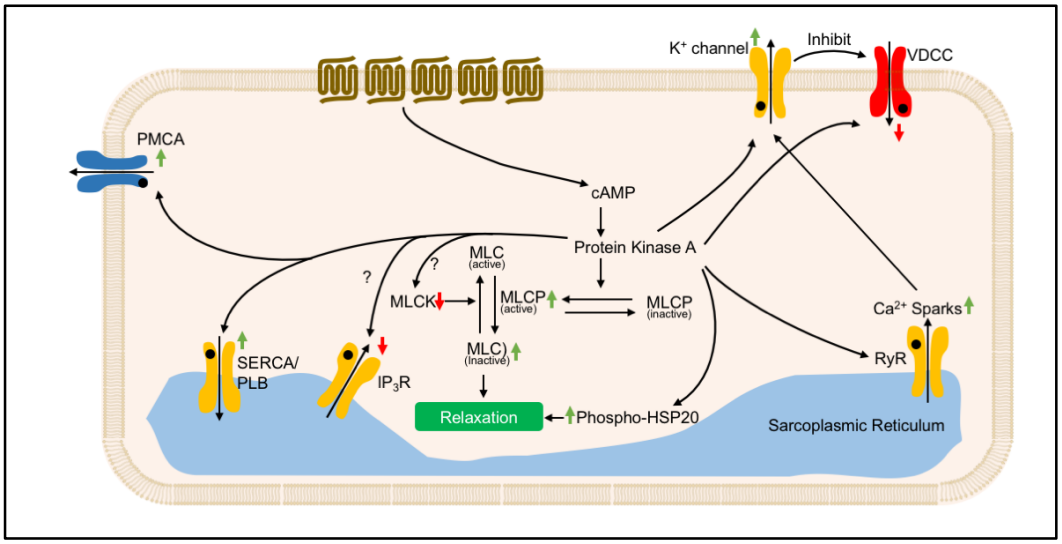


Figure 1.8- PKA-dependent relaxation pathways in smooth muscle cells. PKA activation produces vasodilatory mechanisms through numerous pathways including a direct effect on the contractile machinery and through decreasing available Ca^2. The contractile machinery is also modulated by decreasing the level of MLC phosphorylation. PKA-dependent phosphorylation of HSP20 causes relaxation through actin filament regulation. PKA lowers Ca^{2+} by through phosphorylation of many downstream targets such as PMCA, PLB, IP_3R, RyR, K^+ and Ca^{2+} channels.

1.6.3.2 *Cyclic Nucleotide gated ion channels in vasodilation*

The cyclic nucleotide gated ion channel encoded by CNGA2 has been recently shown to mediate numerous agonist-induced vasorelaxation effects. Adenosine and adrenaline-induced relaxations were both attenuated by inhibition of CNGCs using L-diltiazem and specific siRNA. CNGCs produces relaxation by increasing the Ca^{2+} current through CNGCs in endothelial cells, which produces an activation of the Ca^{2+}-sensitive enzyme, eNOS in endothelial cells, and with it increases NO. Indeed, inhibition of NO synthase

with L-NAME removed the effect of CNGC inhibition on adenosine vasorelaxation (K. T. Cheng et al. 2008).

1.6.3.3 *Epac effect on contractility*

Considerably less is known about the functional role of Epac in regulating vascular tone. However, in cardiomyocytes Epac activation has an effect on calcium handling by increasing store Ca^{2+} release, through Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-dependent phosphorylation of downstream targets, including ryanodine-sensitive channels (RyR) and phospholamban (PLB). This Ca^{2+} modulation by Epac is responsible for such effects as chronic inotropy, cardiac hypertrophy and arrhythmia (Ruiz-Hurtado et al. 2012; Pereira et al. 2013; Pereira et al. 2007).

Epac activation in the vasculature has been shown to produce relaxation through the desensitization of the contractile mechanism to Ca^{2+}. *Zieba et al.* (2011) showed that Epac activation led to Rap1 activation, which suppressed RhoA activity and with it ROCK (Zieba et al. 2011). In permeabilised preparations of various arteries and smooth muscle preparations, Epac activation produced relaxation that was not susceptible to PKA inhibition with H89 (Zieba et al. 2011). The lack of dependence on PKA was confirmed through Epac1 knockdown, after which no Rap1 activation could be detected in response to isoprenaline application (Zieba et al. 2011). The integral involvement of Rap1B in aortic vessel tone was further shown through Rap1B knock out mice, vessels from which showed an increased susceptibly to contraction and a decreased acetylcholine response, due to a decrease in NO synthesis (Lakshmikanthan et al. 2014).

Recently Stott *et al.* (2017) showed that Epac activation caused relaxation in mesenteric and renal arterial preparations, mediated by K_v7 channel activation (Stott et al. 2016). In both these preparations, BK_{Ca} channel blockade blunted Epac-mediated relaxation. Interestingly, cAMP-mediated relaxation in response to isoprenaline was inhibited by the Epac inhibitor, ESI-09, in mesenteric artery preparations but not renal (Stott et al. 2016). A concern is that ESI-09, a nonspecific inhibitor shown to produce its effect via protein degradation, was used at 100nM, whereas the published concentration required for 50% inhibition (IC_{50}) of Epac1 and Epac2 is 10.8μM and 4.4μM, respectively (Zhu et al. 2015). The same group further explored the effect of combining the inhibitors, CE3F4 and HJC0350. Again, CE3F4 was used at 1μM, whereas its published IC_{50} is 10.7μM. The Epac2 inhibitor HJC0350 was used at 1μM, whereas its published IC_{50} is 0.3μM (Chen et al. 2013; Courilleau et al. 2013). Using these inhibitors at between 10 and 100 fold their IC_{50} it is hard to understand how the effect on relaxation can be attributed to Epac inhibition. Secondfold the non-specificity of these inhibitors is of concern, with the mechanism of action being general protein instability in one instance (Rehmann 2013). Experiments previously undertaken in our laboratory show that ESI-09, when used at a near IC_{50} concentration inhibits constriction of the vessel (Personal Communication. C Dart & T Kamishima).

While Epac activation has predominately been shown to affect vasodilatory mechanism, one intriguing effect would oppose vasodilation. Purves *et al.* (2009) reported that Epac activation in aortic smooth muscle cells inhibited K_{ATP} current, with the mechanism activating the Ca^{2+}-sensitive protein phosphatase, calcineurin. These results are difficult to understand, however

they could implicate Epac as a negative feedback effector in conduit arteries following excessive cAMP generation, or a possible involvement in pathophysiology. Interestingly, stimulating cAMP synthesis with increase forskolin produced no inhibition, whereas forskolin treatment in the presence of PKA inhibitors reduced pinacidil-evoked K_{ATP} currents to the same extent as specific Epac activation. (Purves et al. 2009).

Selective Epac activation in mesenteric artery produces a relaxation that is partially endothelium-dependent (Roberts et al. 2013). Endothelium removal or NO synthase produced the same level of inhibition on Epac mediated relaxation. NO synthase inhibition was shown by the application of the NOS inhibitor, L-N^G-Nitroarginine methyl ester (L-NAME). Indeed, Ca^{2+} imaging in endothelial sheets showed a transient increase in $[Ca^{2+}]_i$ upon Epac activation. Ca^{2+} transients such as these can activate NO synthesis via Ca^{2+}/calmodulin binding to eNOS (Förstermann & Sessa 2012).

Within smooth muscle cells, Epac activation increased Ca^{2+} spark frequency, but did not change any other parameter of sparks i.e. amplitude/rise time/decay half-life (Roberts et al. 2013). As expected this increase in Ca^{2+} spark frequency translated to an increase in STOC frequency under Epac activation **(Figure 1.9)**. In the same cells STOC amplitude was increased, due to an increase in the probability of larger STOCs. Confirmation of recorded currents being STOCs (i.e. activated by Ca^{2+} sparks) was shown by both ryanodine and iberiotoxin (a BK_{Ca} channel blocker) inhibiting the currents. This increase in STOCs caused hyperpolarization of the cell membrane, confirmed by current-clamp recordings. It is important to note that selective Epac activation was confirmed in mesenteric artery smooth muscle cells by

assessing both the level vasodilatory-associated stimulated phosphoprotein (VASP) phosphorylation by immunoblotting, a well know PKA phosphorylation substrate, and with electrophysiological experiments being undertaken in the presence of the selective PKA inhibitor, PKI-(Myr-14-22)-amide (Roberts et al. 2013) therefore negating PKA activation in cells.

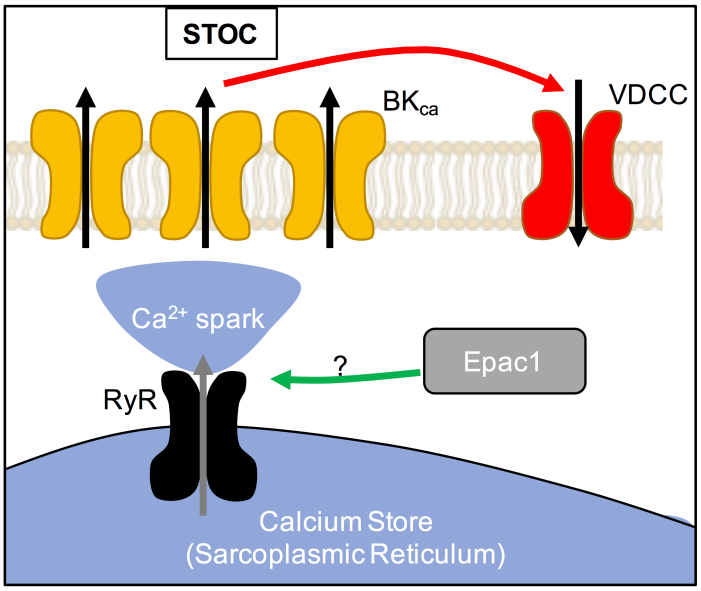


Figure 1.9- Epac activation increases Ca^{2+} spark frequency and STOC frequency. Epac activation using the specific Epac activation 8-pCPT-AM increases Ca^{2+} spark frequency. Ca^{2+} sparks are localised, high concentrated (>10μM) releases of Ca^{2+} from ryanodine receptors (so called due to being sensitive to ryanodine). This release of Ca^{2+} causes activation of BK_{ca} channels, or Ca^{2+} activated K^+ channels producing a transient outward current of potassium. This current causes a decrease in the membrane potential and decreases/inhibits voltage dependent Ca^{2+} entry, thereby causing relaxation of the vascular smooth muscle cell.

1.7 Aims

The aim of this thesis is to further elucidate Epac-mediated signaling that lends to vasodilation in mesenteric artery smooth muscle cells. Using both biochemical and electrophysiological techniques I aim to elucidate the signaling pathway by which Epac increases STOCs in freshly isolated mesenteric artery smooth muscle cells. Our starting hypothesis is that, similar

to cardiomyocytes, Epac activation leads to activation of CaMKII, with this leading to phosphorylation of downstream signaling components.

2 Materials and Methods

2.1 Animals

Tissues were obtained from adult male Wistar rats (175-225g; Charles River Laboratories, Wilmington, USA) and wild-type, CaMKIIδ and CaMKIIγ knockout mice (C57B/6 background; provided by Dr Tim Curtis, Queens University, Belfast, UK). Wild-type mice were littermates of the homozygous knockouts. All animals were euthanized by a rising concentration of CO_2 followed by cervical dislocation. The care and euthanasia of animals conformed to the requirements of the UK Animals (Scientific Procedures) Act 1986, and were approved by the University of Liverpool and Queens University, Belfast local ethics committee.

2.2 Chemicals

2-[N-(2-hidroxyethyl)-N-(4-methoxy-benzenesulfonyl)]-amino-N-(4-chloro-cinnamyl)-N-methylbenzylamine (KN-93), 2-[N-(4'- Methoxybenzenesulfonyl)] amino-N-(4'-chlorophenyl)-2-propenyl-N-methylbenzylamine (KN-92), carbamimidothioic acid, 3-[3-[2,5-dihydro-4-(1-methyl-1H-indol-3-yl)-2,5-dioxo-1H-pyrrol-3-yl]-1H-indol-1-yl]propyl ester, monomethanesulfonate (BIM IX) (Cambridge Bioscience, Cambridge, UK), KN93 and KN92 were prepared as 500μM stocks in 100% DMSO (Final DMSO 0.1%). BIM-IX was prepared in DMSO at a stock concentration of 500μM (Final DMSO 0.05%) 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate-AM (8-pCPT-2'-*O*-Me-cAMP-AM; Biolog Life Science Institute, Bremen, Germany) was prepared as a 5mM stock in DMSO (stored at -80 ^{o}C). Thapsigargin (Merck (Calbiochem), Darmstadt, Germany) stock was made up in DMSO. All other chemicals were purchased from Sigma Aldrich (St.Louis, USA).

2.3 Cell Isolation

Proximal first-order branches of rat superior mesenteric arteries were dissected and placed in low Ca^{2+} buffer containing (in mM): 134 NaCl, 6 KCl, 0.42 Na_2HPO_4, 0.44 NaH_2PO_4, 10 HEPES, 10 glucose, 1.0 $MgCl_2$, 0.1 $CaCl_2$ (pH 7.4) at 35 ^oC for 10 minutes. After incubation, branches were moved to the first stage digestion buffer consisting of low Ca^{2+} buffer containing papain (1.4mg/ml; P4762 Papain from Papaya Latex), 4-dithioerythritol (0.9 mg/ml A7030 fatty acid free) and bovine serum albumin (BSA; 0.9mg/ml) for 8 to10 minutes at 35 ^oC. Branches were then washed 3 times in pre-warmed, low Ca^{2+} buffer and transferred to second stage digestion buffer, consisting of low Ca^{2+} buffer containing hyaluronidase (0.9mg/ml), collagenase (1.4 mg/ml) and BSA (0.9mg/ml) at 35 ^oC. The incubation period for second stage digestion varied from 8 to 12 minutes. Branches were subsequently transferred to fresh ice-cold, low Ca^{2+} buffer and carefully washed before dispersion of cells by gentle trituration of the tissue through a heat-polished glass pipette. Dispersed rat mesenteric artery smooth muscle cells (RMASMCs) were stored in low Ca^{2+} buffer on ice and used in experiments between 1 and 8 hours after digestion. All chemicals were from Sigma-Aldrich.

2.4 Cell Culture

Chinese hamster ovary (CHO) cells were grown in Dulbecco's Minimum Essential Medium containing 10% (v/v) foetal bovine serum and 4% (v/v) non-essential amino acids supplemented with 50U/ml penicillin and streptomycin (Thermo Fisher Scientific (Invitrogen), Carlsbad, USA). Cells were kept at 37 ^oC in 5% CO_2 humidified atmosphere and passaged at a 1 in 8 dilution into fresh media when ~ 90% confluent.

2.5 Electrophysiology

Spontaneous transient outward currents (STOCs) were recorded from single freshly isolated RMASMCs using the whole-cell configuration with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, USA) as previously described (Roberts et al. 2013). Currents were filtered at 5 kHz and digitized at 10 kHz using a Digidata 1320A interface (Molecular Devices, Sunnyvale, USA) after which they were stored on a HP desktop PC. The pipette-filling solution contained (in mM): 140 KCl, 3 $MgCl_2$, 0.2 EGTA, 10 HEPES, 3 Na_2ATP, pH 7.2 with KOH. Extracellular recording solutions contained (in mM): 6 KCl, 134 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, pH 7.4 with NaOH. STOCs were recorded at a holding potential of -20 mV and defined as events deviating from the baseline by a factor of 4 standard deviations above baseline noise. For experiments using the CamKII inhibitor peptide, Autoinhibitory-2-related peptide (1μM) was placed in the intracellular solution and allowed to dialyse into the cell for 5 minutes before the start of recording.

2.6 Ca^{2+} Fluorometry: Measurement of SR Ca^{2+} content

Transient Ca^{2+} release from the SR was triggered by pulse applications of the RyR activator caffeine, in single freshly-isolated. voltage-clamped and fura-2-dialyzed RMASMCs, as previously described (Kamishima & McCarron 1996). Fura-2-pentapotassium was purchased from Thermo Fisher Scientific (Invitrogen, Carlsbad, USA). Cells were suspended in low Ca^{2+} extracellular solution containing (in mM): 145 NaCl, 6 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4 with NaOH) and placed in a perspex perfusion chamber with size 0 glass coverslip as a base. The cell was illuminated alternately with 340 and 380 nm light (bandpass 5 nm) using a PTI DeltaRAM

Illuminator (Horiba Scientific, Canada), and the emission signal was detected at 510 nm (40 nm bandpass). Background fluorescence counts for dual excitation wavelengths were taken after establishing a giga seal but before achieving the whole-cell clamp configuration. On achieving the whole-cell configuration, the cell was dialyzed with pipette-filling solution containing (in mM): 145 CsCl, 3 $MgCl_2$, 3 Na_2ATP, 10 HEPES and 0.05 fura-2 pentapotassium (pH 7.2 with CsOH). The cell membrane potential was held at -60 mV. Cells were superfused with extracellular solution, in which $CaCl_2$ has been raised to 3 mM. Changes in photon counts were measured, background counts subtracted, and the ratio 340:380 obtained at 50 Hz. Fluorescence signals and cell membrane potentials were acquired using PTI Computer Software FelixGX (Horiba Scientific, Kyoto, Japan). RyRs were activated by rapid application of 5 mM caffeine using a U tube superfusion system. Caffeine was applied for 100 ms by closing a solenoid valve placed downstream of the U tube ejection hole. Valve closure was triggered by the patch clamp software producing a transient hyperpolarisation to -90mV (pCLAMP, version 10.2, Molecular Devices, Sunnyvale, USA). Caffeine application was terminated by restoration of the membrane potential to -60 mV (i.e. holding potential), which causes a closure of the valve. The brief membrane hyperpolarization thus served to accurately mark the duration of caffeine exposure on the fluorescence recording. Valve closure was normally triggered once every 60 seconds with cells superfused in normal Ca^{2+} extracellular solution. After the 3rd or 4th caffeine application, the extracellular solution was switched to 3mM Ca^{2+} containing solution containing either vehicle control (DMSO) or 8-pCPT-AM (5μM), whilst caffeine transients were

continuously triggered at regular intervals. To convert the fluorescence ratio to cytoplasmic calcium concentration $[Ca^{2+}]_i$, *in vitro* calibration was carried out using EGTA-buffered intracellular solutions containing a range of free $[Ca^2]$. The free $[Ca^{2+}]$ was calculated with MAXchelator (#46 v8;standford.edu). the fluorescence ratio in the absence of added $CaCl_2$ (R_{min}) and in the presence of saturating Ca^{2+} (R_{max}) were decreased by 15% to account for viscosity (Poenie 1990), and the K_d of fura-2 was determined as 160 nM using the equation –

$$[Ca^{2+}] = \frac{kd \times \beta \times (R - R_{min})}{(R_{max} - R)}$$

β = the ratio of fluorescent count at 340 nm / 380 nm.

R= ratio at specific calcium concentration

To profile Ca^{2+} transients, three parameters were examined: amplitude, duration and rate of cytosolic Ca^{2+} increase. Amplitude was measured as the $[Ca^{2+}]_i$ difference between resting and peak $[Ca^{2+}]_i$. Duration was determined as the time that $[Ca^{2+}]_i$ remained above half amplitude. To examine the early stage of the cytosolic Ca^{2+} rise, data points from baseline to $[Ca^{2+}]_i$ of 200 nM were fit with a third order polynomial using Sigmaplot 13 (Systat Software, San Jose, USA). The rate of Ca^{2+} increase was determined as the time taken to reach a $[Ca^{2+}]_i$ of 100 nM. For each parameter, the value of the 3rd Ca^{2+} transient after switching to the test solution was measured relative to that of the Ca^{2+} transient immediately before solution change.

2.7 1D-gel electrophoresis & Immunoblotting

First- or second-order branches of rat superior mesenteric arteries (approximately 5-8 branches) were homogenized in a pre-cooled hand-held

homogenizer, using 100-500 μL of ice-cold lysis buffer of composition (in mM): 20 Tris-HCl; 250 NaCl, 3 EDTA, 3 EGTA, 0.5% (v/v) Triton-X 100, pH 7.6, 1% (v/v) mammalian protease inhibitor cocktail (Sigma Aldrich, St Louis, USA) and 1X PhosSTOP (Roche, Basel, Switzerland). Resultant lysates were placed on ice for 10 minutes then centrifuged at 16,100 x *g* for 10 minutes at 4 ^{o}C . Supernatants were removed and mixed 1:3 (v/v) with 4 times sodium dodecyl sulfate (SDS) sample buffer (2% SDS final concentration), before heating to 98 ^{o}C for 10 minutes. Lysates for RyR, PLB and Epac1 immunoblots utilised RIPA buffer (in mM) 50 Tris-HCl (pH 7.4 at 21^{o}C), 150 NaCl, 0.1% SDS, 1% NP-40, 1% protease inhibitor or 1x SDS sample buffer to maximise phosphorylation preservation and increase protein solubilisation. RIPA lysates were then mixed 1:3 with 4 times sample buffer as with Triton X-100 based lysates. Samples were kept at -20 ^{o}C until use. Proteins within the lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on either 5%, 10% or 15% polyacrylamide-Tris-glycine gels (dependent on the protein of interest, and transferred electrophoretically onto nitrocellulose membranes (Hybond ECL, GE Healthcare (Amersham), Little Chalfont, UK). When immunoblotting for either phospholamban or RyR, proteins were transferred to 0.22μm or 0.44μm PVDF membrane, respectively. Immunoblotting was performed as previously described (Purves et al. 2009). Where indicated, membranes were stripped at room temperature for 1 hour in 50mM Tris (pH 6.8 at 21^{o}C), 1% SDS or using Abcam's recommended mild stripping buffer (1.5g glycine, 0.1g SDS, 1ml Tween per 100mL; pH 2.2).

2.8 2D-gel electrophoresis

Seven cm, pH 3-10 immobilised pH Gradient (IPG) strips were used to separate proteins based on their Pi (BioRad, Hercules, USA) as per the BIO-RAD ReadyStrip^{TM} IPG strip instruction manual. IPG strips were rehydrated overnight in 135 μL rehydration buffer (8 M urea, 5 % (v/v) beta-mecaptoethanol, 2% NP-40, 0.2 % (v/v) ampholytes (IPG) and a few grains of bromophenol blue (approx. 5mg). Samples were cup-loaded (10 μL) at the cathode end of the IPG strip. The electrophoresis run was a linear ramp preset method. Parameters for the S1-conditioning step were 250 V for 15 minutes; for S2-voltage ramping step the voltage increased towards a maximum of 4,000 V using linear ramping. The S3-final focusing step continued with a voltage step to a maximum voltage of 4,000V by rapid ramping (the limiting factor is current per strip set at 50μA). Focusing was stopped once approx. 16,000 Vh had been reached. IPG strips were then removed from the cup loading tray, drained, and transferred into a 15 ml tube where the strips were equilibrated in SDS-containing buffers. First the IPG strips were placed in equilibration buffer (6 M urea, 2 % (w/v) SDS, 50mM Tris-HCl, 10 % (v/v) glycerol and a few grains of bromophenol blue) with 10 mg/ml dithiothreitol (DTT) for 15 minutes and placed on an orbital shaker. Strips were then drained and placed in equilibration buffer with 30 mg/ml iodoacetamide (IAA) and gently shaken for a further 15 minutes. IPG strips were run in the second dimension on 10 % SDS-PAGE gels (as per immunoblots) at 150 V (max 40 mA per gel) until the dye front reached the end of the gel. Gels were then stained with colloidal or silver staining, or transferred to nitrocellulose membranes as described in Immunoblot section **(Section 2.7)**.

2.9 Colloidal/ Coomassie Staining

Depending on the quantity of sample loaded, one or two staining methods were used, standard Coomassie staining or colloidal Coomassie staining (standard being used first, after which colloidal was used). Standard Coomassie staining was undertaken on gels as follows. SDS-PAGE gels were removed from the running cassette and rinsed in MilliQ water, quickly followed by immersion in staining solution (0.1% (w/v) Coomassie (R-250), 7% acetic acid, 50% methanol) and stained, with agitation, for 1 hour. Gels were then rinsed in MilliQ water and placed in de-staining solution (50% methanol, 10% acetic acid, 40% MilliQ water), with agitation until the background was clear. Colloidal Coomassie staining was undertaken as described in Kang *et al.* (2002). SDS-PAGE gels were twice rinsed in MilliQ water to remove SDS followed by overnight incubation, with agitation in staining buffer (0.02% (w/v) colloidal brilliant blue (G-250), 5% w/v aluminium sulphate, 10% v/v ethanol and 2% phosphoric acid). Gels were de-stained in 10% v/v ethanol and 2% phosphoric acid until the background was removed.

2.10 Silver Staining

Silver staining was undertaken using the ProteoSilver Plus Silver Stain Kit (Sigma Aldrich, St Louis, USA). After complete de-staining of Coomassie gels, SDS-PAGE gels were fixed in 50% (v/v) ethanol, 40% (v/v) MilliQ water and 10% (v/v) acetic acid for between 40 mins and overnight. Gels were then transferred to 30% (v/v) ethanol for 10 minutes, washed in MilliQ water for 10 minutes and placed in sensitisation buffer for 10 minutes. Gels were then washed twice for 10 minutes in MilliQ water, followed by a 10 minute incubation in silver staining solution. A short rinse for less than 1 minute in water followed by incubation in Developing Buffer (kit). Development of gels took approx. 5

minutes, after which it was terminated using Stop Buffer. The gel were then rinsed and imaged by a standard PC scanner (Epson, Hemel Hempstead, UK). All solutions compositions are proprietary and undisclosed by the provider Sigma-Aldrich.

2.11 Immunoprecipitation

Tissue was homogenised in modified immunoprecipitation (IP) buffer (in mM) 50 Tris-HCl (pH 7.4 at 21^{o}C), 150 NaCl, 1% (v/v) Triton X-100, 1% (v/v) protease inhibitor unless otherwise stated, followed by end-over-end incubation at 4 ^{o}C for 1 hour. Homogenates were spun at 16,100 x *g* for 10 minutes after which homogenates were equally split (approx. 250μl per IP condition), with approx. 10% of total homogenate retained as an input reference. Homogenates were then incubated overnight on an end-over-end rotator with 2 μg of antibody or same species IgG control. 25 μL per sample of protein A/G agarose slurry (Thermo Fisher Scientific (Pierce), Carlsbad, USA) was prepared the following morning by 3 washes in IP buffer. Following washing, beads were suspended in IP buffer and divided equally between samples, followed by 2 hours incubation at 4 ^{o}C. Following this final incubation, beads were spun and washed three times in 1ml wash buffer containing (in mM) 50 Tris-HCl (pH 7.4), 150 NaCl , 0.1% (v/v) Triton X-100 with 5 minutes agitation between washes. After the final wash, beads were pelleted and two times sample buffer (final concentration 2% SDS) was added, followed by 10 minutes heating at 98 ^{o}C. Samples were then loaded on SDS-PAGE for either immunoblotting or Coomassie/colloidal/silver staining. Proteins expected at ~55kDa and ~25kDa used immunoprecipitation specific secondary antibodies. This eliminates the cross reaction of secondary antibodies with the IgG heavy

and light chains from antibodies used to pull down the protein of interest during the IP.

2.12 Antibodies

For immunoblotting, the primary antibodies used were phospho-CaMKII T^{286} (D21E4; 1 in 1000), phospholamban (D9W8M; 1:500) and phospho-serine/threonine (9631), all from Cell Signaling Technology (Danvers, USA). CaMKIIδ (EPR13095; 1:2500) and CaMKII (pan; EP1829Y; 1:2500) antibodies were from Abcam (Cambridge, UK). The phospho-phospholamban (T^{17}: A010-13; 1 in 5000) antibody was from Badrilla (Leeds, UK). Anti-BK_{ca} (APC-021; 1:250) was from Chemicon International (Merck, Darmstadt, Germany). Anti-PKA Regulatory II (ab75993) was from Abcam (Cambridge, UK). Anti-Epac1 antibodies used were as follows: Abcam (EPR6172; Cambridge, UK), Cell Signalling Technology (5D3; Danvers, USA) and Santa Cruz Biotechnology (A-5; Dallas, USA). Figure legends indicate which antibody was used. The secondary antibodies used were anti-mouse IgG (H+L) HRP conjugated polyclonal antibody and anti-rabbit IgG (H+L) HRP conjugated polyclonal antibody (Stratech Scientific Ltd., Newmarket, U.K.). For immunoprecipitation samples, secondary antibodies were: Veriblot for IP mouse (Abcam) and mouse anti-rabbit (conformational; Cell Signaling Technology).

2.13 Expression of GST-RalGDS-RBD

GST-RalGDS-RBD fusion protein, consisting of glutathione *S*-transferase (GST) fused to the Rap binding domain (RBD) of Ral guanine nucleotide dissociation stimulator (RalGDS); was expressed from the pGEX4T3-GST-RalGDS-RBD plasmid, kindly provided by Prof. Johannes Bos (University Medical Centre, Utrecht, the Netherlands), in the BL21 strain of *Escherichia*

coli as described previously (Van Triest et al. 2001). Following induction cells were spun down and placed overnight at -20 ^{o}C to aid lysis, followed by 3 bouts of sonication for 30 seconds in lysis buffer containing (in mM) 150 NaCl, 1% (v/v) Triton X-100, 50 Tris (pH 7.5 at 21^{o}C) and 1% (v/v) protease inhibitor (Sigma Aldrich, St.Louis, USA). Lysates were spun to remove insoluble material before incubation at 4 ^{o}C for 1 hour with 1ml glutathione-coated sepharose bead slurry (GE Healthcare, Little Chalfont, UK). The bead/lysate mixture was then poured into an empty PD-10 column followed by 30mL phosphate-buffered saline (PBS). Bound GST-RalGDS-RBD fusion protein was eluted using elution buffer containing (in mM) 50 Tris-HCl pH 8.0, 10 reduced glutathione. Elutants containing GST-RalGDS-RBD were pooled, followed by buffer exchange with a solution containing (in mM) 50 Tris-HCl (pH 7.2 at 21^{o}C), 150 NaCl, 0.5% (v/v) Triton X-100, 5 $MgCl_2$, 1 dithiothreitol (DTT), 1% protease inhibitors and 10% glycerol using a P10 column (Thermo Fisher Scientific (Pierce), Carlsbad, USA).

2.14 Expression of PKA Regulatory IIα (His Tagged)

PKA RIIα-His fusion protein, consisting of protein kinase A (PKA) RIIα subunits possessing a his-tag, was expressed from the PET280-PKAR2α plasmid (kindly provided by John Scott (University of Washington), in the pLysS strain of *Escherichia coli*. A starter culture of 50ml Luria-Bertani (LB) w/chloramphenicol (35 μg/ml) + kanamycin (50 μg/ml) (LB+Chl/Kan) was grown overnight and 5ml was used to inoculate 750ml LB +Chl/Kan. This culture was grown until an optical density at 600nm (OD_{600}) of 0.6. Cells were then induced overnight with 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 ^{o}C. Cells were pelleted (10 mins at 5000 x *g*) followed by

resuspension in 40ml ice cold lysis buffer containing (in mM) 50 Tris-HCl (pH7.4 @ 21 ^{o}C), 300 NaCl, 1 DTT, 10% (v/v) glycerol, 10 imidazole, 0.1 EDTA, 0.1 EGTA and 1% (v/v) protease inhibitor. Lysis by sonication with five 15 second bursts was undertaken followed by filtration of the lysate through a 0.45 μM filter. Filtered lysates were spun at 8000 x *g* for 1 hour at 4 ^{o}C before the supernatant was mixed with Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 1 hour at 4 ^{o}C. Beads were sedimented on a column then washed with 30 ml of binding buffer containing (in mM) 50 Tris-HCl pH 7.4, 500 NaCl, 10% (v/v) glycerol and 20 imidazole. PKA RIIα was eluted in 0.5 ml fractions with elution buffer (in mM) 50 Tris-HCl (pH7.4 @ ^{o}C), 300 NaCl, 10% (v/v) glycerol and 500 imidazole. Fractions were assessed by SDS-PAGE before pooling of PKA RIIα containing fractions, followed by protein concentration determination by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific (Pierce), Carlsbad, USA).

2.15 Rap1-GTP pull-down assay

50 μL of Glutathione-sepharose 4B beads (Thermo Fisher Scientific (Pierce), Carlsbad, USA) at 10% (v/v) in PBS (GE Healthcare), were washed three times in wash/lysis/binding buffer (Thermo Fisher Scientific (Pierce), Carlsbad, USA) followed by incubation with 20 μg GST-RalGDS-RBD fusion protein for 1 hour at 4 °C. First-order proximal rat superior mesenteric arteries were incubated with 5 μM 8-pCPT-2'-*O*-Me-cAMP-AM in extracellular electrophysiology recording solution (2mM Ca^{2+}) at room temperature (18–22 ^{o}C) or the equivalent volume of vehicle (dimethyl sulfoxide, DMSO) for 5 minutes, homogenised in 300 μl of ice-cold lysis buffer per artery and centrifuged at 16,100 x *g* for 10 minutes at 4°C. One-tenth of the supernatant

was removed and heated at 98 ^{o}C for 5 minutes after mixing 3:1 with 4 times SDS sample buffer for later assessment of total Rap1 levels. The remaining supernatant was mixed with washed GST-RalGDS-RBD-bound sepharose beads for 1 hour at 4 ^{o}C. After washing the beads four times in ice-cold lysis/binding/wash buffer, proteins were eluted in two times SDS sample buffer and heated at 98 ^{o}C for 5 minutes. Proteins were resolved by SDS-PAGE followed by immunoblotting as described above.

2.16 8-AHA-2'-O-Me-cAMP-Agarose Pulldowns

After homogenisation, lysates were mixed end-over-end at 4 ^{o}C for 1 hour, followed by centrifugation at 16,100 x *g* for 10 minutes. 8-AHA-2'-O-Me-cAMP agarose beads (Biolog, Bremen, Germany) were prepared by washing 3 times in lysis buffer. Lysate supernatant and beads were combined and mixed at 4 ^{o}C overnight (18 hours). Beads were pelleted and washed three times in 1ml lysis buffer with 5 minutes agitation between washes. After the final wash beads were pelleted and suspended in two times sample buffer and heated at 98 ^{o}C for 10 minutes.

2.17 Total RNA extraction and reverse transcription

Total RNA was extracted and purified from first- or second-order branches of rat superior mesenteric arteries using an RNeasy Mini Kit® (Qiagen, Hilden, Germany), as per the manufacturer's instructions. Extracted RNA was treated with DNase I by incubating total RNA with 10x DNase I buffer and DNase I (1 U/µl; Invitrogen) at room temperature to 15 minutes. EDTA (25 mM) was then added and the reaction heated to 65 ^{o}C for 10 minutes. First strand cDNA was synthesized using SuperScript® III reverse transcriptase (Thermo Fisher Scientific (Invitrogen), Carlsbad, USA). In brief for each reaction, 1 Oligo (dT),

total DNase I-treated RNA, dNTPs (10 mM each) and nuclease free H_2O were heated at 65 ^oC for 5 minutes. Reaction tubes were quickly chilled on ice before the addition of five times reaction buffer, dithiothreitol (0.1 M) and RNase Out (40U/µl). The samples were then incubated at 42 ^oC for 2 minutes. SuperScript® III reverse transcriptase (200 U/µl) was added to the reaction tubes, then incubated at 50 ^oC for 50 minutes. Reverse transcriptase was inactivated by heating at 85 ^oC for 15 minutes. The purity and concentration of the resulting cDNA template was determined by measuring absorbance at 260 and 280 nm using a NanoDrop spectrophotometer, with a 1.8 ratio indicating pure DNA with minimal protein contaminants (Thermo Fisher Scientific, Carlsbad, USA).

2.18 Polymerase Chain Reaction (PCR)

Touchdown PCR (decreasing the annealing temperature per PCR cycle from high to low) was carried out using HotStarTaq Master Mix Plus (Qiagen, Hilden, Germany), following the manufacturer's instructions. Primers used to amplify specific CaMKII isoforms were:

CaMKIIδ

- Forward (5-GCATGTGGCGTCATCCTCTA-3)
- Reverse (5-TCCTTTACCCCATCCGGTT-3)

CaMKIIγ

- Forward (5-CATCCACCAGCATGACATCG-3)
- Reverse (5-CTTTCCTCAAGACCTCAGG-3).

CaMKIIδ Splice Variation

- Forward (5- CGGAAATTGAAGGGTGCCATC-3)

- Reverse (5-CCCTACCAGGTGTACGTGAG-3)

CaMKIIγ Splice Variation

- Forward (5-GAAGGGTGCCATCCTCACAA-3)
- Reverse (5-GTTACCAAGGGCTTCTGGCT-3)

Following an initial 5 minute denaturing step at 95 ^{o}C, each reaction went through the following touchdown PCR protocol for 15 cycles: 94 ^{o}C for 1 minute, annealing temperature (ranging from 65 to 50 ^{o}C – decreasing by 1 ^{o}C each cycle) for 30 seconds, extension at 72 ^{o}C for 1 minute. After the touchdown element 25 cycles at the lowest annealing temperature took place. At the end of the cycles, final extension was at 72 ^{o}C for 5 minutes. Products were analysed by running on a 3 % (w/v) agarose gel containing Midori Green (1:10,000; GC Biotech, Alphen aan den Rijn, Netherlands) for ~1 hour at 80 V. A 100 bp DNA ladder (New England Biolabs, Ipswich, USA) was used Gels were viewed under ultraviolet (UV) light and analysed using Quality 4.6.4 and ChemDoc software (Bio-Rad, Hercules, USA). Bands were excised under UV light and products purified using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Briefly, bands were weighed and Buffer QC was added at a 1:6 ratio, after which bands were dissolved at 50 ^{o}C for 10 minutes. Isopropanol was added at a 1:1 weight of band to volume of isopropanol. The dissolved gel was then bound to a QIAquick column using centrifugation (10,000g). The column was washed with 750 μl Buffer PE and centrifuged (10,000g), before being left to dry for 2 minutes. DNA was eluted using 30μl water. Products were verified by sequencing (GATC Biotech, Germany).

2.19 IP_3 Competitive ELISA Assay

An IP_3 rat ELISA assay was purchased from Bluegene Biotech (Shanghai, China), all buffers used were included with the kit. One whole aortae, or one mesenteric bed, was homogenised in 150 µL ice-cold PBS containing (in mM) 137 NaCl, 2.7 KCl, 10 Na_2PO_4,1.8 K_2PO_4 (pH7.4 @ 21 ^oC), followed by 2 rounds of sonication. Homogenates were centrifuged at 1500 x *g* for 15 minutes at 4 ^oC. After pelleting cell debris, the supernatant was incubated in pre-coated anti-IP_3 wells along with 10 µL Balance Solution and 50 µL Conjugate Solution for 1 hour at 37 ^oC. This was followed by 3 washes with wash buffer, then a final drying of the wells by inverting the wells onto a paper towel. To dry wells, 50 µL of Solution A and 50 µL of Solution B were added and the wells mixed, followed by incubation for 30 minutes at 37 ^oC Stop Solution was then added, wells were mixed well and the optical density at 450 nm ($O.D_{450}$) was measured on a spectrophotometer. Known concentration standards were also treated as the samples apart from the addition of balance solution. These were used to produce a standard curve to quantify IP_3 levels in tissue lysates.

2.20 Statistical Analysis

Results are expressed as the mean ± S.E.M. Intergroup differences were analysed using repeated measures ANOVA followed by Newman-Keuls post-hoc test for experiments where a single cell was exposed to three treatments, or Student's t-test for simple comparisons between two groups; levels of significance were * $p < 0.05$, ** $p < 0.01$. All data was tested for normality using a Shapiro-Wilk normality test.

Results

3 Epac activates Ca^{2+}/calmodulin-dependent kinase 2 (CaMKII) in arterial smooth muscle

3.1 Introduction

Following the identification of Epac as a downstream target of cAMP, much research has been undertaken to explore it's involvement in a number of cAMP-mediated signalling pathways (de Rooij et al. 1998; Kawasaki et al. 1998). Our group has previously explored the role of Epac in vasculature, showing Epac activation produces relaxation of rat mesenteric arteries (Roberts et al. 2013). Epac specifically modulates Ca^{2+} sparks in vascular smooth muscle cells, but the mechanism by how it does this is unknown. In line with the aims of this thesis, we explored possible mechanisms by how Epac increases Ca^{2+} sparks, and with it STOCs. Very little research has been undertaken on Ca^{2+} spark modulation in smooth muscle, however an abundant amount of research exists on Ca^{2+} spark modulation in cardiomyocytes. Indeed, a protein commonly shown to modulate Ca^{2+} sparks in cardiomyocytes is calcium/calmodulin-dependent protein kinase II (CaMKII), which has been shown downstream on Epac activation in cardiomyocytes (Oestreich et al. 2007; Pereira et al. 2007).

3.1.1 Ca^{2+}/calmodulin-dependent kinase 2 (CaMKII)

CaMKII, one of the 74 kinases within the Serine/Threonine Ca^{2+}/CaM-dependent kinase family, is a large multifunctional kinase that is made up of 12 subunits that stack in 2 hexameric rings (Rellos et al. 2010; Manning et al. 2002). CaMKII is produced from 4 closely-related genes (α, β, γ and δ) that produce 4 isoforms (Swulius & Waxham 2013). In addition, CaMKII genes

undergo numerous alternative splicing events, giving rise to a number of different variants 50-68kDa (Swulius & Waxham 2013; Toussaint et al. 2016). Each subunit of the CaMKII holoenzyme consists of 3 domains, an N-terminal catalytic region, an autoregulatory domain and a C-terminal association domain responsible for forming the dodecameric structure (Hoelz et al. 2003; Hudmon & Schulman 2002) **(Figure 3.1).**

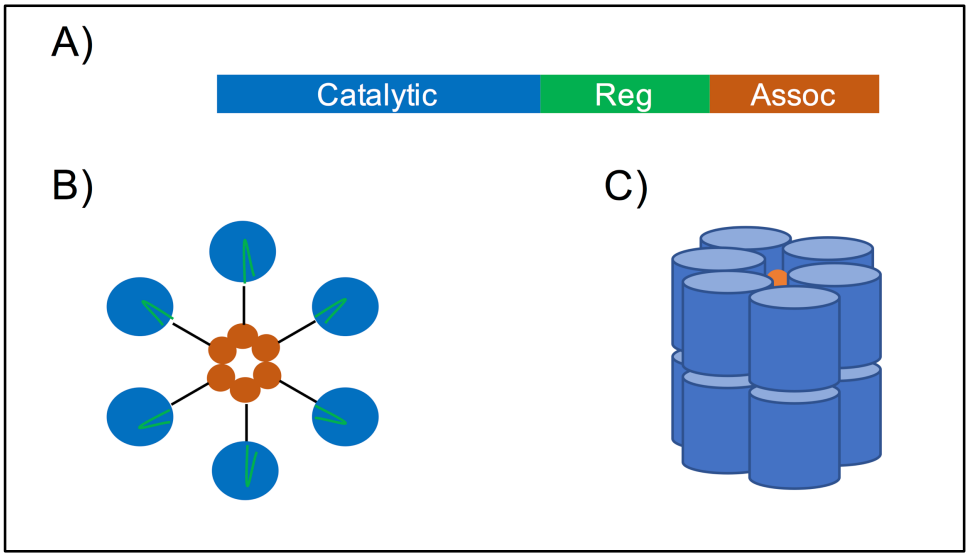


Figure 3.1– CaMKII Structure. A) CaMKII consists of three domains, catalytic, autoregulatory and association. **B)** Schematic of CaMKII forming a hexameric ring. **C)** Two hexameric rings form to produce the dodecameric CaMKII holoenzyme.

Under resting conditions, the autoregulatory domain inhibits the activity of the catalytic region through tight affinity interactions (Soderling & Stull 2001; Payne et al. 1988). Binding of Ca^{2+}/CaM "opens up" the structure and disinhibits CaMKII (Payne et al. 1988). Upon this activation, a single CaMKII subunit can phosphorylate a neighbouring CaMKII subunit at a threonine residue $T^{286/7}$, known as autophosphorylation (Meyer et al. 1992). Threonine 286 (287 in the β,γ,δ isoform) is conserved in all four α, β, γ and δ. This phosphorylation produces autonomous activity, where the removal of Ca^{2+}/CaM does not reduce activation. This autonomous activity is due to the

position of the phospho-threonine, which inhibits interactions between the catalytic and the regulatory domains and thus reformation of the closed/inactive state. This activation continues until the phosphate is removed by a phosphatase (Erickson 2014). This gives the kinase so-called 'memory' of previous Ca^{2+} events. See *Coultrap and Bayer* (2012) for an in-depth review on CaMKII (Coultrap & Bayer 2012).

In differentiated vascular smooth muscle, which relies on complex patterns of Ca^{2+} signalling, surprisingly little is known about the functional role of this Ca^{2+}-sensing enzyme. The main CaMKII holoenzymes found in vascular smooth muscle form from splice products of CaMKIIγ and CaMKIIδ genes (Singer 2012). CaMKIIγ expression dominates in contractile smooth muscle, with CaMKIIδ expressed as a relatively minor isoform (Kim et al. 2000; Marganski et al. 2005). CaMKIIδ is up-regulated in synthetic/proliferating smooth muscle and is required for injury-induced neointima formation, largely through its regulation of the expression and activity of cell cycle activators (Li et al. 2011). In large blood vessels (ferret aortae and pig carotid artery) CaMKII is activated in response to contractile stimuli, with activation maintained throughout tonic contraction (Kim et al. 2000; Rokolya & Singer 2000) . However, in mesenteric arteries, CaMKII deletion has no effect on vascular tone in response to high extracellular K^+, angiotensin II, or phenylephrine (Prasad et al. 2013). Instead, CaMKII supports intracellular Ca^{2+} homeostasis by phosphorylating and maintaining the activity of voltage-dependent Ca^{2+} channels and phospholamban (Prasad et al. 2013).

3.2 Aim

The aim of this part of the study was to:

1. Determine the expression of CaMKII isoforms within mesenteric artery;
2. Assess isoform-selective activation by Epac;
3. Establish the role of CaMKII activation in Epac-induced STOC activity in rat mesenteric artery smooth muscle cells.

3.3 Results

3.3.1 Epac1 is widely expressed in vascular beds

As a starting experiment, we first assessed the relative expression of Epac in a range of vascular beds, including aorta, cerebral, femoral, mesenteric and renal beds, using immunoblot analysis (**Figure 3.2**). Immunoblots of arterial lysates, using antibodies against Epac1, produced an immunoreactive doublet at ~100 kDa, the predicted molecular weight for Epac1 (Kawasaki et al. 1998). Epac1, the product of the *RAPGEF3* gene, possesses several splice variants explaining the visualisation of a doublet (Isoform 1- 104kDa, Isoform 2 – 100 kDa (Uniprot Database (Q9Z1C8). Immunoblots using antibodies against Epac2 produced no immunoreactive bands for arterial lysates, however the positive control (rat ventricular tissue) produced a clear doublet at ~120 kDa, the predicted molecular weight for Epac2 (Purves et al. 2009). Expression levels of Epac1 varied between different vascular beds, with the highest expression found in cerebral arteries and the lowest in femoral arteries (**Figure 3.2;** equal protein loading 20 μg per lane).

Previous work from our lab using a combination of myography, Ca^{2+} imaging and electrophysiology has shown that selective activation of Epac in rat mesenteric artery induces vasorelaxation, by increasing Ca^{2+} spark and STOC

activity (Roberts et al. 2013). Since we have significant background information in this artery, and the mesenteric bed provides relatively large amounts of tissue (compared to for example cerebral arteries), we chose to conduct subsequent studies on these resistance vessels.

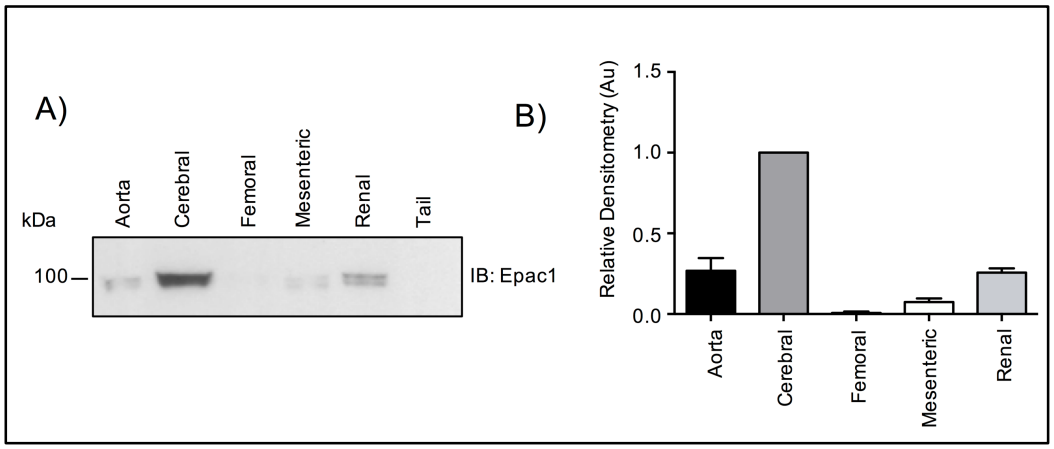


Figure 3.2- Epac1 is not equally expressed throughout the arterial vasculature. A) Proteins within lysates from different vascular beds (lysates equalized for protein content; 20 μg per lane) were separated on 8% polyacrylamide gels. Membranes probed using anti-Epac1 (Cell Signaling Technology 1:1000) showed variation in Epac1 expression in different vascular beds. **B)** Summary of relative Epac expression in various artery beds, with cerebral lysates being given a value of 1 due to the highest intensity.

3.3.2 mRNAs for multiple splice variants of CaMKII exist in the vasculature

We next assessed the expression of CaMKII within different vascular beds. Reverse transcription-PCR, using cDNA derived from arterial lysates as a template and primers designed to detect CaMKII isoforms, showed clear δ and γ isoform expression in all vascular beds (**Figure 3.3A**). CaMKIIβ was also present. This isoform is predominantly expressed in neuronal and endothelial tissue, as assessed by immunocytochemistry and immunoblotting (Soderling & Stull 2001; Toussaint et al. 2015). Whole arterial lysates contain a combination of different cell types, and we believe that the presence of the β isoform likely represents the presence of cDNA originating from nerve and

endothelial cell-derived mRNA. Primers designed to amplify Von Willeband factor, an endothelial cell marker, also produced clear PCR products, confirming the presence of cDNA originating from the endothelium within our cDNA library. Alternative splicing of the δ and γ genes produces numerous splice variants (Toussaint et al. 2016). Thus, we next designed PCR primers to detect δ and γ splice variants. These splice variant primers were designed so that different splice variants produced different sized PCR products. This was confirmed by amplifying numerous variants from other tissues (**Figure 3.3C**).

RT-PCR using splice variant-specific primers confirmed the presence of mRNA for at least two γ variants and two δ variants in mesenteric arteries (**Figure 3.3B**). Sequencing of these products revealed them to be γ predicted isoforms X18 and X16, and δ isoforms 2 and/or 3.

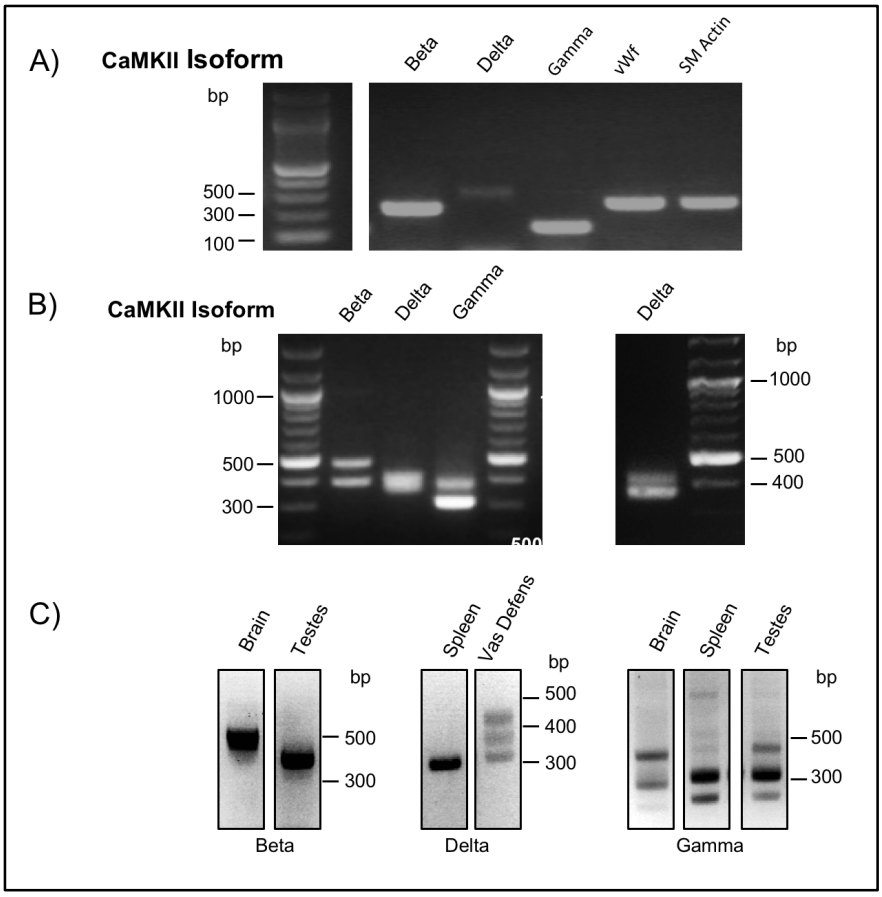


Figure 3.3– Mesenteric artery cDNA library contains numerous CaMKII Isoforms and Splice Variants. A) Mesenteric artery cDNA library was examined for CaMKII β, δ and γ expression. Primers designed to amplify Von Willeband Factor (endothelial cell marker) and smooth muscle α actin (smooth muscle marker) were also used. PCR products were separated on a 1.5% agarose gel **B)** The same cDNA library as in A was used to examine which β, δ and γ splice variants were expressed. Following PCR, 3% agarose gels were used to allow separation of products **C)** Numerous rat tissues cDNA libraries were examined to confirm that splice variant primers detect multiple variants. PCR products were separated on 3% agarose gels.

3.3.3 Multiple splice variants of CaMKII are expressed in the vasculature

Consistent with the expression of multiple CaMKII mRNA isoforms, immunoblots of arterial lysates using pan-specific CaMKII antibodies, which bind all four isoforms of CaMKII, produced three or four distinct immunoreactive bands (**Figure 3.4**). Interestingly, different arterial beds appeared to produce different banding patterns in relation to the relative density between certain bands. Aortic preparations possessed a more intense

lower molecular weight (MW) band in contrast to cerebral, in which the higher MW band was prominent (**Figure 3.4C**).

3.3.4 Selective Epac activation causes autophosphorylation of specific CaMKII isoforms in rat mesenteric artery

To assess the effect of Epac activation on CaMKII activity, we incubated arteries in the selective Epac activator 8-pCPT-2`-*O*-Me-cAMP (8-pCPT; 10μM) or vehicle control (DMSO). As a positive control, we also incubated mesenteric arteries in the Ca^{2+} ionophore A23187 (12.5μM), which would be expected to globally raise intracellular [Ca^{2+}] and activate CaMKII. Immunoblot analysis of 8-pCPT-treated arterial lysates indicated phosphorylation of CaMKII at $pThr^{286}$, an autophosphorylation site that indicates CaMKII activation, following 8-pCPT exposure (n=3; **Figure 3.4**). Of the three or four immunoreactive bands detected by pan-specific anti-CaMKII, one band (second from bottom) strongly bound phospho-CaMKII antibodies against $pThr^{286/7}$ following 8-pCPT-AM treatment. Exposure to A23187 induced phosphorylation of all immunoreactive bands detected by pan-specific anti-CaMKII (**Figure 3.4**).

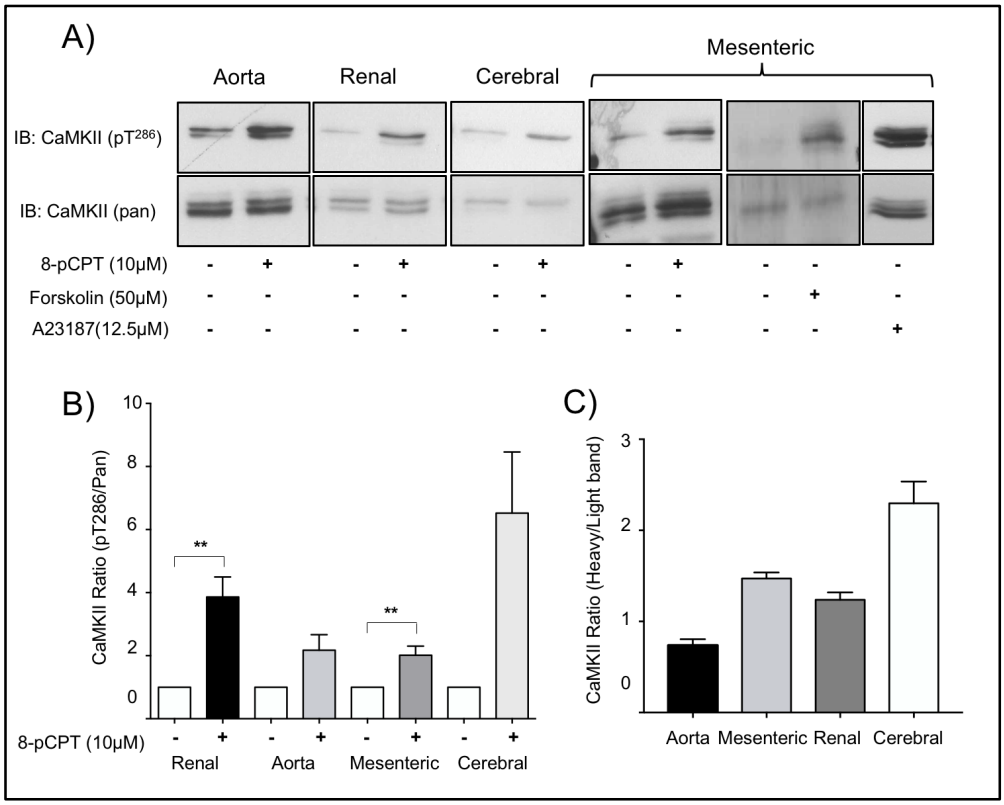


Figure 3.4- Epac activation induces autophosphorylation of CaMKII. A) Representative immunoblots of CaMKII activation in aorta, renal, cerebral and mesenteric vasculature in response to 8-pCPT. **B)** Histogram of CaMKII pT^{286}/Pan ratio in vascular beds in control and stimulated vessels (n=3 animals). **C**) The two major CaMKII bands present in the vasculature are expressed at different proportions in various vascular beds.

3.3.5 Selective Epac activation causes autophosphorylation of predominantly CaMKII γ isoforms

To identify the CaMKII isoform/s differentially phosphorylated by Epac activation, we used immunoblotting to analyse arterial tissue obtained from mice in which the gene encoding either CaMKIIδ or CaMKIIγ had been globally knocked out (Backs et al. 2009; Backs et al. 2010). Immunoblots of arterial lysates from wildtype mice using CaMKII antibodies showed a similar pattern of three/four immunoreactive bands as observed in rat tissue (**Figure 3.5**). In arterial lysates obtained from CaMKIIδ knockout animals, the lowest molecular weight band was absent. In contrast, in CaMKIIγ knockout lysates, the lower

band remained but the higher molecular weight bands were missing (**Figure 3.5)**

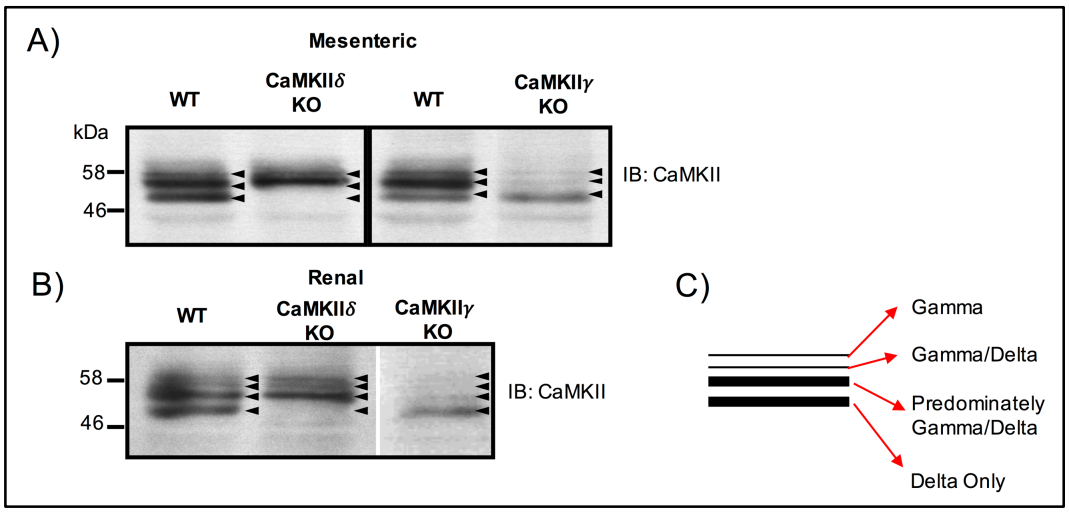


Figure 3.5–Arterial lysates from knockout mice map immunoreactive bands to CaMKIIδ and γ isoforms. A) Representative immunoblots of mesenteric artery lysates from wildtype mice, CaMKIIδ and CaMKIIγ knockout mice. **B**) Representative immunoblots of renal artery lysates from wildtype mice, CaMKIIδ and γ knockout mice **C)** Simplified diagram showing which isoform is responsible for each band in CaMKII immunoblots.

The potential involvement of CaMKIIδ downstream of Epac activation was confirmed by 2D western blot, which separated isoforms on that basis of isoelectric point (P_I; 3-10 IPG Strip) and molecular weight **(Figure 3.6)**. Immunoblots for CaMKII (pT^{286}) and CaMKIIδ showed that the highest intensity "dot" when probed with CaMKII (pT^{286}) was the same "dot" as that when probed with an anti-CaMKIIδ antibody (**Figure 3.6).** Knock out (KO) animals were not used for functional studies as δ and γ isoforms are known to functionally compensate for each other (Kreusser et al. 2014).

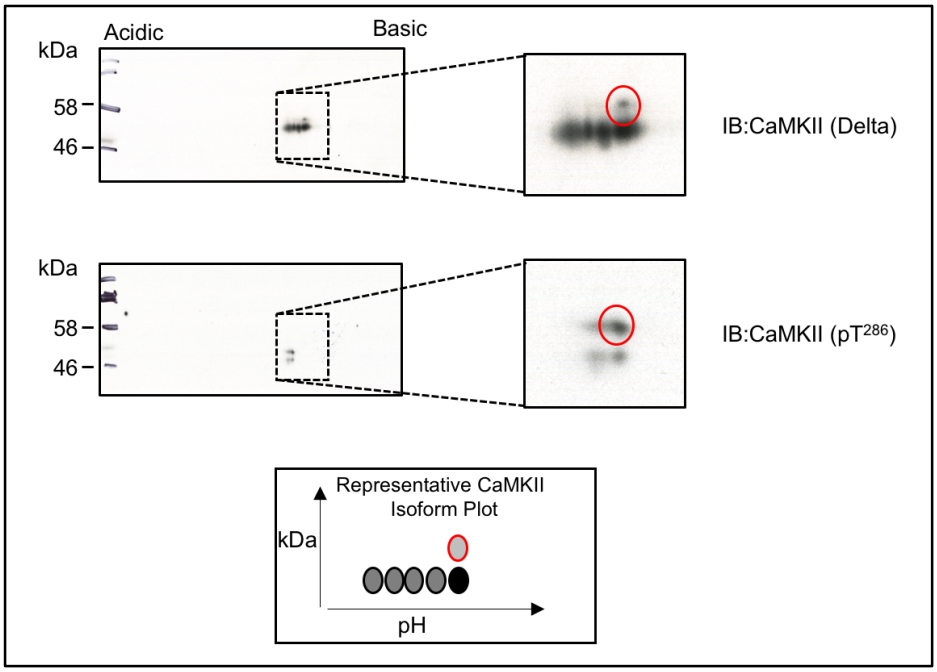


Figure 3.6- CaMKIIδ cannot be ruled out of Epac mediated CaMKII activation. Shown is an immunoblot of 8-pCPT-AM stimulated rat mesenteric arterial lysates separated using 2D electrophoresis and western blotting. During 2D electrophoresis proteins are simultaneously separated by molecular weight and isoelectric point. Proteins were subsequently immunoblotted against CaMKIIδ and CaMKII (pT^{286}). Bottom is a schematic of the observed CaMKII immunoblots. Circled in red is the protein "dot" which is present in both immunoblots and overlaps in both immunoblots.

3.3.6 Inhibition of CaMKII attenuates the increase in STOC frequency mediated by Epac in rat mesenteric myocytes

Selective activation of Epac with 8-pCPT-AM induces vasorelaxation by increasing both the frequency and amplitude of STOCs in rat mesenteric beds **(Figure 3.7)** (Roberts et al. 2013).

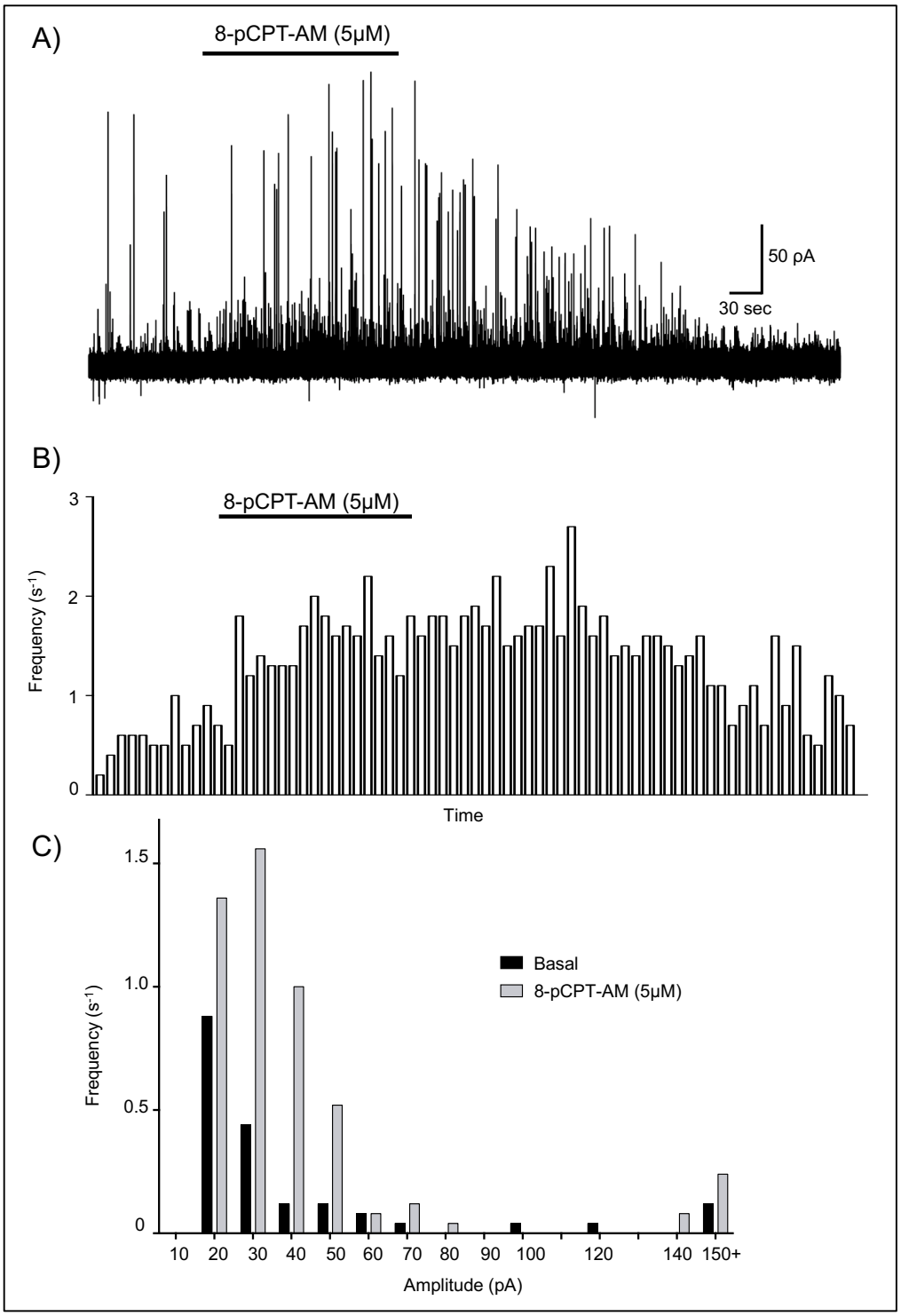


Figure 3.7- Epac activation with 8-pCPT-AM produces an increase in STOC frequency and a higher probability of larger STOCs. A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole cell configuration. 8-pCPT-AM was applied before wash out **B)** Frequency histogram of the representative cell above. **C)** STOC amplitude probability in basal and 8-pCPT-AM incubated conditions.

Several parameters were explored in basal and stimulated (8-pCPT-AM) conditions: frequency, STOC duration, STOC rise time (baseline to peak), STOC decay time (peak to return of baseline), charge per STOC, total outward charge per analysed segment and average STOC amplitude. Decay and rise rate were also obtained by dividing amplitude by rise or decay time. No difference was seen in STOC duration, rise time, decay time. This is in line with the Ca^{2+} sparks analysed by Roberts *et al.* (2013) who saw no difference in decay rate. An increase in charge was expected as it is this charge that produces the hyperpolarisation of the cell, as seen by Roberts *et al.* 2013. Epac mediated STOCs are larger in amplitude under Epac activation compared to control **(Table 3.1)**.

Parameter	Control	8-pCPT-AM	P-value
Frequency (s^{-1})	0.62 ± 0.11	1.71 ±0.22	**0.013**
Amplitude (pA)	19.24 ±1.92	25.52 ± 1.72	**0.043**
Duration (s)	0.031 ± 0.003	0.036 ± 0.003	ns
Duration/Amplitude (s/pA)	0.0017 ± 0.0001	0.0014 ± 0.00007	**0.0326**
Rise Time (s)	0.013 ± 0.001	0.015 ± 0.001	ns
Decay Time (s)	0.018 ± 0.002	0.021 ± 0.003	ns
Rise Rate (pA/s)	1637 ± 183.5	2241 ± 191.7	ns (0.073)
Decay Rate (pA/s)	1404 ± 201.4	1629 ± 113.7	Ns
Charge- Sum	2.30 ± 0.68	8.59 ± 1.46	**0.023**
Charge- per STOC	0.46 ± 0.07	0.66 ± 0.06	Ns (0.077)

Table 3.1– Table summarising the effects of Epac activation on STOC kinetics: Epac activation significantly effects Frequency, Amplitude, Duration/Amplitude and Charge per cell condition. Epac activation does no significantly affect other parameters such as total duration, rise/decay time, rise/decay rate and charge per STOC

Furthermore, 8-pCPT-AM-induced STOCs were inhibited by pre-incubation of cells in the RyR inhibitor, ryanodine, confirming that they are triggered by underlying RyR/Ca^{2+} spark activity. In other experiments STOC frequency was significantly attenuated by the application of ryanodine after 8-pCPT-AM induced STOCs were activated (2.54 ± 0.59 STOCs s^{-1} vs. 0.58 ± 0.18 STOCs s^{-1}, mean ± SEM, $p<0.05$, paired t-test, n=4 **(Figure 3.8)**.

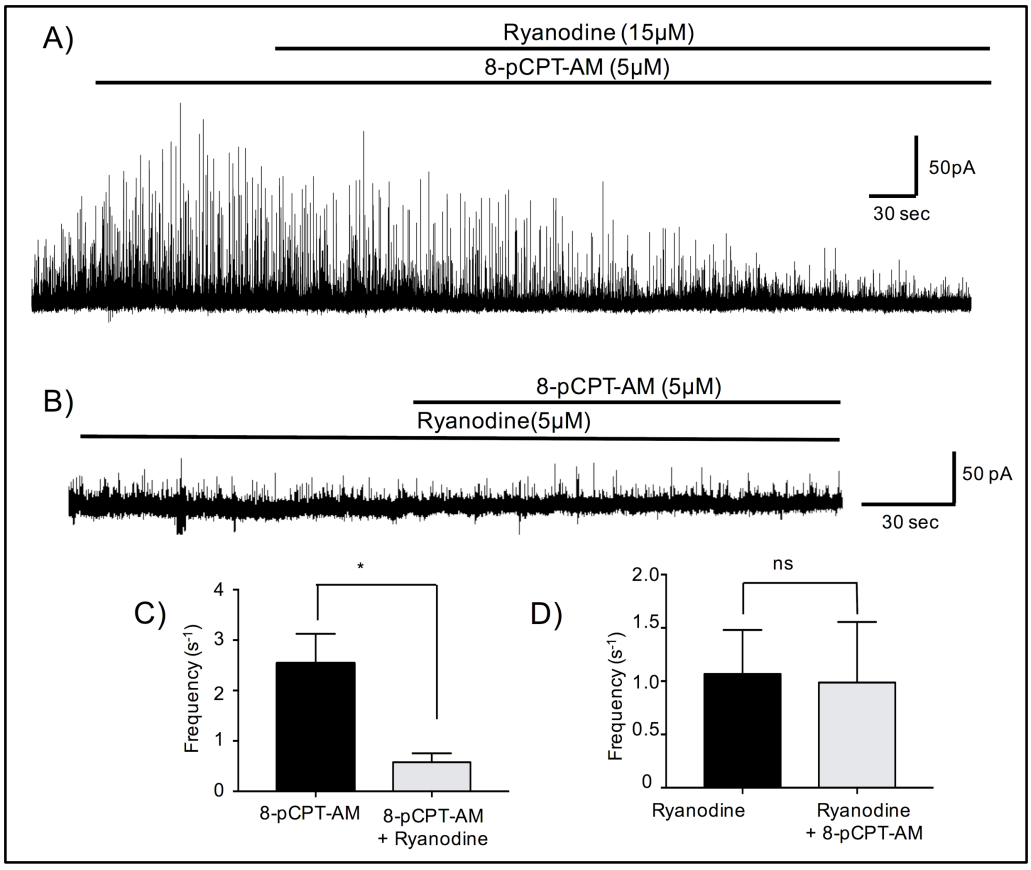


Figure 3.8– Epac activation produces its effects through ryanodine receptors in RMASMCs. A) STOCs recorded in a freshly isolated rat mesenteric artery smooth muscle cell under whole-cell configuration. Application of ryanodine reversed the increase in STOC frequency and amplitude induced by 8-pCPT-AM. **B**) In cells pre-incubated in ryanodine (15 μM), application of 8-pCPT-AM does not increase STOC activity (n = 3). **C)** Summary of STOC frequency for experiments shown in (A). **D)** Summary of STOC frequency for experiments shown in (B).

To assess whether 8-pCPT-induced activation of CaMKII is involved in the activation of STOC activity, we used the whole-cell recording technique to record STOCs in mesenteric artery myocytes following activation of Epac in

the presence and absence of pharmacological inhibitors of CaMKII. Application of the CaMKII inhibitor KN-93 (500nM) reversed the increase in STOC frequency (1.71 ± 0.22 STOCs s^{-1} to 0.61 ± 0.18 STOCs s^{-1}; mean ± SEM) induced by 8-pCPT-AM (5 μM; $p<0.05$; n=5; paired t-test; **Figure 3.9**). The mean amplitude of STOCs in the presence of 8-pCPT-AM was significantly different to the mean amplitude in the presence of 8-pCPT-AM + KN93 (32.6 ± 3.00 pA vs. 25.86 ± 2.43 pA, mean ± SEM, $p<0.01$, paired t-test, n=5). To further assess and confirm it is the inhibition of CaMKII that reverts STOC frequency to baseline, KN-92, the inactive analogue of KN-93, was used.

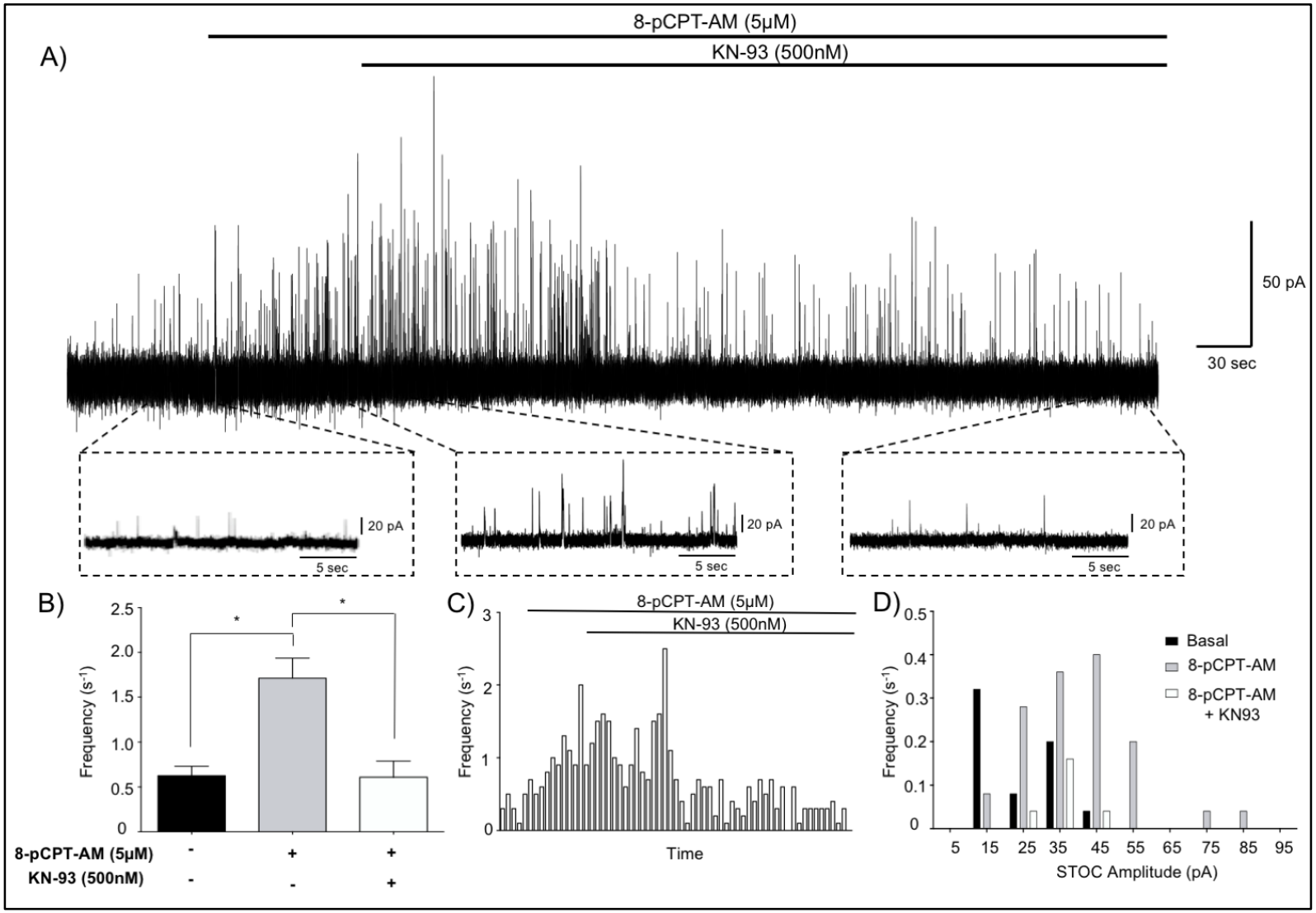

A)
8-pCPT-AM (5μM)
KN-93 (500nM)
50 pA
30 sec
20 pA
5 sec
20 pA
5 sec
20 pA
5 sec
B)
Frequency (s-1)
2.5
2.0
1.5
1.0
0.5
0
*
*
8-pCPT-AM (5μM) - + +
KN-93 (500nM) - - +
C)
8-pCPT-AM (5μM)
KN-93 (500nM)
Frequency (s-1)
3
2
1
0
Time
D)
Frequency (s-1)
0.5
0.4
0.3
0.2
0.1
0
Basal
8-pCPT-AM
8-pCPT-AM + KN93
5 15 25 35 45 55 65 75 85 95
STOC Amplitude (pA)

Figure 3.9– Epac-mediated increase in STOCs are attenuated by CaMKII inhibition (KN-93). A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle in whole cell configuration exposed to 8-pCPT-AM (5 μM), followed by addition of the CaMKII inhibitor, KN-93 (500 nM). 25 second inserts highlight analysed areas. Bar indicated 20 pA**. B)** Summary of STOC frequency for experiments as in A (n=5) **C**) STOC frequency histogram of A. **D)** Probability frequency histogram of the STOC amplitude from A.

Unlike KN-93, addition of KN-92 (500 nM) did not reverse the increase in STOC frequency induced by 8-pCPT-AM back to basal STOC frequency (1.05 ± 0.36 STOCs s^{-1} vs. 1.61 ± 0.38 STOCs s^{-1}, mean ± SEM, $p>0.05$, paired t-test, n=3) **(Figure 3.10)**. To further confirm the involvement of CaMKII, we also undertook experiments using autocamtide-2-inhibitory peptide (AIP), a highly specific peptide inhibitor of CaMKII that corresponds to its autoinhibitory domain (Ishida et al. 1995).

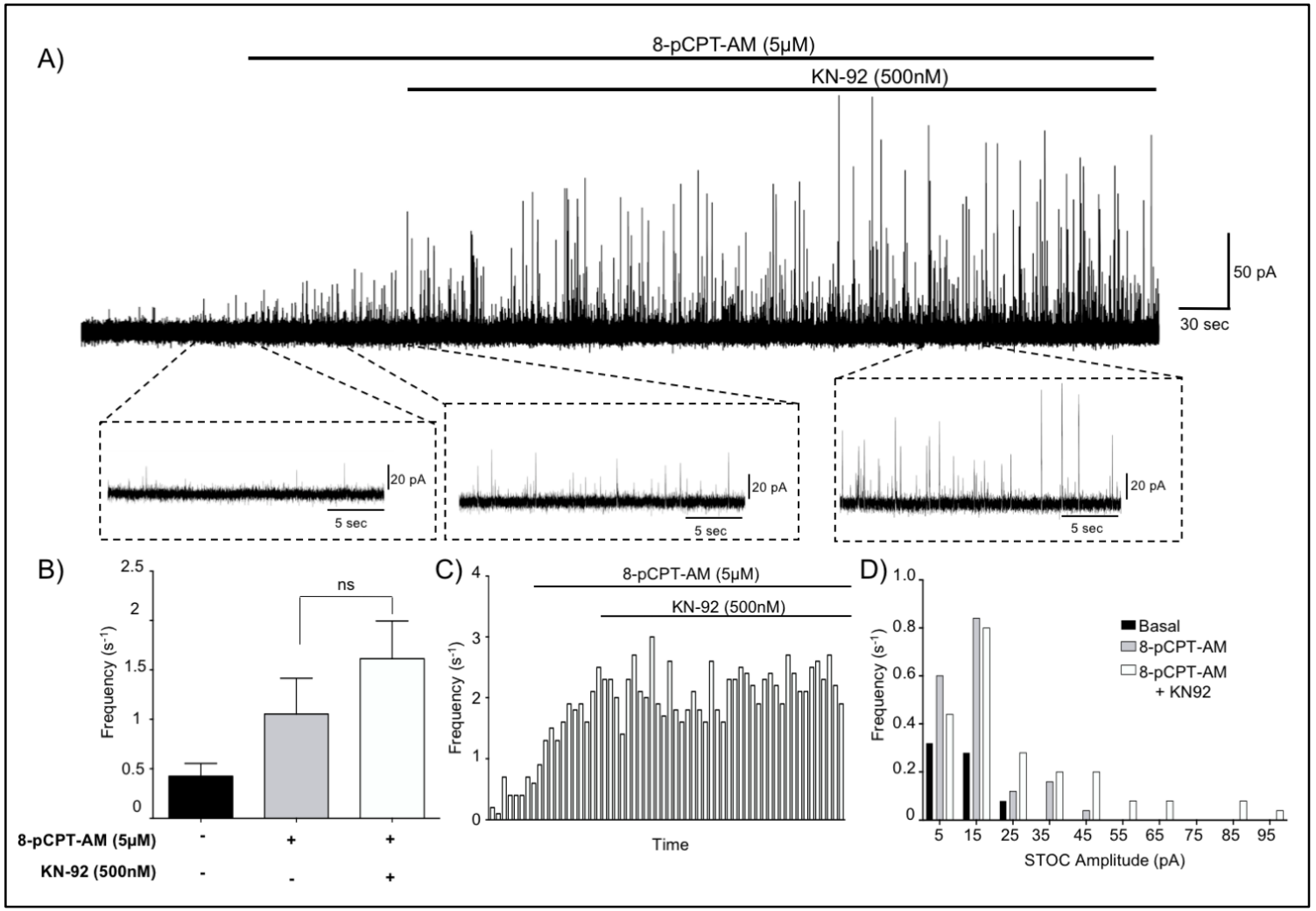

A)
8-pCPT-AM (5μM)
KN-92 (500nM)
50 pA
30 sec
20 pA
5 sec
20 pA
5 sec
20 pA
5 sec
B)
ns
Frequency (s-1)
2.5
2
1.5
1
0.5
0
8-pCPT-AM (5μM) - + +
KN-92 (500nM) - - +
C)
8-pCPT-AM (5μM)
KN-92 (500nM)
Frequency (s-1)
4
3
2
1
0
Time
D)
Basal
8-pCPT-AM
8-pCPT-AM + KN92
Frequency (s-1)
1.0
0.8
0.6
0.4
0.2
0
5 15 25 35 45 55 65 75 85 95
STOC Amplitude (pA)

Figure 3.10- Epac-mediated increase in STOCs are not affected by KN-92, the inactive analogue of KN93. A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole-cell configuration exposed to 8-pCPT-AM (5 μM), followed by addition of KN-92 (500 nM), an inactive analogue of KN-93. Inserts highlight analysed areas. Bar indicates 20 pA. **B)** Summary of STOC frequency for experiments shown in A (n=3) **C)** Frequency histogram of the above representative trace against time. **D)** Probability frequency histogram of STOC amplitude of the cell in A.

Inclusion of this peptide in the pipette-filling solution (1μM), followed by an incubation to allow cell dialysis, prevented Epac-mediated STOC frequency increases (1.08 ± 0.23 STOCs s^{-1} vs. 0.59 ± 0.12 STOCs s^{-1}, mean ± SEM, $p<0.05$, paired t-test, n=4; **Figure 3.11)**. The statistical analysis in-fact showed a decrease in STOC frequency in the presence of Epac activation with inclusion of AIP. This we believe is due to delayed inhibition of basal CaMKII activity by AIP during the experiment. This basal CamKII activation can be seen with a degree of CamKII autophosphorylation in control arteries (**Figure 3.4)**.

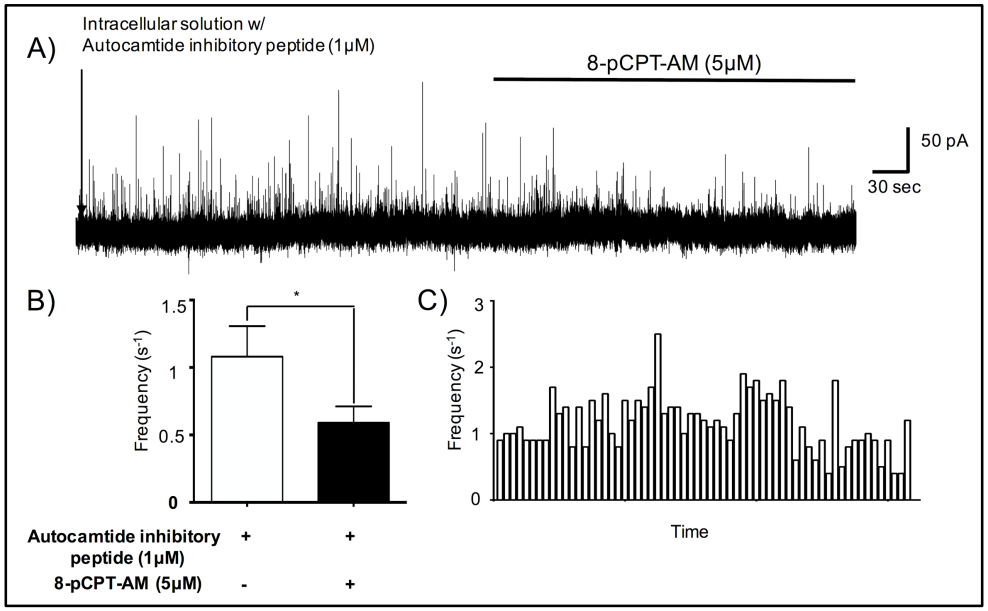


Figure 3.11– CaMKII inhibition by an intracellular blocking peptide inhibits Epac mediated STOC increases. A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole-cell configuration exposed to 8-pCPT-AM (5μM) after dialysis of autocamtide inhibitory peptide (1μM), the CaMKII inhibitor peptide. **B)** Summary of STOC frequency from experiments as in A (n=4). **C)** Frequency histogram of the above representative trace.

3.4 Discussion

The key findings of this chapter are:

1. Vascular tissue expresses several different isoforms of CaMKII that may vary in relative levels between different vascular beds;
2. Selective activation of Epac causes autophosphorylation (activation) of CaMKII, primarily the γ isoforms;
3. Activation of CaMKII is required to mediate Epac-driven increases in STOC activity.

The main downstream targets for CaMKII phosphorylation in the heart are RyRs, a major class of Ca^{2+} release channel on the SR, and phospholamban, a protein that controls the activity of the SERCA pump. CaMKII phosphorylation of these targets increases store Ca^{2+} release by increasing both cardiac RyR2 Ca^{2+} sensitivity and Ca^{2+} content of the store (Grimm et al. 2015; Ai et al. 2005; X. H.T. Wehrens et al. 2004).

The main CaMKII holoenzymes found in vascular smooth muscle form from splice products from CaMKIIγ and CaMKIIδ genes (Singer 2012). Consistent with this we found messenger RNA encoding at least two CaMKIIγ variants and two CaMKIIδ variants in rat mesenteric artery. The expression of multiple CaMKII isoforms in this tissue was confirmed by immunoblots of arterial lysates using pan-specific CaMKII antibodies that produce three or four immunoreactive bands, only one of which was significantly phosphorylated following Epac activation. By immunoblotting arterial tissue obtained from mice in which the gene encoding either CaMKIIδ or CaMKIIγ had been globally deleted (Backs et al. 2010; Backs et al. 2009), we were able to determine that Epac activation predominantly induced phosphorylation of CaMKIIγ variants.

This is consistent with the finding that CaMKIIγ is the most abundant isoform in differentiated smooth muscle. Studies in ferret aortae suggest it regulates contractile pathways (Kim et al. 2000; Marganski et al. 2005). We cannot entirely rule out the possibility that δ variants made up a minor component of the band that strongly bound phospho-CaMKII antibodies following 8-pCPT-AM treatment. Faint anti-CaMKII immunoreactive bands persisted at this molecular weight (approx. 50kDa) in CaMKIIγ knockout lysates, and it is possible that δ and γ subunits exist together in the functional holoenzyme complex. A CaMKIIγ specific antibody was not used due to cross-detection of CaMKII isoforms from all commercially available CaMKIIγ antibodies. Using 2D electrophoresis to further separate CaMKII isoforms, based on isolectric point and molecular weight (Rabilloud & Lelong 2011), we also saw an overlap between CaMKIIδ and CaMKII pT^{286} 2D immunoblots. Interestingly, the two major PCR products we detected using primers designed to amplify CaMKIIγ isoforms are predicted splice variants of the CaMKIIγ gene, whose expression has never been reported. Thus, the CaMKII isoforms involved in this pathway may be novel.

Since these biochemical experiments were conducted in whole arteries we cannot fully distinguish whether the isoforms were detected in smooth muscle cells, endothelial cells or innervating nerves. Involvement of CaMKII in Epac-mediated STOCs was therefore confirmed using patch-clamp electrophysiology on single isolated arterial myocytes. Epac activation increased STOC frequency and amplitude in freshly isolated mesenteric artery smooth muscle cells, which was attenuated to baseline by addition of the CaMKII inhibitor KN-93. KN-93 inhibits CaMKII by competitively blocking CaM

binding to the kinase (Pellicena & Schulman 2014), but can produce off-target effects, mostly notably on voltage-gated K^+ channels and I_{ca} (L-type calcium current from ventricular cardiomyocytes) (Anderson et al. 1998; Rezazadeh et al. 2006) . Important to note is that the use of 500 nM KN-93, along with a -20mV holding potential minimised these off-target effects (Pellicena & Schulman 2014; Anderson et al. 1998). KN-92, the inactive analogue of KN-93, did not attenuate Epac-mediated STOCs, while intracellular inclusion of the CaMKII inhibitor peptide, autocamtide related peptide 2 (AIP) prevented Epac-mediated STOC frequency increases.

4 CaMKII activation by Epac is mediated through IP_3 dependent Ca^{2+} release

4.1 Introduction

CaMKII is activated by the binding of Ca^{2+}/calmodulin to the regulatory domain of the enzyme. CaMKII possesses a K_{CaM} (concentration of CaM needed to half activate CaMKII) of ~25 nM at saturating Ca^{2+} concentrations. A half maximal activation is produced at 0.5-1 μM Ca^{2+} in non-saturating conditions (Soderling & Stull 2001; Hudmon & Schulman 2002). *In vitro* application of ATP allowing CamKII activation, and with it autophosphorylation, reduces this half maximal activation to 150pM (Hudmon & Schulman 2002). This reduction in half maximal activation allows prolongation of activation, commonly also called activation memory. The [Ca^{2+}] elevations responsible for activation of CaMKII are produced through Ca^{2+} release from localised subcellular stores, such as the nucleus and sarcoplasmic reticulum (X. Wu et al. 2006; Mishra et al. 2011) or potentially through a novel extracellular Ca^{2+} influx (Chandra & Angle 2005).

4.1.1 Epac activation by CaMKII in Cardiomyocytes

Two models currently exist. One proposes that Epac-mediated CaMKII activation is activated by inositol trisphosphate (IP_3) -mediated Ca^{2+} release (Ruiz-Hurtado et al. 2013; Pereira et al. 2012), the second proposes that CaMKII is activated in a Ca^{2+}-independent manner, through phosphorylation by protein kinase C (PKC), possibly increasing CaMKII Ca^{2+} sensitivity by phosphorylation of $pT^{286/7}$ (Oestreich et al. 2009). Both models place the enzyme phospholipase C (PLC) upstream of CaMKII activation, since PLC

forms both IP_3 and diacylglycerol (DAG), which subsequently activates PKC (Katan 2005).

A role for IP_3 was proposed as CaMKII activation could not be produced by Epac activation in the presence of 2-aminoethoxydiphenylborate (2APB), an IP_3 receptor antagonist (Pereira et al. 2007). Protein kinase C has also been shown, *in vitro* and *in vivo*, to activate CaMKII (Waxham & Aronowski 1993; Oestreich et al. 2007), however Pereira *et al.* saw no inhibition of Epac mediated Ca^{2+} spark activity in the presence of chelerythrine, a PKC inhibitor, suggesting no role of PKC in Epac mediated spark modulation.

In opposing results, Oestreich *et al.* (2009) showed that 8-pCPT addition increased electrically-evoked Ca^{2+} transients in cardiomyocytes, which could be inhibited by bisindolylmaleimide IX, a PKC inhibitor. Unlike Pereira *et al.*, Oestreich *et al.* saw no effect of 2-APB on the Ca^{2+} transient evoked by Epac activation (Oestreich et al. 2009) **(Figure 4.1)**.

4.1.2 Phospholipase C involvement in Epac-mediated signalling

Upstream in both models lies the IP_3 and DAG-producing enzyme, phospholipase C. The phospholipase C family is a class of membrane-associated enzymes that cleave phosphatidyl 4,-5 inositol bisphosphate (PIP_2) to release IP_3 (the head group), and diacylglycerol (DAG). There are thirteen kinds of mammalian phospholipase C that are classified into six isotypes (β, γ, δ, ε, ζ, η) according to structure (Katan 2005). PLCε contains a PIP_2 catalytic domain, a CDC25-HD domain and a Ras Association (RA) domain (Song et al. 2001; Kelley et al. 2001). The PIP_2 catalytic domain is responsible for the cleavage of PIP_2 to IP_3 and DAG, responsible for Ca^{2+} release through IP_3

receptors and PKC activation, respectively. PLCε is an unusual PLC isoform in that it possesses an N-terminal Ras GEF domain and two C-terminal Ras-binding domains (Kelley et al. 2001). PLCε can be activated by Rap1 and Ras when they are active (i.e. GTP bound), through its RA domain. The CDC25 domain of PLCε appears however to be specific for Rap1 activation, with other small G proteins such as Rap2 and Ha-Ras not activated by PLCε (Jin et al. 2001).

IP_3 produced by PLC hydrolysis of PIP_2 activates IP_3 receptors present on numerous Ca^{2+} stores such as the sarcoplasmic reticulum, with IP_3R1 being the predominant receptor isoform in mesenteric vascular smooth muscle cells (Narayanan et al. 2012). This IP_3-mediated Ca^{2+} release can activate CaMKII through Ca^{2+}/calmodulin binding.

Protein Kinase C isoforms reside predominately within the cytoplasm at rest, with most isoforms translocating to the membrane fraction upon activation (Morgan & Claypool 2010). Certain isoforms reside within the perinuclear region, entering the nucleus under activation (Khalil & Morgan 1991). The PKC isoforms predominating in the vasculature are α, β, δ, ε, θ, η μ and γ. In vascular smooth muscle, they have been shown to be involved in both contraction and relaxation.

- **Contraction** - PKC phosphorylates and inhibits K^+ channels, K_{ATP} (Sampson et al. 2007; Y. Hayabuchi et al. 2001; Kubo et al. 1997) , BK_{Ca} (Barman et al. 2004) and K_v channels (Y Hayabuchi, Standen, et al. 2001; Aiello et al. 1996), which are involved in generating hyperpolarizing membrane currents. It also phosphorylates and

activates the 17-kDa PKC potentiated inhibitor protein (CPI-17), which inhibits MLCP (Somlyo & Somlyo 2003).

- **Relaxation**. PKC phosphorylates MLCK and PMCA (responsible for **transient** contraction by PKC) (Inagaki et al. 1987; Khalil 2010).

PKC isoforms α,β,γ known as classical isoforms, are activated by diacylglycerol and phosphatidylserine. Activation is dependent on Ca^{2+}, with Ca^{2+} increasing the affinity for negatively charged lipids (however Ca^{2+} produces minimal activation on its own). Novel isoforms $\delta, \varepsilon, \theta, \eta, \mu$ do not require Ca^{2+} (Newton 1995). Atypical PKC isoforms λ and ζ do not bind DAG, but PIP_3 and ceramide (Steinberg 2008). While CaMKII has been shown to be phosphorylated *in vitro* by PKC, little *in vivo* mechanistic data is available (Waxham & Aronowski 1993; O-Uchi et al. 2008). However, Oestreich *et al.* (2009) showed a clear decrease in Epac-mediated CaMKII autophosphorylation under PKC inhibition, as shown by CaMKII immunoblots (Oestreich et al. 2009) (summarised in **Figure 4.1**).

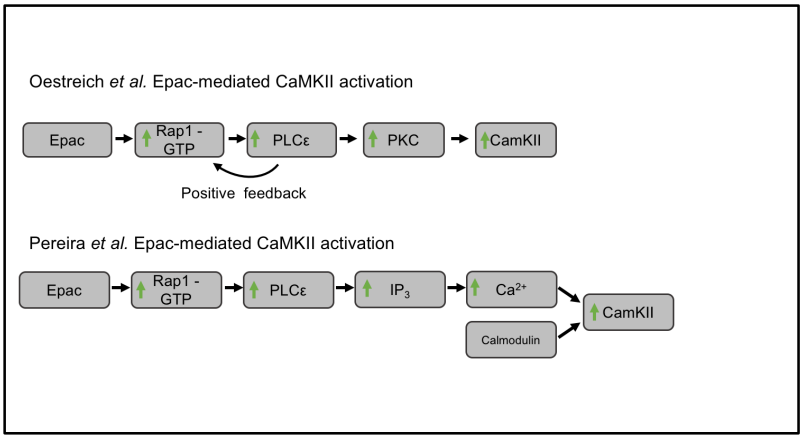


Figure 4.1- Comparison of Epac mediated CaMKII activation. Schematic comparison of two signalling pathways, which both activate CaMKII following Epac activation.

4.2 Aim

The aim of this part of the study was to assess the upstream signalling pathway responsible for Epac-induced activation of CaMKII in rat mesenteric vascular smooth muscle. This involved determining:

1. The involvement of PLC;
2. The role of IP_3-mediated Ca^{2+} release;
3. The role of PKC.

4.3 Results

4.3.1 Phospholipase C-mediated feedback is essential for sustained Epac-induced Rap activity

To assess PLC involvement in Epac signalling in the vasculature, we utilised a Rap1 activation assay. Epac directly activates Rap1, which in turn can activate PLCε. Activated PLCε has two roles, further activation of Rap1 through its unusual GEF activity and phosphoinositol hydrolysis to produce IP_3 and DAG (Oestreich et al. 2007). Active Rap levels were determined by incubating arterial lysates with fusion proteins comprising glutathione-S-transferase and the Rap-binding domain of Ral-guanine nucleotide-dissociation stimulator (GST-RalGDS-RBD), which only bind the active GTP-bound form of Rap1. Glutathione-sepharose beads were then used to specifically pull-down Rap1-GTP from arteries preincubated with or without 8-pCPT-AM (5 minutes) and the PLC inhibitor, U73122 (10 minutes). Levels of captured Rap1-GTP were then quantified by immunoblot analysis **(Figure 4.2**). 8-pCPT-AM increased active Rap1 (Rap1-GTP), which was inhibited by PLC inhibition using U73122.

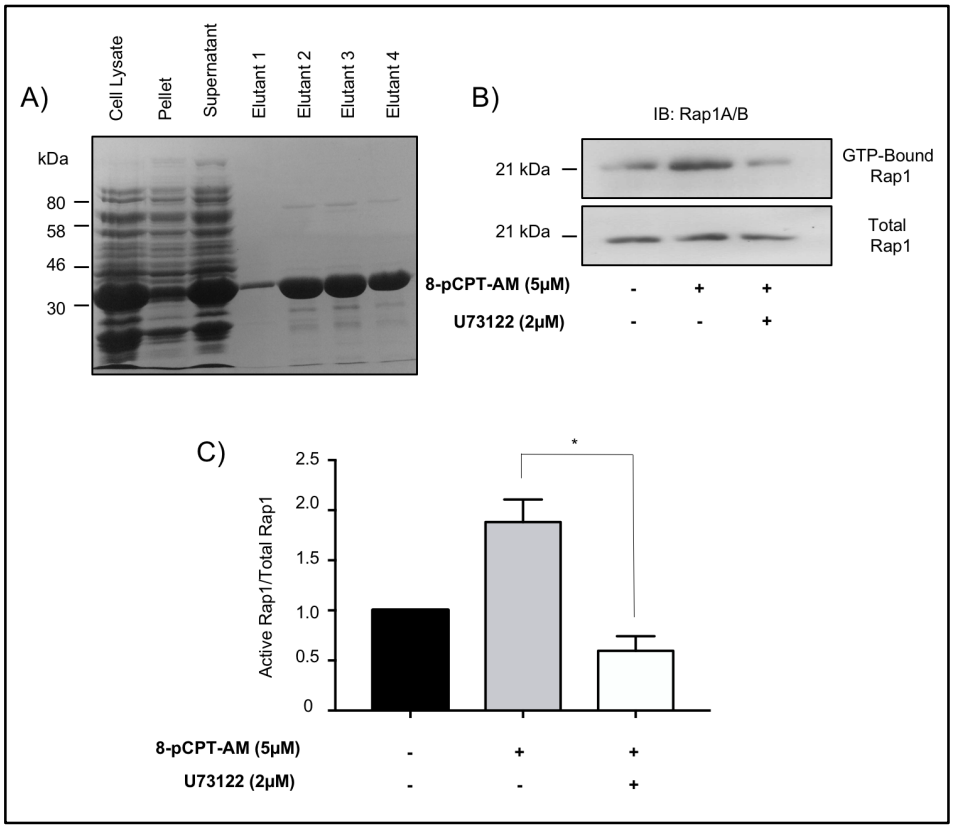


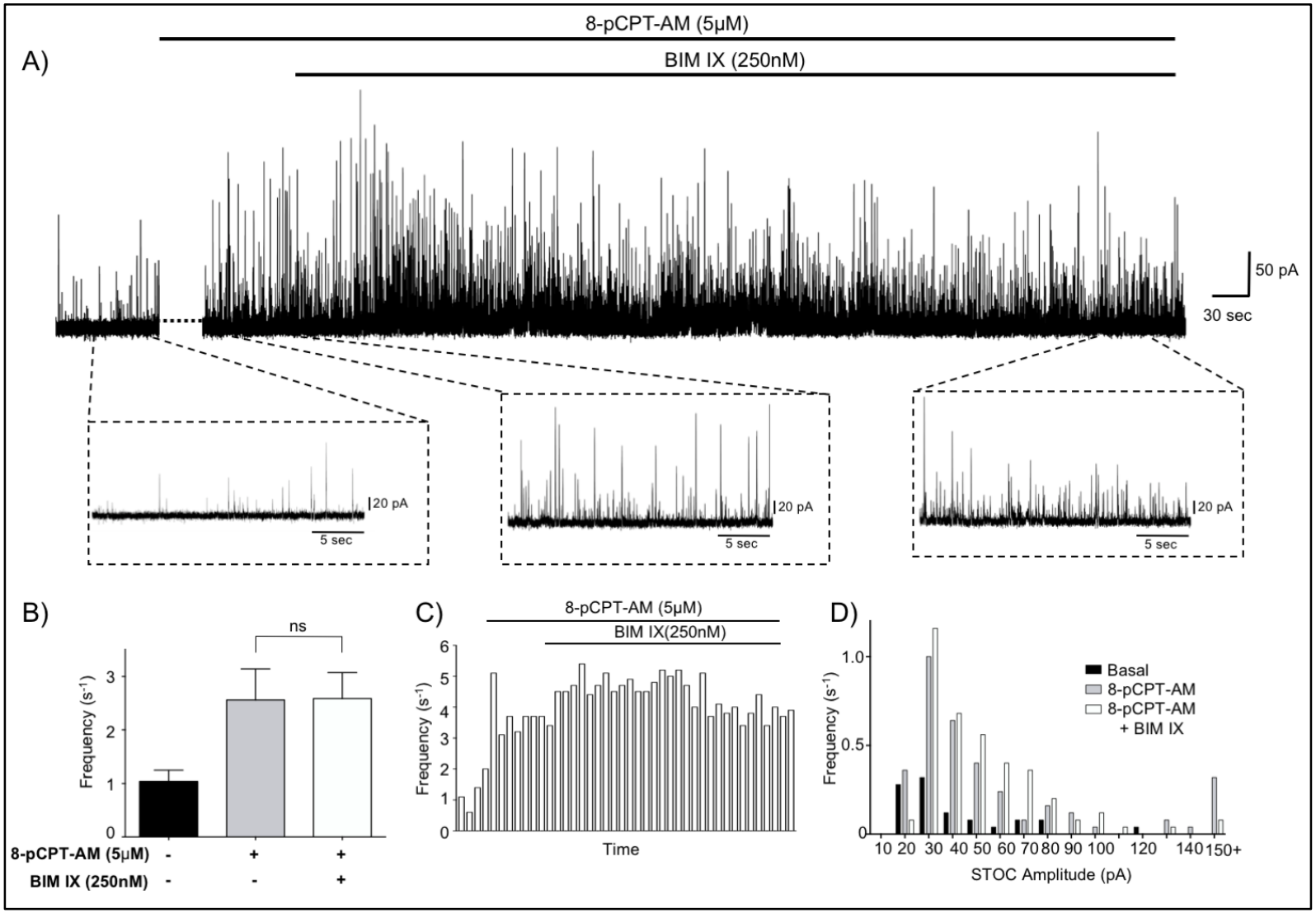
Figure 4.2- Inhibition of PLC inhibits Epac-mediated Rap1 activation in rat mesenteric artery. A) SDS-PAGE gel showing various stages of RBD-RalGDS-GST purification **B)** Representative immunoblot of active Rap 1 (top) and total Rap1 levels (bottom) following exposure to 8-pCPT-AM and U73122 (PLC inhibitor). **C)** Histogram of Active Rap1/Total Rap1 following exposure to 8-pCPT-AM and U73122 (n=2).

4.3.2 Protein kinase C is not involved in Epac-mediated changes in STOC activity

In cardiomyocytes, CaMKII may be activated by Ca^{2+}/calmodulin or via phosphorylation by PKC in a Ca^{2+} independent manner (Oestreich et al. 2009; Pereira et al. 2012). We therefore explored the role of PKC in Epac-mediated STOC frequency increases. PKC was inhibited with the potent PKC inhibitor, bisindolylmaleimide IX (BIM IX), also known as RO 31-8220, which has a K_i for PKC isoforms ranging from 5 to 28 nM (Wilkinson et al. 1993). Application of 8-pCPT-AM to freshly isolated, voltage-clamped rat mesenteric artery myocytes increased STOC frequency, which application of BIM IX (500nM) did not attenuate (1.91 ± 0.25 STOCs s^{-1} vs. 2.05 ± 0.11 STOCs s^{-1}, mean ± SEM,

paired t-test, p>0.05, n=3; **Figure 4.3**). STOC amplitude was also not affected by PKC inhibition (47.13 ± 15.01 pA vs. 43.2 ±12.47 pA, mean ± SEM, paired t-test, p>0.05, n=3).

Figure 4.3- Inhibition of PKC does not attenuate Epac-mediated STOC increases. A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole-cell configuration exposed to 8-pCPT-AM (5 μM), followed by addition of BIM IX (250nM), a PKC inhibitor. Inserts: Highlight analysed areas (25 seconds). Bar indicates 20 pA. **B)** Histogram summarising STOC frequency (n=3). **D)** Probability frequency histogram of STOC amplitude from A.


A)
8-pCPT-AM (5μM)
BIM IX (250nM)
50 pA
30 sec
20 pA
5 sec
20 pA
5 sec
20 pA
5 sec
B)
ns
Frequency (s-1)
8-pCPT-AM (5μM) - + +
BIM IX (250nM) - - +
C)
8-pCPT-AM (5μM)
BIM IX(250nM)
Frequency (s-1)
Time
D)
Basal
8-pCPT-AM
8-pCPT-AM
+ BIM IX
Frequency (s-1)
10 20 30 40 50 60 70 80 90 100 120 140 150+
STOC Amplitude (pA)

4.3.3 Inhibition of IP_3 receptors blocks Epac-mediated CaMKII activation and increases in STOC activity

Since our data suggest the upstream involvement of the IP_3-generating enzyme PLC, we next assessed whether Ca^{2+} from IP_3-releasable stores could activate CaMKII to affect STOC activity. Epac activation with 8-pCPT-AM (5 μM), was unable to increase STOC activity in rat mesenteric artery myocytes pre-incubated in 2-aminoethoxydiphenyl borate (2-APB; 100 μM), an IP_3 receptor (IP_3R) inhibitor. It was noted during experiments that 2-APB application alone caused a rapid increase in STOC activity that declined to baseline under prolonged application. Importantly however, after return to basal STOC frequency, application of 8-pCPT-AM produced no effect on STOCs **(Figure. 4.4).**

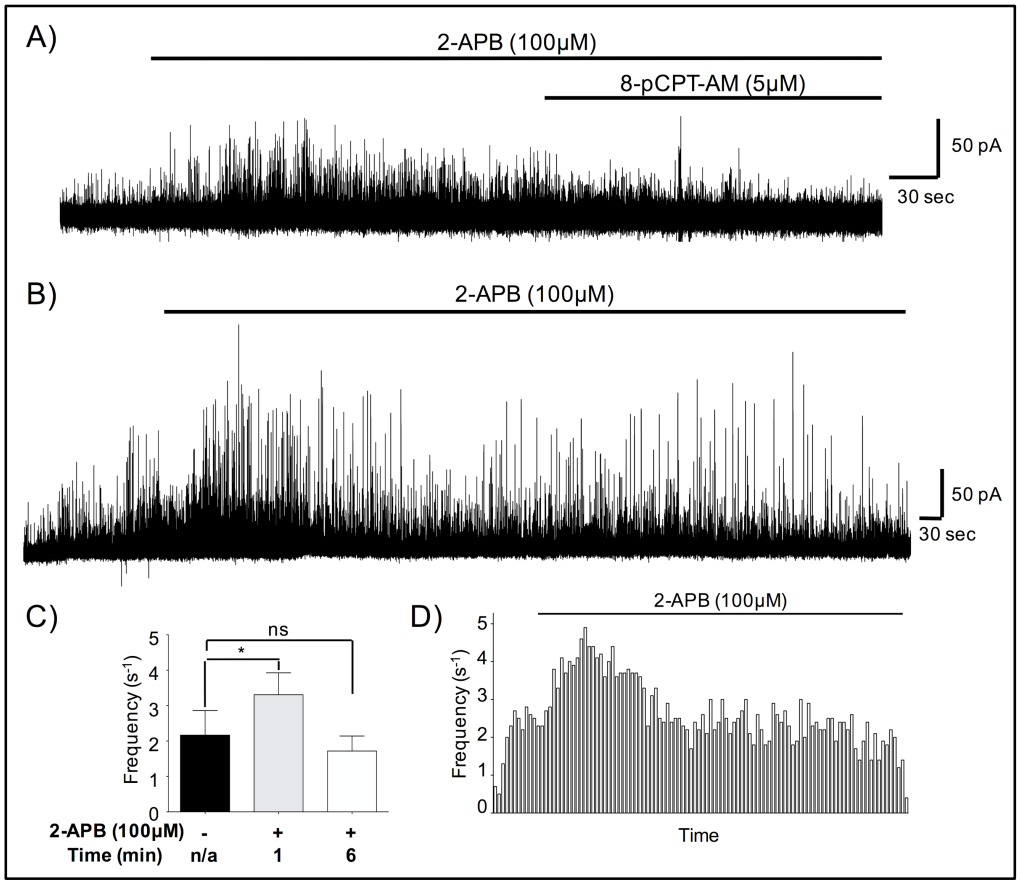
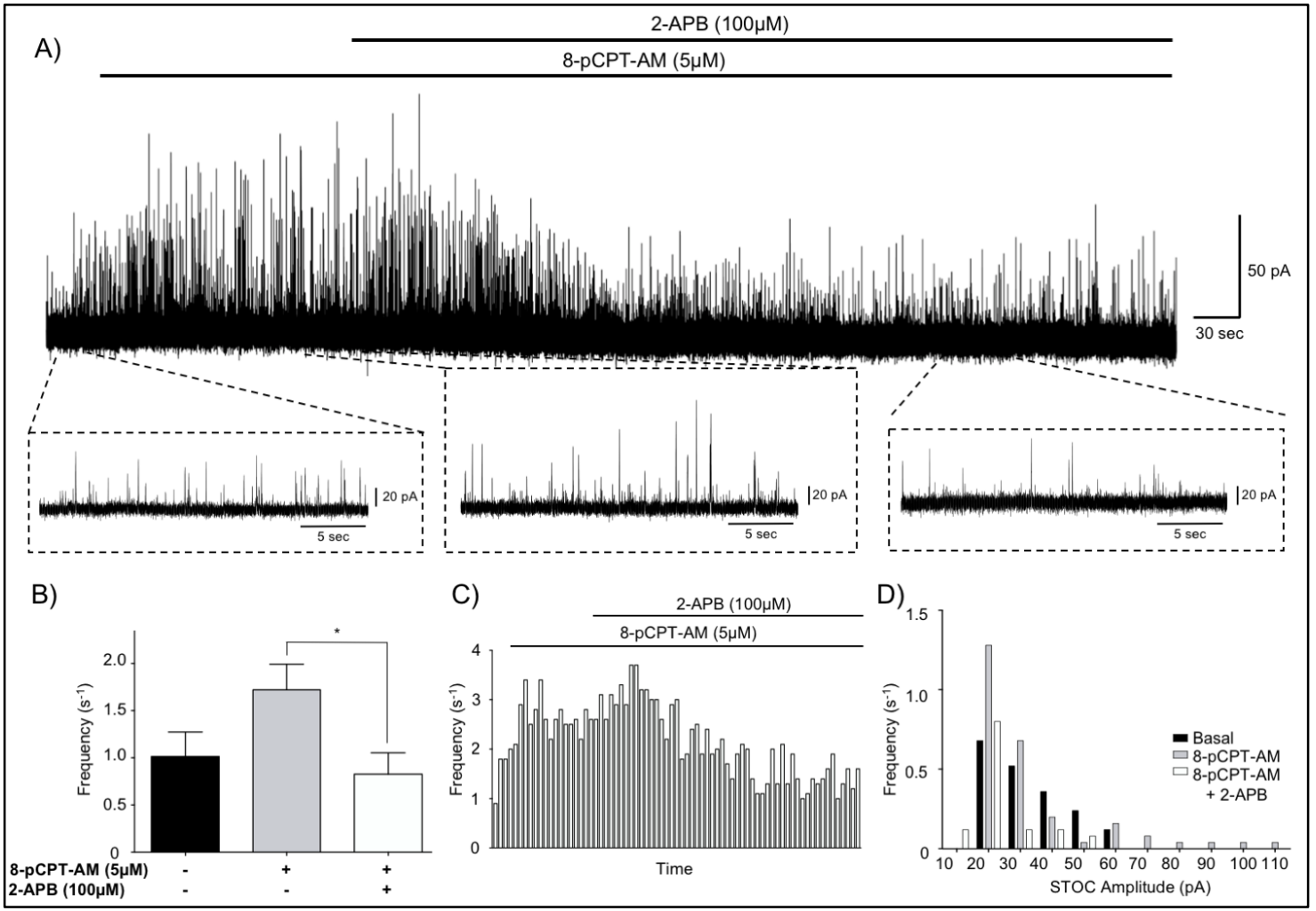


Figure 4.4- Activation of Epac does not increase STOC activity following inhibition of IP_3Rs: A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole cell recording exposed to 8-pCPT-AM (5μM) following 2-APB (100μM) pre-incubation. **B)** Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole-cell recording configuration exposed to IP_3R block with 2-APB (100 μM). **C)** Histogram summarising STOC frequency. The effect of 2-APB was measured at 1 minute and 6 minutes after initial application. **D)** STOC frequency histogram for cell B.

Consistent with these data, 2-APB application following STOC activation with 8-pCPT-AM caused an initial rapid increase in STOC frequency, followed by a decline to levels significantly below those measured in 8-pCPT-AM alone (from 1.72 ± 0.27 STOCs s^{-1} to 0.83 ± 0.23 STOCs s^{-1}; mean ± SEM, $p<0.05$; paired t-test; n=6; **Figure 4.5**).

Figure 4.5- Inhibition of IP_3R attenuates Epac-mediated STOC increases. A) Representative trace of a freshly isolated rat mesenteric artery smooth muscle cell in whole cell configuration exposed to 8-pCPT-AM (5 μM), followed by addition of 2-APB (100 μM) an IP_3 receptor antagonist. Inserts highlight analysed areas. Bar indicates 20 pA. **B)** Histogram summarising STOC frequency (n=6) **D)** Probability frequency histogram of STOCs from the above representative cell.


A)
2-APB (100μM)
8-pCPT-AM (5μM)
50 pA
30 sec
20 pA
5 sec
20 pA
5 sec
20 pA
5 sec
B)
Frequency (s-1)
2.0
1.5
1.0
0.5
0
*
8-pCPT-AM (5μM) - + +
2-APB (100μM) - - +
C)
2-APB (100μM)
8-pCPT-AM (5μM)
Frequency (s-1)
4
3
2
1
0
Time
D)
Frequency (s-1)
1.5
1.0
0.5
0
Basal
8-pCPT-AM
8-pCPT-AM
+ 2-APB
10 20 30 40 50 60 70 80 90 100 110
STOC Amplitude (pA)

To directly link the effects of 2-APB to CaMKII activity, we stimulated rat mesenteric arteries with 8-pCPT-AM (5 μM) in the presence and absence of 2-APB (100μM). Then homogenised the tissue and quantified the changes in CaMKII phosphorylation by immunoblot analysis, using phospho-specific antibodies against the CaMKII $Thr^{286/7}$ autophosphorylation site. In the presence of 2-APB, 8-pCPT-AM was unable to induce phosphorylation at $Thr^{286/7}$ (**Figure 4.6**, n=3). Consistent with the idea that store Ca^{2+} is needed to activate CaMKII, pre-treatment with thapsigargin (500nM), which depletes intracellular Ca^{2+} stores by blocking Ca^{2+} uptake via SERCA, also abolished 8-pCPT-AM-induced phosphorylation of CaMKII at $Thr^{286/7}$ (**Figure 4.6,** n=3). Thapsigargin was not used for STOC electrophysiology experiments since store depletion with this drug automatically inhibits Ca^{2+} sparks and thus STOCs (Nelson et al. 1995).

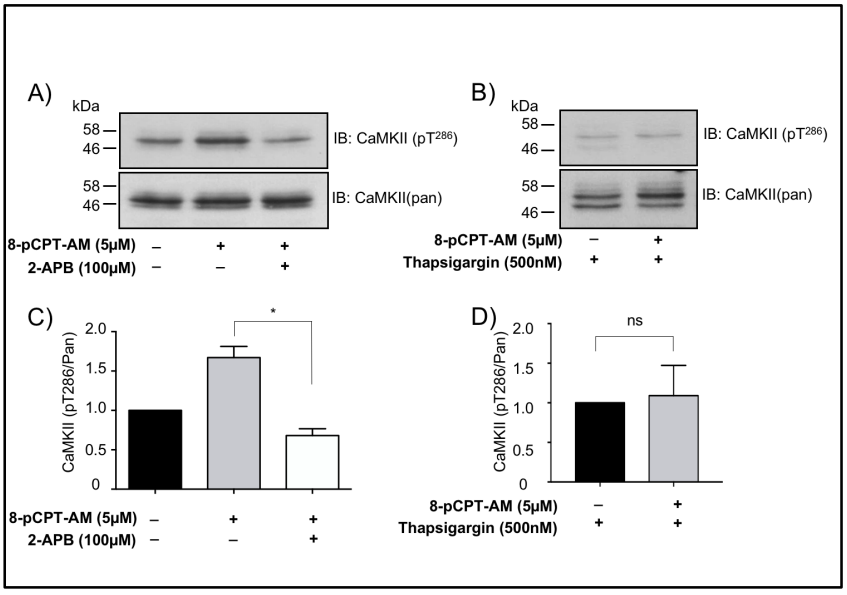


Figure 4.6- Intracellular stores are vital for Epac-mediated CaMKII activation. A) Prior to homogenisation branches of rat mesenteric artery were incubated with vehicle control (DMSO) or 8-pCPT-AM (5 μM) for 5 min, or incubated with 8-pCPT-AM (5 μM) for 5 min following prior application of 2-APB (100 μM) for 15 min. Proteins were separated on 10% polyacrylamide-Tris gels and probed using CaMKII pT^{286} and CaMKII (pan) antibodies **B)** Representative immunoblot of Epac activation in the presence of thapsigargin. As in A) branches were incubated with 8-pCPT-AM in the absence or presence of thapsigargin, followed by separation and immunoblotting. Histogram summarising the level of CamKII activity in experiments **A)** and **B)**

Having demonstrated that Epac is likely activated by Ca^{2+} released from IP_3 receptors, we aimed to quantify the level of Epac-induced IP_3 in vascular smooth muscle. An IP_3 ELISA was used, however the concentration of global IP_3 induced by the application of 8-pCPT-AM was below the sensitivity of the ELISA **(Figure 4.7)**.

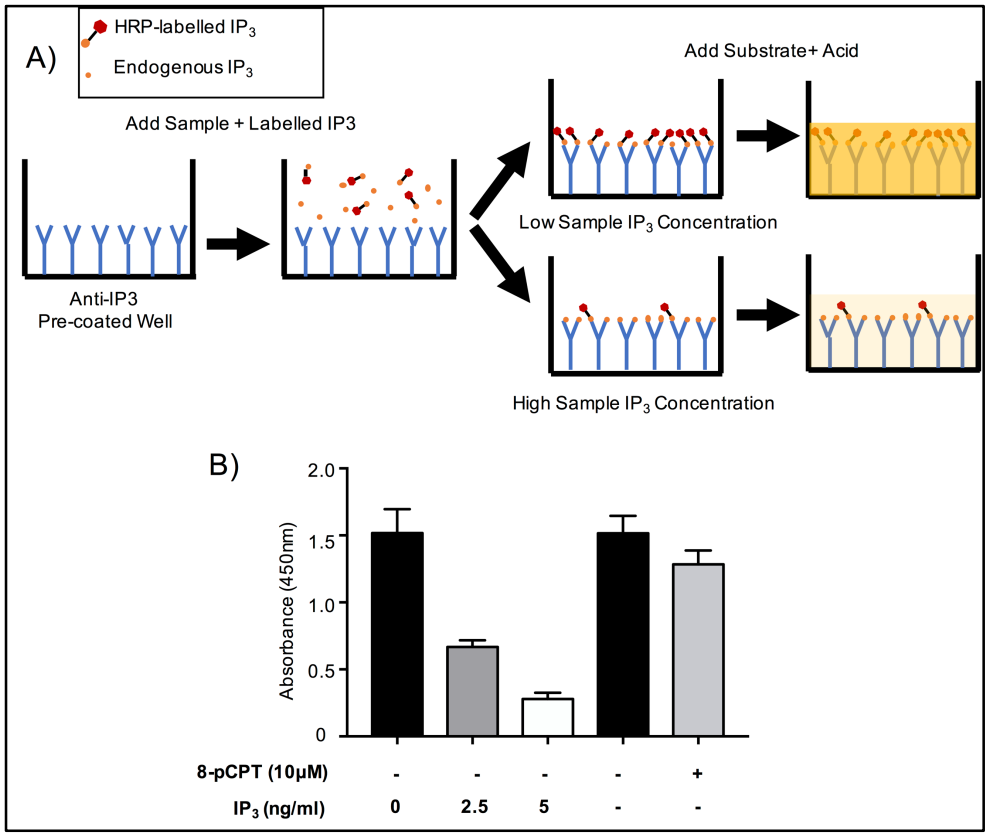


Figure 4.7- IP_3 concentrations in rat mesenteric artery beds under basal and stimulated conditions are below the sensitivity of ELISA detection. A) Illustration of competitive ELISA, where a high endogenous IP_3 concentration causes a low absorbance due to less labelled IP_3 binding to the well. The opposite is the case for low endogenous IP_3 levels. After addition of IP_3 wells were incubated in an acidic substrate to allow development of the colour **B)** 0ng/ml, 2.5ng/ml and 5ng/ml produced reproducible standard curves. Control and 8-pCPT-AM stimulated arteries had IP_3 levels below the sensitivity of the ELISA (2.5ng/ml). To note between DMSO and 8-pCPT a paired t-test shows $p>0.05$.

4.4 Discussion

Our data suggest that CaMKII activity in vascular smooth muscle is triggered by Epac-induced Ca^{2+} efflux through IP_3Rs. Epac's immediate downstream target is the small-Ras-related G-protein Rap1. We propose that following activation, Rap interacts with the C-terminal Ras binding domains of PLCε (Kelley et al. 2001). Subsequent activation of PLCε results in two separate events: phosphoinositol hydrolysis leading to the formation of IP_3, and further activation of Rap through PLCε's N-terminal Ras GEF domain. Consistent with this model, the PLC inhibitor U73122 significantly reduced the ability of the selective Epac activator, 8-pCPT-AM, to induce sustained activation of Rap1. Indeed, the abolition of 8-pCPT-AM-induced Rap activation by U73122 suggests that potentially as in cardiomyocytes, a PLC-mediated feedback mechanism is essential for sustained Rap activation in vascular smooth muscle. Further investigation downstream of PLC using U73122 (i.e. its effect on Epac-induced STOCs) was not undertaken due to U73122's known side effect of phospholamban inhibition (Macmillan & McCarron 2010). We also found that in the presence of the IP_3 receptor inhibitor 2-APB, 8-pCPT-AM is unable to induce phosphorylation of CaMKII at $Thr^{286/7}$, or increase STOC frequency/amplitude, which would be consistent with IP_3-released Ca^{2+} being essential for activation of the CaMKII holoenzyme. Pre-treatment with thapsigargin, which depletes intracellular Ca^{2+} stores by blocking Ca^{2+} uptake via SERCA, also abolished 8-pCPT-AM-induced CaMKII phosphorylation. During experiments, we noticed that 2-APB application following STOC activation with 8-pCPT-AM had a bi-phasic effect on STOC activity, causing an initial rapid increase in STOC frequency followed by a decline to levels significantly below those measured in

8-pCPT-AM alone. Further experiments showed application of 2-APB alone caused a transient increase in basal STOC frequency, which may indicate a constant Ca^{2+} leak from a common Ca^{2+} store via IP_3Rs which, when blocked, alters store content and RyR activity **(Figure 4.8)**. We see some evidence of basal phosphorylation of CaMKII in immunoblots, which may be consistent with this idea of basal leak through IP_3 receptors activating CaMKII.

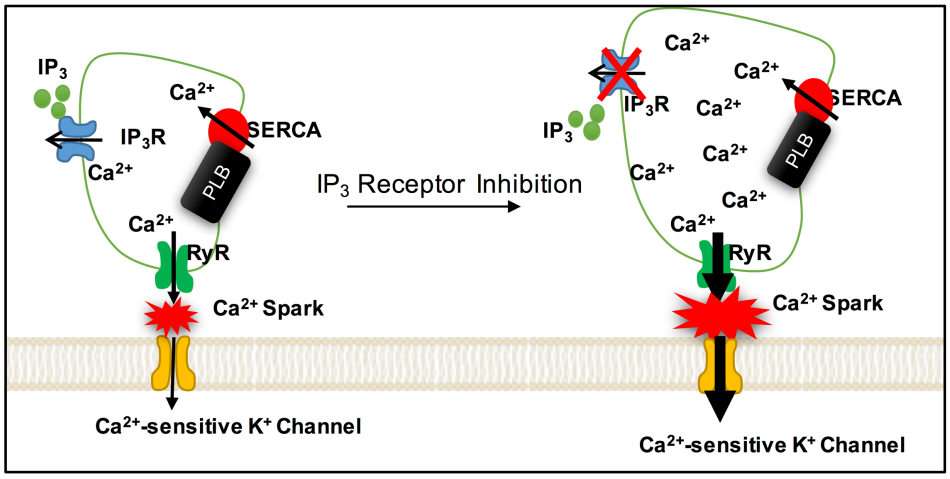


Figure 4.8- IP_3R inhibition hypothesis. Under IP_3R inhibition in electrophysiological recordings we observe an increase in STOC frequency. We hypothesis that this IP_3R inhibition limits basal IP_3-mediated calcium release, increasing the store content and therefore increasing leak mediated through RyRs.

In Pereira *et al.* and this thesis the use of 2-APB as an IP_3 receptor inhibitor could be problematic as this compound has been shown to be a promiscuous blocker of many Ca^{2+}-related ion channels, such as TRPCs and store operated Ca^{2+} entry (SOCE) pathways (Missiaen et al. 2001). Controls for the application of 2-APB using STOC recordings showed a transient increase in STOCs, which we hypothesise is due to a "push" towards RyR dependent Ca^{2+} leak. The possibility of CaMKII being directly activated by 2-APB dependent Ca^{2+} leak exists (Lievremont et al. 2005). However, after 8-pCPT-AM addition in STOC recordings, at which point we propose CaMKII is activated due to the effects of KN-93 addition and immunoblots, we still see a transient increase in STOCs by

2-APB followed by reversal. Due to this we favour the theory of pushing Ca^{2+} leak to RyR dependent pathways, however we cannot rule out 2-APB directly further activating CaMKII in this transient increase period. Further experiments in rat cardiomyocytes utilising the novel CaMKII FRET-biosensor, Camui, showed that 2-APB inhibition of Ca^{2+} sparks were due to CaMKII inhibition by 2-APB, not a possible off-target effect, such as 2-APB mediated calcium store leak (Pereira et al. 2015).

In our experiments, specific inhibition of PKC with BIM IX produced no significant decrease in STOCs induced by Epac activation, suggesting that PKC does not play a significant role in the Epac-STOC pathway in VSMCs. Thapsigargin inhibited CaMKII activation as assessed by immunoblot of mesenteric arterial lysates, showing a dependency on intracellular calcium stores, such as IP_3-mediated release.

While Ca^{2+} efflux from IP_3-releasable stores is the likely mechanism for Epac-mediated CaMKII activation we cannot rule out other possible regulators of CaMKII. Recently CaMKII autonomous activity, whereby CaMKII activity is maintained in the absence of Ca^{2+}/CaM, has been shown to be produced through various translational modifications, such as oxidation, nitrosylation and O-GlcNAcylation (Toussaint et al. 2016; Erickson et al. 2015; Erickson et al. 2013). Therefore, while we use CaMKII T^{286} phosphorylation as an indicator of CaMKII activity, assessing the proportion of autonomous and total CaMKII activity through radioactive experiments may prove insightful. Here, CaMKII activity would be assessed by phosphorylation of CaMKII substrates to explore total and autonomous activity, in the presence or absence of Ca^{2+}. Indeed, Epac signalling via Akt has recently been shown to activate nitric oxide synthase type 1 (NOS1),

causing an increase in autonomous CaMKII activity by S-nitrosylation of CaMKII (Pereira et al. 2017). This NOS1 activation is required for β-adrenergic-spark responses in cardiomyocytes (Curran et al. 2014). The site responsible for this CaMKII autonomous activity is cysteine 290. Interestingly, pre-incubation with an NO donor inhibited this autonomous activity. The nitrosylation must be after the initial activation of CaMKII by Ca^{2+}/calmodulin. The site responsible for the inhibition of CaMKII is cysteine 273 (Erickson et al. 2015; Pereira et al. 2017). NOS1 and 3, as with CaMKII, require Ca^{2+}/CaM for activation and therefore the potential arises for an elevation of Ca^{2+}/CaM to produce NOS1/3 activation and CaMKII simultaneously. An alternative modulator of CaMKII is glucose, which increases CaMKII autonomous activity by addition of O-linked N-acetylglucosamine on CaMKII at site S279 (Erickson et al. 2013). Inhibition of GlyNAc inhibitors (i.e. increasing glycosylation) increased Ca^{2+} spark frequency in cardiomyocytes (Erickson et al. 2013). This effect of glucose could be a reason why autophosphorylation is seen in control lysates, as all buffers used contained glucose. Oxidation, another post translation modification affecting CaMKII activity, has not been specifically explored in vascular smooth muscle. However, X-ROS (Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produced reactive oxygen species (ROS)) increased STOCs in cerebral vasculature (Pritchard et al. 2016). Inhibition of O_2^- produced by NADPH oxidase (NOX), in response to such events as angiotensin II application, vascular pressure increase and hypoxia, attenuated STOCs (Griendling et al. 1994; Rathore et al. 2008; Vecchione et al. 2009). Could this ROS activate CaMKII and produce its effects on sparks/STOCs via CaMKII as part of a negative feedback mechanism to contraction?

While block of IP_3Rs inhibited CaMKII initial activation, the possible role of other post-translation modifications on the prolonged effect (autonomous CaMKII activity) of Epac-mediated STOCs is still to be elucidated. Indeed recently two pathways both of which increase Ca^{2+} sparks, involving Epac/CaMKII/Sparks and Akt/NOS/CaMKII/Sparks, have been shown to work together in a merged signalling cascade. Experimental data from this pathway showed no autophosphorylation of CaMKII without O-Nitrosylation of CaMKII, showing a clear need for post-translation modifications in Ca^{2+} signalling pathways (Pereira et al. 2007; Curran et al. 2014). Assessing the involvement of Akt in smooth muscle using phospho-Akt antibodies in immunoblots, along with the use of an Akt inhibitor in electrophysiological studies will provide further knowledge.

4.5 Conclusion

Epac-induced CaMKII activation is likely initiated by IP_3-mobilized Ca^{2+}: 8-pCPT-AM failed to induce CaMKII activation following intracellular Ca^{2+} store depletion, and inhibition of IP_3 receptors blocked both 8-pCPT-AM-mediated CaMKII phosphorylation and STOC activity.

5 Downstream targets of Epac-activated CaMKII

5.1 Introduction

Within smooth muscle cells changes in STOC frequency or amplitude can arise from direct changes in the behaviour of BK_{Ca} channels, or indirectly from changes in the behaviour of the underlying RyRs. Both of these routes can be modulated by CaMKII. RyRs respond to changes in both cytosolic and luminal SR Ca^{2+} levels (Zhuge et al. 2002). Increases in RyR activity can in turn come about either through enhancement of their Ca^{2+} sensitivity, or through an increase in the content of the RyR-accessible Ca^{2+} store (Van Petegem 2012). Primary targets downstream of Epac-induced CaMKII activation are therefore BK_{Ca} channels, RyRs, and/or proteins that modify Ca^{2+} uptake into the SR.

5.1.1 Large conductance Ca^{2+} activated K^+ (BK_{Ca}) channels

BK_{Ca} channels are formed from four, seven transmembrane domain α subunits along with β subunits, responsible for modulation of the channel (Humphries & Dart 2015). Like voltage-dependent K^+ channels, BK_{Ca} channels possess a voltage-sensor in the α subunit S4 transmembrane segment, in addition to a "Ca^{2+} bowl" at the C terminus. These are responsible for voltage and Ca^{2+} regulation, respectively (Humphries & Dart 2015). BK_{Ca} channels open in response to depolarising membrane potentials, with activation at lower membrane potentials in the presence of Ca^{2+} (Humphries & Dart 2015) . CaMKII phosphorylation of BK_{Ca} channels has been previously explored in glomerular mesangial and neuronal cells (van Welie & du Lac 2011; Sansom et al. 2000). In neurones, CaMKII phosphorylation of BK_{Ca} channels has been shown to increase their open probability, by shifting their voltage dependence towards more

hyperpolarized membrane potentials (van Welie & du Lac 2011). In contrast it has been shown that somatostatin produces its effect of increased BK_{Ca} activity by dephosphorylating BK_{Ca} channels (White et al. 1991). Over 20 possible phosphorylation sites have been identified on BK_{Ca} channel subunits (Yan et al. 2008) and temporal patterns of phosphorylation are likely to be important, since effects of PKA on BK_{Ca} activity are dependent on previous PKC phosphorylation (Zhou et al. 2010; Yan et al. 2008).

5.1.2 Ryanodine Receptors (RyRs)

Ryanodine receptors coded by RYR1-3 are one of the largest known ion channels, with a single subunit at ~565 kDa forming a homotetramer >2MDa (Lanner et al. 2010). Ryanodine receptors (RyR1-3) are expressed in cardiomyocytes, skeletal tissue, vascular smooth muscle and various non-muscle cells, such as neurones (Lanner et al. 2010). In the vasculature, specifically the cerebral vasculature, all RyR isoforms are expressed, with RyR2 being the predominant isoform, and importantly the most common isoform found at subplasmalemma localisations (Vaithianathan et al. 2010) . Interestingly, although RyR3 has been shown by Vaithianathan *et al.* (2010) to be minimally expressed in the subplasmalemma SR membrane, they still appear to play a pivotal role in spark and STOC formation, as shown by RyR3 KO mice (Löhn et al. 2001). RyRs are susceptible to numerous post-translational modifications and possess multiple phosphorylation sites (Ozawa 2010). In cardiac muscle, PKA can phosphorylate RyR at serine 2808/9 and serine 2030, located in cytoplasmic region of RyR, while CaMKII can phosphorylate the same proteins at ser2808/9 and ser2814/5 (Van Petegem 2012).

Phosphorylation of RyR by PKA at ser2808/9 causes dissociation of FK506 binding protein (FKBP12.6, also known as calstabin 2) from a RyR/FKBP12.6 complex. FKBP12.6 is responsible for regulating RyRs, therefore this loss of FKBP 12.6 causes an increase in open probability (Marx et al. 2000; Ondrias et al. 1994). CaMKII phosphorylation of this site is controversial, however the weight of evidence now suggests that Ser2808/9 can be phosphorylated by CaMKII (Rodriguez et al. 2003; Camors & Valdivia 2014). It is important to note that the effect of PKA phosphorylation and phosphorylation at 2808/9 is still under debate, with a wide range of responses to p2808/9 phosphomimetic knock-in mice. For a full report of the conflicts and contrasting results see *Camors and Valdivia* (2014), while *Dobrev and Wehrens* (2014) present some possible technical reasons that may cause discrepancies (Camors & Valdivia 2014; Dobrev & Wehrens 2014). The effect of phosphorylation of Ser2030 is still unknown, with no effect on the Ca^{2+} sensitivity of RyR induced by phosphomimetic mutation (Xiao et al. 2005).

CaMKII-dependent phosphorylation of RyR at serine 2814/5, produces (by general consensus) an activation of RyR as an increase in open probability (Xander H T Wehrens et al. 2004). This phosphorylation site is commonly associated with heart failure: ablation of the phospho site, or inhibition of CaMKII, reduces heart failure from transverse aortic constriction induced cardiac hypertrophy (Respress et al. 2012; Ling et al. 2009). This is proposed to be due to an increased diastolic Ca^{2+} leak and decreased Ca^{2+} store content, both mediated by CaMKII phosphorylation (Ai et al. 2005). This phosphorylation site has less phosphorylation under basal condition compared to p2808/9 (15% vs. 69%) as shown by Li *et al.* 2013.

5.1.3 Phospholamban (PLB)

Another key Ca^{2+} handling protein involved in STOC production is phospholamban (PLB) (Cheranov & Jaggar 2002). PLB is a 5/6 kDa pentamer (25-30 kDa) present on the sarcoplasmic reticulum membrane, where its role is that of inhibiting the sarco/endoplasmic reticulum ATPase (SERCA) (Hagemann & Xiao 2002). Phosphorylation of PLB disinhibits its control on SERCA, therefore leading to an increase in reuptake of Ca^{2+} from the cytosol, by lowering the K_{Ca} (half maximal activation of Ca^{2+} transport) of SERCA by 50% (Simmerman & Jones 1998). CaMKII and PKA phosphorylate PLB at T17 and S16, respectively (Hagemann & Xiao 2002).

5.1.4 Ca^{2+} Modulation/The SR effect

Ca^{2+} store levels within smooth muscle cells play a pivotal role in Ca^{2+} spark formation, whereas there is less dependency in cardiomyocytes (Essin et al. 2007). In cardiomyocytes, Ca^{2+} enters through Ca^{2+} channels and activates RyR to release more Ca^{2+}. Each opening of an L-type Ca^{2+} channel produces a Ca^{2+} spark, which leads to a twitch response. Vascular smooth muscle in contrast lacks tight coupling between LTCC and RyRs. In vascular smooth muscle cells not every L-type Ca^{2+} channel opening produces a Ca^{2+} spark, indeed it is the net inward flux of Ca^{2+} that regulates Ca^{2+} sparks (Collier et al. 2000; Essin & Gollasch 2009; Essin et al. 2007). In murine arterial smooth muscle cells, ablation of the Cav1.2 α subunit (a major Ca^{2+} entry route) has been shown to reduce spark frequency, due to a decrease in the $[Ca^{2+}]_i$ causing a decrease in intracellular store calcium levels. Reversal of this decrease in cytoplasmic Ca^{2+} by increasing Ca^{2+} influx led to an increase in store content, which restored calcium spark frequency in KO mice (Essin et al. 2007).

5.2 Aims

In this part of the study I aimed to assess:

1. Epac-induced post-translational modifications to RyRs;
2. Epac-induced post-translational modifications to PLB which reverse its inhibition of SERCA;
3. Epac-induced changes in Ca^{2+} store uptake and store content;
4. Epac-induced changes in the activation and phosphorylation of BK_{Ca} channels.

5.3 Results

Epac and subsequent CaMKII activation likely induce changes in RyR activity through phosphorylation of the release channel itself, its associated regulatory proteins, or the SERCA pump regulator PLB. We assessed the effects of Epac activation on each of these targets.

5.3.1 PLB undergoes a post-translational modification in response to Epac activation

To assess the effects of Epac activation on PLB, arteries were incubated in 8-pCPT-AM, lysed and proteins within the lysates analysed by SDS-PAGE and immunoblotting with antibodies against PLB and phosphorylated PLB (phospho-specific antibodies against the CaMKII site Thr17). We detected some evidence of phosphorylation at Thr17 in response to 8-pCPT-AM (**Figure 5.1A**). A faint immunoreactive band is detected by the phospho-antibody (upper panel) and a slightly heavier species of PLB is detected by the pan-PLB antibody (lower panel).

Serendipitously, it was observed that control (DMSO treated) arteries produced a stronger immunoreactive band than both forskolin and 8-pCPT-AM incubated arterial lysates when probed with a PLB (pan) antibody which detects all forms of PLB, phosphorylated and non-phosphorylated (**Figure 5.1C**). Further analysis using α-actin as a loading control showed this not to be a loading error, but due to a decrease in PLB in the soluble fraction of the lysate upon stimulation with forskolin or 8-pCPT-AM. Post translation modification of PLB, such as phosphorylation, has previously been reported to change its susceptibility to detergent extraction from membranes (Bidlack et al. 1982). Indeed, further pan PLB immunoblots, using RIPA buffer for lysis, showed 8-pCPT-AM-mediated changes in PLB mobility within gels, which may indicate PLB phosphorylation following Epac activation **(Figure 5.1B)**.

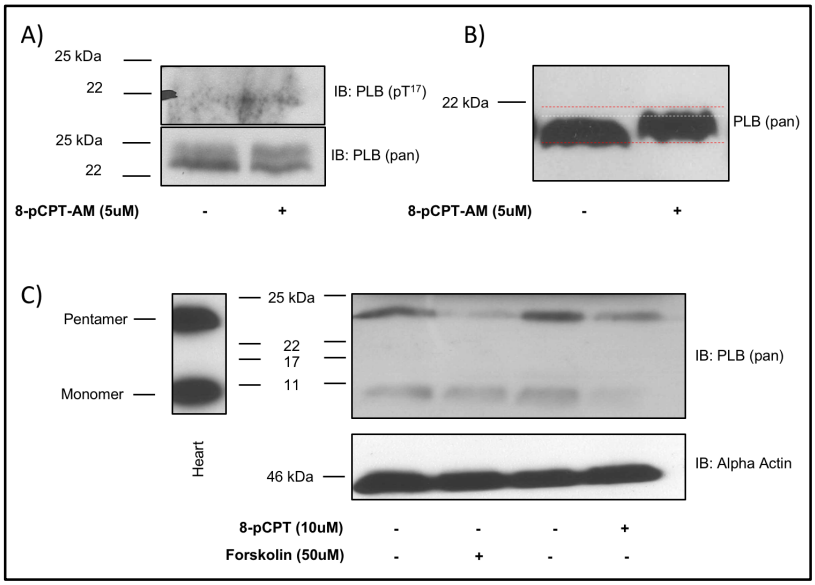


Figure 5.1– Epac activation produces possible post-translational modifications on the Ca^{2+} reuptake protein PLB. A) Immunoblot of rat mesenteric arteries stimulated by 8-pCPT-AM before lysis. PLB (pan) and PLB (T17) antibodies were used to probe membranes **B)** Pan PLB immunoblots of mesenteric artery lysates, lysed in RIPA buffer separated on 15% SDS-PAGE gels **C)** Left: Positive control Heart lysate used to show pentamer and monomer separation on 15% SDS-PAGE immunoblots. Right: Rat mesenteric arteries stimulated with forskolin or 8-pCPT-AM before lysis, followed by separation on SDS-PAGE gels and immunoblotted for PLB. Smooth muscle alpha actin shown as a loading control.

5.3.2 RyR is not detectable via biochemical techniques in a single rat mesenteric artery bed

We next attempted to assess both total RyR and phosphorylated RyR levels in response to 8-pCPT-AM exposure. Firstly, total RyR was assessed by immunoblotting arterial lysates using a commercially available RyR (34C) antibody (Thermo Scientific) and a custom antibody (#2160 RyR(pan)) provided by Prof Tony Lai (University of Cardiff, Wales). Neither antibody produced a clear immunoreactive band from rat mesenteric lysates (**Figure 5.2**). Positive controls, such as the SR fraction of heart tissue and RyR immunoprecipitation from heart lysates, were used and showed a clear band at ~500kDa, consistent with the expression of RyR (Van Petegem 2012). A phospho-specific antibody against the CaMKII site (ser2808/9) did not detect any band at ~500 kDa.

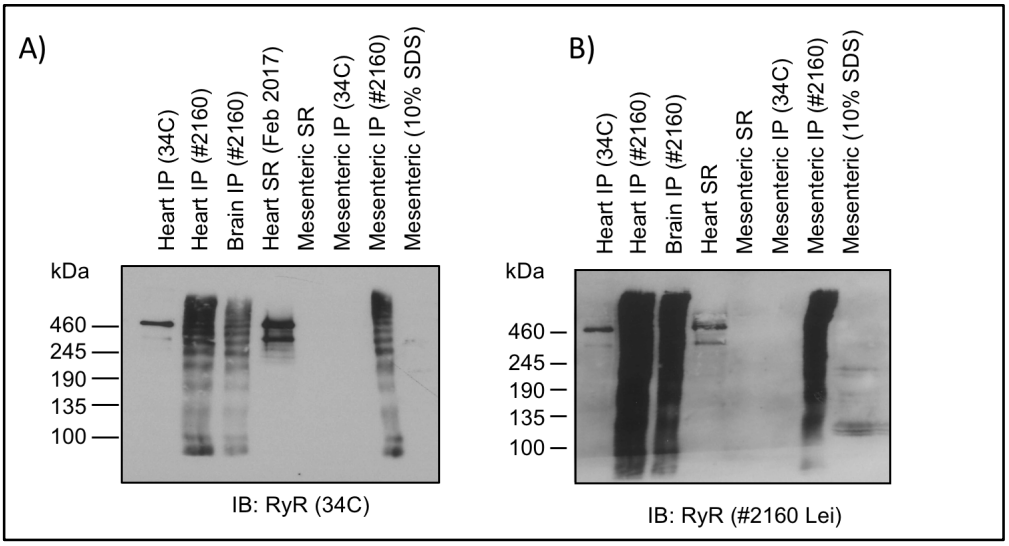


Figure 5.2– RyR is undetectable by immunoblotting in mesenteric arterial lysates. A) Immunoblot of rat tissues using the anti-RyR 34C (Developmental studies Hybridoma Bank). **B**) Immunoblot of rat tissues (as in A) using the anti-RyR antibody #2160 kindly provided by Prof. Tony Lai (Cardiff, Wales). Tissues are: Lane 1–Heart IP using anti-RyR 34C, Lane 2–Heart IP using anti–RyR #2160, Lane 4–enriched sarcoplasmic reticulum (Heart), Lane 5–enriched sarcoplasmic reticulum (Mesenteric), Lane 6–Mesenteric Heart IP using anti-RyR 34C, Lane 7–Mesenteric IP using anti –RyR #2160, Lane 8–Mesenteric artery lysed in 10% SDS containing lysis buffer.

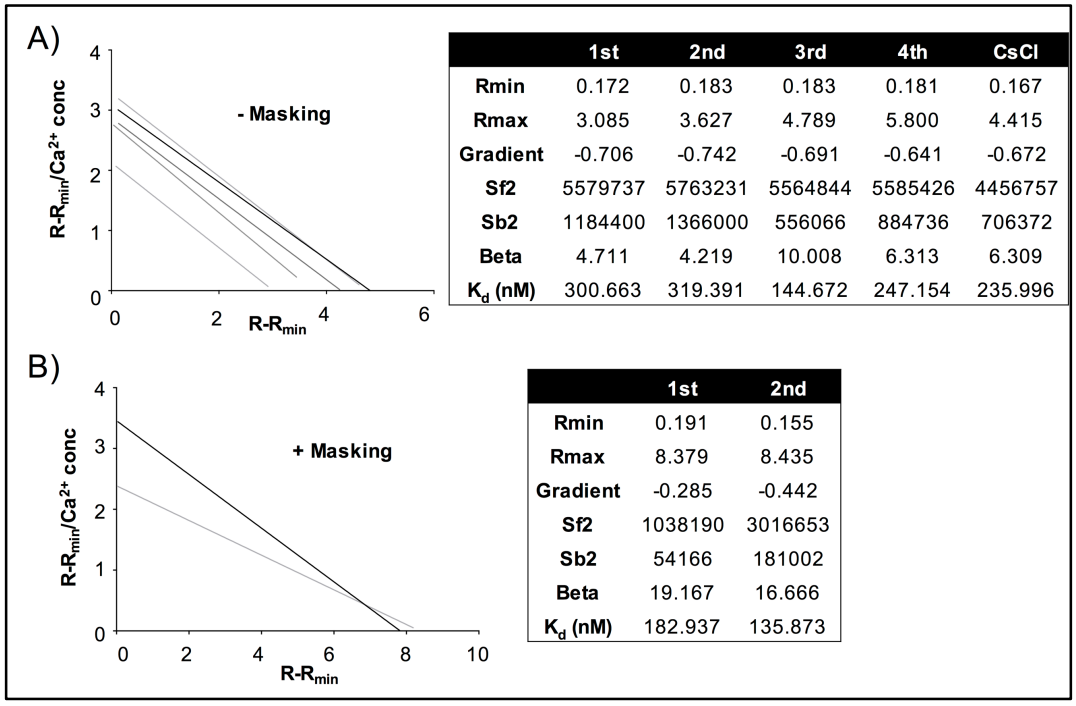
Since our results suggest possible Epac/CaMKII effects on PLB we investigated whether Epac activation alters the Ca^{2+} content of the RyR-accessible Ca^{2+} store. We therefore measured the effect of Epac activation on Ca^{2+} store content and the rate of Ca^{2+} clearance from the cytosol. Transient Ca^{2+} release from the SR was induced using pulsed applications of the RyR activator caffeine, in voltage-clamped and fura-2-dialyzed myocytes. Prior to experiments, fura-2 calibration was undertaken to provide accurate Ca^{2+} concentrations within the cytosol, rather than relative fluorescent intensity changes.

5.3.3 In-vitro fura-2 calibration and optimisation of caffeine-induced Ca^{2+} release

Fura-2, a ratiometric Ca^{2+} indicator was used to assess Ca^{2+} concentrations in the cytosol. A non-membrane-permeant version was used to avoid both leakage out of the cell and the loading of fura-2 into intracellular organelles during experiments (10-15 mins). Fura-2 is a dual excitation dye that allows ratiometric measurements. Ca^{2+}-bound fura-2 absorbs at approx 340nm, free unbound fura-2 absorbs at approx. 380nm. Excitation at both these wavelengths emits light at ~510nm, therefore allowing a ratio of bound to free indicator that is independent of dye contraction/cell size to be recorded.

A range of calibration solutions buffered with 10 mM EGTA and containing free Ca^{2+} concentrations of 15nM, 40nM, 100nM, 225nM, 250nM, 600nM, 1.35μM and 20mM were used. A minimum free Ca^{2+} solution was produced by using a 10mM EGTA, with no $CaCl_2$ added. Experiments were firstly undertaken without image masking, which delineates a small area of recording to minimise background; However, this produced dissociation constant (K_d) values of

~300nM that are high compared to previously published values (Grynkiewicz et al. 1985). It was also noted that the β values (ratio of the photon count with R_{min} and R_{max} solutions at 380 nm) were lower than expected and varied (Grynkiewicz et al. 1985). A single rat mesenteric artery smooth muscle cell occupies a fraction of the viewing field and therefore the interference by background will be substantial. K_d values were therefore obtained with image masking. For this the area of measuring is decreased to only surround the cell of interest. This is undertaken as it is not possible to draw a region of interest using a PTI system. These results allowed us to determine the K_d for fura-2 in our specific set up i.e. temperature/solutions/imaging system. This was averaged from two separate recordings of 183nM and 136nM, therefore 160nM was our average K_d (Grynkiewicz et al. 1985) (**Figure 5.3)**. This value is as expected with the level of free Mg^{2+} present in the intracellular solution.



	1st	2nd	3rd	4th	CsCl
Rmin	0.172	0.183	0.183	0.181	0.167
Rmax	3.085	3.627	4.789	5.800	4.415
Gradient	-0.706	-0.742	-0.691	-0.641	-0.672
Sf2	5579737	5763231	5564844	5585426	4456757
Sb2	1184400	1366000	556066	884736	706372
Beta	4.711	4.219	10.008	6.313	6.309
K_d (nM)	300.663	319.391	144.672	247.154	235.996

	1st	2nd
Rmin	0.191	0.155
Rmax	8.379	8.435
Gradient	-0.285	-0.442
Sf2	1038190	3016653
Sb2	54166	181002
Beta	19.167	16.666
K_d (nM)	182.937	135.873

Figure 5.3– Fura-2 *In-Vitro* Calibration. **A)** Initial experiments were undertaken without image masking, however produced high K_d values and low β-values. Graphs were produced by plotting $R\text{-}R_{min}/Ca^{2+}$ Concentration vs. $R\text{-}R_{min}$ **B)** Experiments with image masking

produced lower K_d values, higher β-values and showed maximal R_{max} compared to previous experiments. Graphs were plotted as in A. K_d values are in nM.

Following accurate dissociation constant evaluation, which gave measured resting state $[Ca^{2+}]_i$ that was close to previously published concentrations (Kamishima & McCarron 1998), preliminary experiments were undertaken to assess the concentration of caffeine needed to evoke multiple Ca^{2+} release events. Initial experiments showed that while 10mM caffeine evoked transients, Ca^{2+} handling was not reliable over the time of the experiment, leading to altered transient shape and cell death during the experiment (**Figure 5.4**). In contrast, 1 and 3mM caffeine produced tolerable transients i.e. reliable transient shape/Ca^{2+} handling but did not consistently evoke Ca^{2+} release. Therefore, it was decided to use 5 mM caffeine as the Ca^{2+} release stimulant, with this concentration producing tolerable and consistent release events. Initial experiments further showed that allowing an incubation period following drug application, in which no transients were produce, reverted the steady decrease in transient amplitude seen with repetitive stimulations. This was not reliable, with transient recovering being varied, while transient amplitude rundown was reliable when evoked every 1 minute. Therefore, it was decided to constantly evoke transients by application of caffeine at regular intervals (60 seconds).

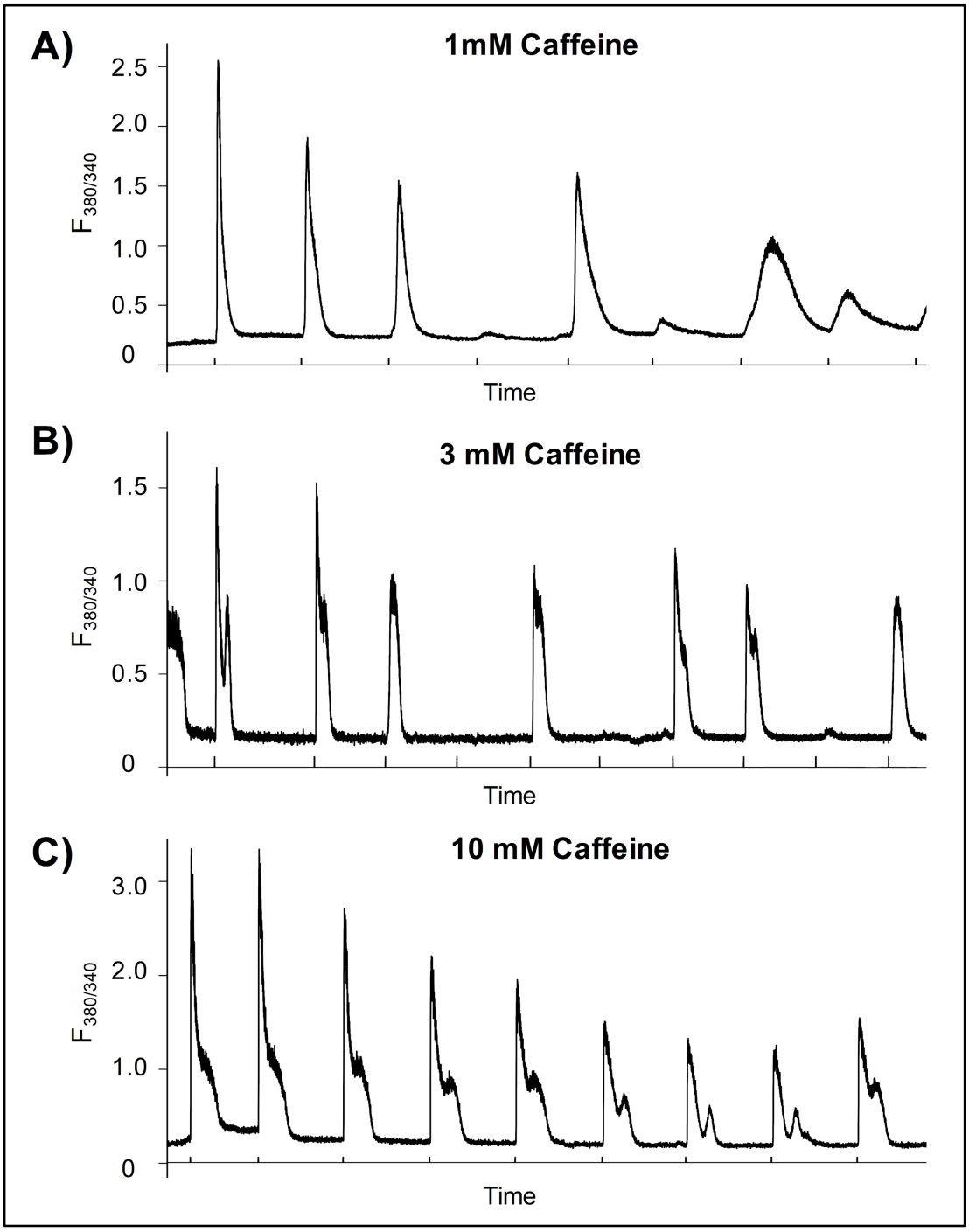


Figure 5.4- Optimisation of Caffeine induced Ca^{2+} transients. A) Repetitive application of 1mM caffeine application produced inconsistent transients. **B)** Using 3mM caffeine produced inconsistent transients as in A. **C)** The use of 10mM caffeine produced transients that were not tolerated by rat mesenteric artery smooth muscle cells. This was seen through altered calcium handling through experiments as seen in C. Upper deflections along x-axis indicate caffeine application. Experiments were undertaken by Dr. Tomoko Kamishima.

5.3.4 Epac activation affects SR kinetics with no effect on store Ca^{2+} load

Caffeine activates RyRs and induces a bulk release of store Ca^{2+} into the cytosol. This Ca^{2+} is subsequently cleared from the cytosol by uptake and extrusion pumps (Kamishima & McCarron 1998). The effects of 8-pCPT-AM and DMSO (control) on 3 parameters of caffeine-induced Ca^{2+} release events were assessed: transient amplitude, transient duration (50% to 50%) and initial rise time (baseline to 200nM cytosolic Ca^{2+}; **Figure 5.5)**.

- Transient amplitude reflects Ca^{2+} SR content where SR containing higher levels of Ca^{2+} produce a larger initial transient. We assessed whether Epac activation modulated SR content in the positive or negative direction.
- Duration (50% to 50%) assesses possible Epac involvement in Ca^{2+} clearance mechanisms. Phosphorylation of PLB, for example, would be expected to increase the activity of SERCA, increase Ca^{2+} reuptake into stores and shorten the duration of transients.
- Initial rise time was used to assess if Epac activation altered RyR activation kinetics. This was calculated using a third order polynomial fit of Ca^{2+} rise between baseline and 200nM. Using this fit we calculated the time to 100nM from baseline. Use of fitted data avoided any bias because of a delay in Ca^{2+} rise due to the application of caffeine or specific cellular effects.

All Ca^{2+} transient data is normalised to the last control transient of the recording (i.e. before DMSO or 8-pCPT-AM application), with this transient given a value of 1 in respect to all measurements. This was undertaken due to the highly variable Ca^{2+} transient profile we observed in rat mesenteric artery smooth muscle cells.

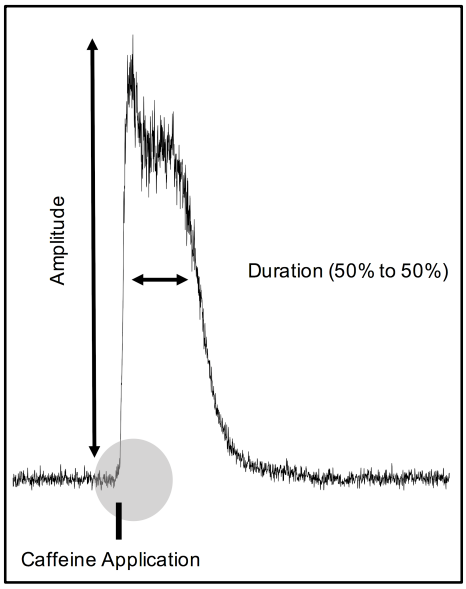


Figure 5.5– Caffeine-induced Ca^{2+} Transient Parameters. Amplitude indicative of SR content is assessed from baseline to peak. Duration upon elevate Ca^{2+} of 50% caffeine peak is 50% to 50% duration. Baseline to 100nM $[Ca^{2+}]_I$ as indicated by the silver circle.

Repeated 100ms application of caffeine (5mM; indicated by circles) at 60 second intervals triggered a train of Ca^{2+} release transients (**Figure 5.6A**).

At the point indicated by the bar above the trace, the extracellular superfusing solution was switched to test solutions containing either vehicle control (DMSO; upper panel) or 8-pCPT-AM (5 μM; lower panel). In both cases, the amplitude of the caffeine-induced Ca^{2+} transients decreased during the course of the experiment, suggesting that the SR Ca^{2+} is being slowly depleted. Epac activation with 8-pCPT-AM had no significant effect on Ca^{2+} transient amplitude, amplitude for DMSO-treated cells 0.68 ± 0.07 and for 8-pCPT-AM treated cells 0.80 ± 0.03, n=5, 4 respectively; $p>0.05$; unpaired t-test; **Figure 5.6Cii**). There was however a significant shortening of the relative duration of the transient following Epac activation (DMSO treated cells 1.39 ± 0.15 and 8-pCPT-AM treated cells 0.98 ± 0.08, n=5, 4 respectively; $p<0.05$; unpaired t-test; **Figure 5.6Ciii**).

During the experiments, it was noted that the initial upward deflection of the Ca^{2+} transient appeared slower following 8-pCPT-AM application. Thus, the early period of $[Ca^{2+}]_i$ increase in response to caffeine was more closely examined. The initial rate of Ca^{2+} rise (from baseline to a $[Ca^{2+}]_i$ of 100 nM) was virtually indistinguishable before and after DMSO application, despite the latter Ca^{2+} transients being smaller in amplitude (**Figure 5.6Bi**). The initial Ca^{2+} rise was however considerably slower after application of 8-pCPT-AM (5 μM; **Figure 5.6Bii**). The relative initial Ca^{2+} rise was 1.17 ± 0.07 and 2.00 ± 0.41 for DMSO and 8-pCPT-AM treated cells respectively (n=4, 5; $p<0.05$, unpaired t-test; **Figure 5.6Ci**). These results suggest that Epac activation has no significant effect upon store content, although may affect the kinetic behaviour of the RyR release channels.

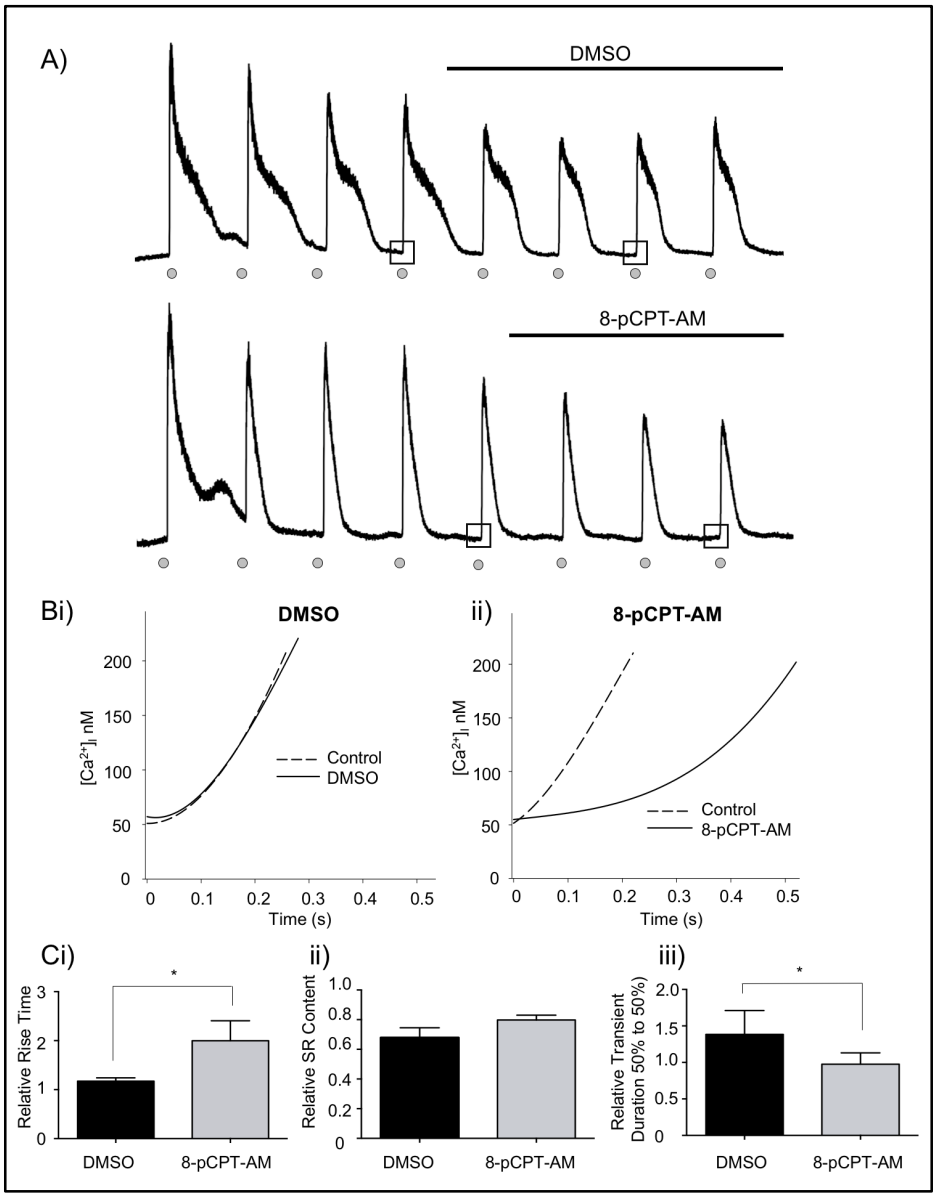


Figure 5.6– Epac activation modulates Ca^{2+} release kinetics, however does not affect Ca^{2+} store content. A) Representative traces of caffeine-induced Ca^{2+} release transients. 5mM caffeine was transiently applied to rat mesenteric artery smooth muscle cells to evoke Ca^{2+} release **B)** Initial Ca^{2+} rise 200nM Ca^{2+} was fit using a third-order polynomial equation. The rise of Ca^{2+} in the last transient before DMSO or 8-pCPT-AM was fit (control), compared to the rise of Ca^{2+} in the third transient of drug application (DMSO or 8-pCPT-AM). **C)** Summary of Epac activation on **i)** initial Ca^{2+} rise, **ii)** store content (transient amplitude) and **iii)** transient duration. Initial Ca^{2+} rise and transient duration were both significantly different, DMSO vs 8-pCPT-AM (n=5,4). Experiments were undertaken by Dr. Tomoko Kamishima.

5.3.5 Epac activation does not directly activate BK_{Ca} channels

In neurones, CaMKII phosphorylation of BK_{Ca} channels has been shown to increase their open probability by shifting their voltage dependence towards more hyperpolarized membrane potentials (van Welie & du Lac 2011). We therefore

investigated whether a component of the 8-pCPT-AM effect on STOC activity could originate from direct effects on BK_{Ca} channels. In whole-cell recordings from isolated mesenteric artery myocytes with ryanodine (15μM) and Ca^{2+} (300nM) included in the pipette-filling solution, application of 8-pCPT-AM (5μM) failed to increase BK_{Ca} channel activity (**Figure 5.7**; n=3). Under these conditions Ca^{2+} release via RyRs will be blocked, but BK_{Ca} channels will still be available for activation. This is demonstrated by the subsequent addition of the selective BK_{Ca} channel activator NS11021 (10 μM), which induced a large outward current (**Figure 5.7**).

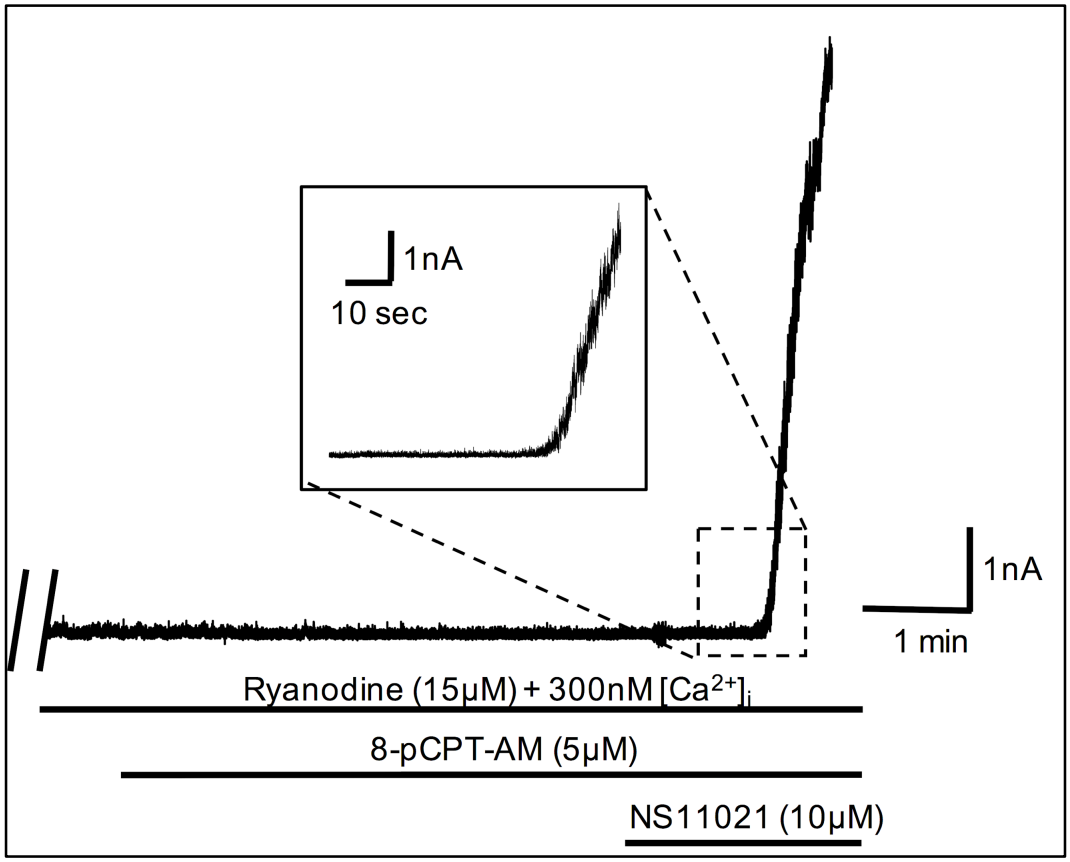


Figure 5.7– Epac activation does not directly activate BK_{Ca} channels. Freshly isolated rat mesenteric artery smooth muscles in the whole cell configuration were perfused with 8-pCPT-AM. Intracellular solution contained Ryanodine (15μM) and 300nM Ca^{2+}. To confirm BK_{Ca} channel expression the opener/Ca^{2+} sensitizer NS11021 was applied.

Using an antibody against phospho-serine/phospho-threonine residues to immunoprecipitate phosphorylated proteins, followed by immunoblotting for BK_{Ca} channels, we aimed to assess the level of BK_{Ca} channel phosphorylation following Epac activation. BK_{Ca} phosphorylation upon Epac activation were varied and showed no consistent trend as seen in **Figure 5.8.**

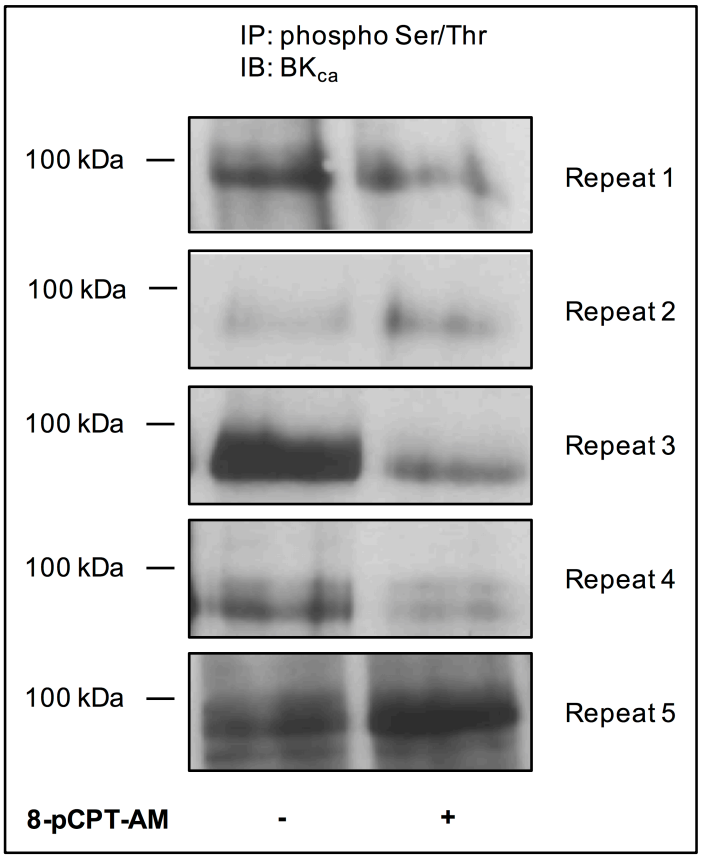


Figure 5.8- Phosphorylation of BK_{Ca} channels following Epac activation. Shown are five individual immunoblots using anti-BK_{Ca} antibodies. Immunoprecipitations were undertaken using phospho Ser/Thr antibodies on freshly lysed rat mesenteric arteries pre-treated in DMSO or 8-pCPT-AM.

5.4 Discussion

To increase STOC activity, Epac may affect Ca^{2+} sparks by directly modulating RyR behaviour, or indirectly by increasing the content of intracellular Ca^{2+} stores. The sarcoplasmic reticulum (SR) in smooth muscle cells contains a large amount of Ca^{2+}, predominantly bound to Ca^{2+} binding proteins such as calreticulin (Akata

2007a). This Ca^{2+} store is involved in IP_3-mediated Ca^{2+} release producing contraction and relaxation through Ca^{2+} spark production, and therefore is pivotal to the generation of force in smooth muscle, and with it vascular tone (Nelson et al. 1995; Khalil 2010). Epac activation significantly slowed the initial global rise in $[Ca^{2+}]_i$ in response to caffeine, but had no effect on the relative transient amplitude. This suggests that Epac activation does not affect store content, but may affect kinetics of the RyR response to caffeine.

A possible explanation for the slowing of the initial global rise in $[Ca^{2+}]_i$ in response to caffeine, could be that the rapid and synchronized opening of RyRs is compromised by Epac activation. CaMKII likely induces changes in RyR activity through phosphorylation of the release channel itself or associated regulatory proteins (Van Petegem 2012). RyR1, 2 and 3 have been reported to be expressed in vascular smooth muscle, with RyR1 and 2 shown to be modulated by post-translational modifications, such as phosphorylation. In cardiomyocytes this phosphorylation via PKA or CaMKII has been associated with an increase in RyR open probability (Li et al. 1997; Marx et al. 2000). Therefore, we assessed whether similar post-translational modifications took place in the vasculature upon Epac activation. We could not assess RyR phosphorylation due to technical limitations, where RyR was not detected in arterial lysates using immunoblot. Every attempt was made to detect RyR, including testing whole tissue lysates, SR fractionation and RyR immunoprecipitations, all of which were confirmed to be technically acceptable in heart tissue, used as a positive control. Immunoblots and immunoprecipitations were performed with two antibodies to exhaust all avenues of detection. #2160 RyR (pan) kindly gifted by Prof. Tony Lai reacts with all RyR isoforms, while 34C

preferentially reacts with RyR2, therefore both were tested to not miss RyR1/3 expression. CaMKII may also directly or indirectly modify the behaviour of regulatory proteins associated with RyR, such as FK-506 binding protein (FKBP). Phosphorylation at ser2808/9 on RyR causes dissocation of the RyR/FKBP complex (Marx et al. 2000). FKBP functions to synchronize the activity of RyRs by coupling the opening and closure of neighbouring channels (Marx et al. 1998). Loss of the ability to simultaneously open in response to caffeine would account for our Epac-mediated slowing of the global $[Ca^{2+}]_i$ rise.

While our data suggest that the Ca^{2+} store content is not affected by Epac activation, we cannot rule out an increase in uptake that balances the increased store leak (spark activity). An analogy for this would be that if the bucket empties as quickly as it fills, the bucket is always full to the same level. Indeed, we show that in the presence of 8-pCPT-AM caffeine-induced Ca^{2+} transients are shorter in duration, which would be consistent with an increase in released Ca^{2+} clearance rates from the cytosol. Within vascular smooth muscle cells and cardiomyocytes there are numerous Ca^{2+} reuptake and extrusion mechanisms (plasma Na^+/Ca^{2+} exchanger, SERCA, PMCA and mitochondrial Ca^{2+} reuptake) (Kamishima & McCarron 1998). *Kamishima and McCarron* (1998) showed, using various inhibitors (SERCA inhibitors and mitochondrial ionophores) and different buffer compositions (removal of Na^+), that the Na^+/Ca^{2+} exchanger is not responsible for the decay of Ca^{2+} transients at any time point in cerebral myocytes (Kamishima & McCarron 1998; Kamishima et al. 2000). While we could not reliably detect phosphorylation of the SERCA pump regulator PLB at Thr^{17}, a key CaMKII target in the heart, we did see that 8-pCPT-AM-mediated changes in both PLB mobility within gels and susceptibility to detergent extraction from

membranes, which may indicate protein modification such as phosphorylation following Epac activation (Bidlack et al. 1982). Together, these results suggest that possible modification of PLB changes Ca^{2+} reuptake in to stores and decreases the duration of the caffeine-induced Ca^{2+} transient.

CaMKII activation has been shown to increase the probability of BK_{Ca} channel opening, as has the application of the adenylyl cyclase activator, forskolin, to arterial myocytes (Porter et al. 1998). In contrast, dephosphorylation of BK_{Ca} channels has been shown in pituitary tumour cells to increase their open probability (White et al. 1991). In whole-cell voltage-clamp experiments, we inhibited Ca^{2+} sparks by including ryanodine in the pipette-filling solution to clamp the Ca^{2+} present at the BK_{Ca} channel. The pipette-filling solution contained 300 nM free Ca^{2+} to allow activation of BK_{Ca} channels. Under these conditions, we saw no significant BK_{Ca} channel activation by 8-pCPT-AM, even though BK_{Ca} channels was still susceptible to activation by NS11021. This is in-line with BK_{Ca} Ca^{2+} sensitivity not being altered, as STOC length was no longer in the presence of 8-pCPT-AM. A longer STOC would be expected if BK_{Ca} channels activated faster to Ca^{2+} and deactivated more slowly. Biochemical analysis of BK_{Ca} channel phosphorylation provided variable results, with both increases and decreases in total phosphorylation seen following Epac activation. Importantly, our functional data suggest that these variable Epac-induced changes in phosphorylation, if real, do not translate directly in to changes in channel activity. Overall these results are in line with those in **Chapter 3** where Epac-mediated STOCs were inhibited by ryanodine. This suggests that STOC activity results from changes in the underlying RyR activity, as opposed to direct changes in BK_{Ca} behaviour.

Acknowledgement: Within this chapter all caffeine evoked transient experiments were undertaken by Dr. Tomoko Kamishima using cells isolated by myself. All other work was undertaken by myself.

6 Insights into Epac-based signalosomes

6.1 Introduction

With cAMP being a common second messenger for several vasoactive transmitters the question quite often posed is, why do not all cAMP elevating coupled receptors ($G_{\alpha s}$) produce the same physiological response? This was first shown in cardiomyocytes, where activation of β-adrenergic receptors by isoprenaline produced cAMP increases and PKA activation to a similar level as prostaglandin, acting on PGE1 receptors. However, only isoprenaline produced inotropic effects in myocytes (Lefkimmiatis & Zaccolo 2014). This phenomenon was shown in whole tissue by Hayes *et al.* (1980) and in cell preparations by *Buxton & Brunton* (1983) (Hayes et al. 1980; Buxton & Brunton 1983). However, nearly 20 years went by before protein-based probes such as ICUE1 (indicator for cAMP based on EPAC) and AKAR1 (a PKA activity biosensor) were developed and cAMP compartmentalisation was confirmed (DiPilato et al. 2004; Zhang et al. 2001). Studies with these proposed that signalling specificity is maintained by distinct pools of cAMP (controlled by barriers of phosphodiesterases) and/or compartmentalization of signalling molecules by protein scaffolds. *Zaccolo & Pozzan* (2002) used neonatal cardiomyocytes transfected with fluorescently-labelled catalytic and regulatory subunits of PKA (YFP-PKA catalytic and CFP-PKA RII). With these they showed that treatment

with IBMX, the broad-spectrum phosphodiesterase inhibitor, or Ht-31, a AKAP-PKA RII disruptor peptide, produced a generalized spread of cAMP throughout the cell i.e abolishing discrete microdomains with high concentration of cAMP (Zaccolo & Pozzan 2002).

6.1.1 AKAPs

A-Kinase anchoring proteins (AKAPs) are a large family (~50 members) of scaffolding proteins expressed in various tissues/cells with differing intracellular localisations (Beene & Scott 2007; Esseltine & Scott 2013; Scott & Santana 2010). AKAPs act as scaffolding proteins that control the localization of PKA and other signalling proteins within the cell. These signalling complexes often contain the cAMP-destroying PDE enzymes, thus producing highly-defined cAMP gradients around the signalosome **(Appendix. 1, Section 8.1)**. AKAPs are structurally diverse, but have in common a 14-18 residue amphipathic helix that predominately binds RII domains via a hydrophobic groove produced by the two anti-parallel PKA RII subunits (Welch et al. 2010). RI selective AKAPs and AKAPs that interact with both RI and RII have also been discovered, such as sphingosine kinase interacting protein (SKIP) and AKAP1 (Eggers et al. 2009; Means et al. 2011). Further analysis of AKAP specificity using various cAMP analogue-bound agarose beads showed that AKAP9,11,7,5,14 and MAP2 were RII specific, AKAP3,4,12,2,1 and 10 were dual specific in that they bound both RI and RII (Aye et al. 2009).

6.1.2 Epac: Involvement in signalosomes

Epac has been explored in a number of signalosomes, predominately in relation to the migration of endothelial cells (Sehrawat et al. 2011; Rampersad et al. 2010;

Wilson et al. 2011). Importantly, most of this work was conducted in cell lines and very little research on *ex-vivo* tissue has been undertaken, possibly due to the numerous cell types present with tissues and limitations on tissue/protein amount. Indeed, immunoprecipitations generally require highly concentrated protein lysates. A number of Epac signalosomes are shown in **Table 6.1**. Epac complexes shown in HEK293 cells only are not shown in the table.

Table 6.1- Epac involving Signalosomes

Proteins Involved	Proposed Function	Cell line	Citation
Epac1/PDE3B/p84-p110y	Inhibit HAEC migration leading to angiogenesis	Human Aortic endothelial cell	(Wilson et al. 2011)
Epac1/PDE4D/β-cateninin/PKARII/ PKAC/VECAD/Rap1	Vascular permeability	Human arterial endothelial cells	(Rampersad et al. 2010)
Epac1/PKA/mAKAP/ PDE4D3/ERK5	Hypertrophy	Ventricular myocytes/heart tissue	(Dodge et al. 2001)
Epac1/Importin β1	Neuronal outgrowth	N2A cells	(Baameur et al. 2016)
Epac1/ HSP70 or HSP71 or HSP90	N/A (Mass Spec Hits)	N/A	(Baameur et al. 2016)
Epac1/Ran	Nucleus Rap1 activation	HEK293	(Liu et al. 2010)
Epac1/GRK2	Pain (hyperalgesia)	Neuronal cells	(Singhmar et al. 2016; Singhmar et al. 2014)
Epac1/β-arrestin/CamK	Beta adrenergic signalling	Heart/HEK293	(Mangmool et al. 2010)
Epac1/AKAP9	Microvascular Permeability	HUVECs	(Sehrawat et al. 2011)
Epac1/2 with SUR1/2	Insulin/Vascular	Pancreatic B-cells Vascular smooth muscle	(Purves et al. 2009; Kang et al. 2006)
Epac1/ERM	Signalling localisation	ACHN Cells	(Gloerich et al. 2010)

Importantly, to our knowledge there is no research presenting Epac-containing signalosomes in vascular smooth muscle.

6.1.3 Signalosomes in smooth muscle

In smooth muscle, AKAP5, also known as AKAP79 (human) and AKAP150 (rodent), is the most researched. AKAP150 has been shown to be involved in the formation of Ca^{2+} sparklets. Sparklets in vascular smooth muscle cells, which are local [Ca^{2+}] increases, are mediated through voltage-dependent Ca^{2+} channels (Cav1.2) or TRPV4 ion channels, with Cav1.2 sparklets smaller in amplitude than TRPV4 but possessing a higher frequency. This higher frequency means Cav1.2 sparklets control global Ca^{2+} within the cell, whereas TRPV4 sparklets produce localised increases (Mercado et al. 2014). When not colocalised with PKCα sparklets through Cav1.2 channels are in “low-activity” mode, where rare stochastic openings of channels takes place. When colocalised with PKCα Ca^{2+} sparklets are in “high activity” mode, through sustained channel opening. This sustained activation places Ca^{2+} sparklets as a major Ca^{2+} entry route into cells when PKCα is activated (Navedo et al. 2008; Navedo et al. 2006). Deletion of AKAP150 disrupts PKC recruitment to Cav1.2 channels, resulting in a lower I_{ca} in AKAP150 $^{-/-}$ myocytes (Navedo et al. 2008). Pressurised artery experiments showed smooth muscle cells in AKAP150 $^{-/-}$ vessels have a lower $[Ca^{2+}]_i$ and tone compared to WT, along with a decreased response to the vasoconstrictor, angiotensin II (Navedo et al. 2008; Navedo & Amberg 2013).

To add another level of complexity to Ca^{2+} handling and the regulation of contraction in smooth muscle, AKAP150 has been shown to be involved in both. Endothelium denuded cerebral arteries from TRPV4$^{-/-}$ KO mice are more

sensitive than WT to angiotensin II induced contraction (Mercado et al. 2014). This relaxant pathway through TRPV4 could merge with earlier work by Earley *et al.* 2005 , who showed that TRPV4/BK_{ca} form a novel signalling complex capable of causing hyperpolarisation. TRPV4-mediated relaxation by epoxyeicosatrienoic acids (EETs) was 50% dependent on BK_{ca} channel activation in smooth muscle cells and 50% dependent on endothelium cells (Earley et al. 2005; Earley et al. 2009).

Further studies to explore the endothelium dependent relaxation showed a small number of TRPV4 channels producing Ca^{2+} sparklets were capable of activating IK_{Ca} and SK_{Ca} channels, to produce maximal vasodilation of small mouse mesenteric arteries (Sonkusare et al. 2012). This activation of TRPV4 by acetylcholine was dependent on AKAP150, where it localises PKC to myoendothelial projections (MEPs) containing IK_{Ca}/TRPV4/AKAP150/PKC.

In rat arterial smooth muscle cells, AKAP involvement in the regulation of K_{ATP} currents has been explored. Here Ht-31, the AKAP-PKA disruptor reduced cAMP-mediated K_{ATP} activation, along with calcitonin-gene related peptide mediated K_{ATP} activation (Y Hayabuchi, Dart, et al. 2001). Together these experiments show a major role for AKAP150 in vascular smooth muscle and endothelial cells, with rather contradictory roles (i.e. contraction vs. relaxation) in Cav1.2 vs TRPV4-mediated sparklets, respectively.

Therefore, exploring this tight spatial regulation is of increasing importance to understanding cAMP-mediated vascular smooth muscle signalling; even more so with the recent work showing PKA catalytic subunit remains localised even after activation (Smith et al. 2017).

6.2 Aim

The aim of this part of the study was to optimise conditions that would allow us:

1) To assess the potential complex partners of Epac1 in the vasculature via immunoprecipitation
2) To explore possible AKAP expression in the vasculature and its involvement in Epac-mediated signalling
3) To investigate the recruitment of proteins to Epac1-mediated complexes when Epac1 is active.

6.3 Results

6.3.1 Testing efficiency of Epac1 antibodies for immunoprecipitation

Optimisation of antibodies and lysis conditions for immunoprecipitation of Epac (and potential complex partners) was initially undertaken by transiently transfecting CHO cells with Epac. The Epac expression construct used was H21, a first-generation biosensor where full-length Epac is tagged with fluorescent proteins (CFP – Epac1 –YFP). A clear band at ~150 kDa, the expected size of H21 can be seen when using anti-Epac1 (EPR6172; Abcam) compared to the control lanes containing Epac1 Ab (Abcam) with no lysate or Epac1 Ab (Abcam) only (hashed box). Using anti-Epac1 (SCBT) produced no clear band with silver staining. Immunoblotting for Epac with anti-Epac1 (CST) showed strong efficient precipitation of H21 by anti-Epac1 (Abcam) (**Figure 6.1**).

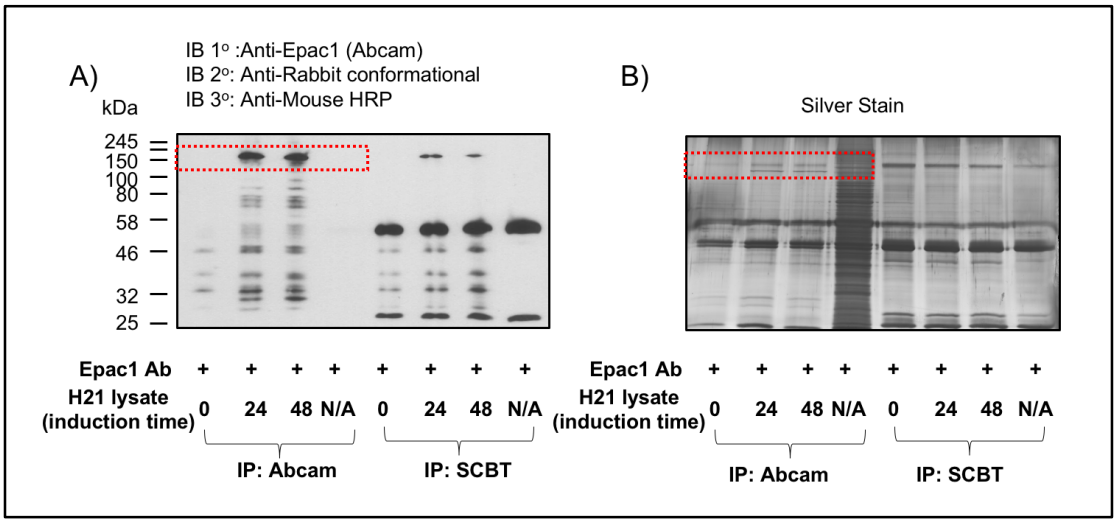


Figure 6.1- Validation of Epac immunoprecipitation using H21 transiently expressed in CHO Cells. A) Immunoblot using an Anti-Epac (CST) antibody on immunoprecipitations from H21 expressing CHO cell lysates. Immunoprecipitations were undertaken using either Abcam or SCBT anti-Epac1 antibodies and seperated by SDS-PAGE. H21 expressing cells were lysed at 0,24 and 48 hours. B) The same lysates as in A were run on a SDS-PAGE gel followed by silver staining.

6.3.2 Optimising Epac1 immunoprecipitations in arterial homogenates

Having established possible antibody combinations we undertook pilot experiments in rat brain tissue, due to abundant material, to optimise endogenous Epac1 immunoprecipitation. Due to initial lysates producing lower than expected levels of Epac pulldown, we undertook a buffer screen to improve efficiency of protein extraction (**Figure 6.2**).

RIPA Buffer (in mM): 10 Tris pH 7.4, 150 NaCl, 0.1% SDS, 1% Triton X-100, 1% Sodium Deoxycholate, 5 EDTA

Triton Based (in mM) : 150 NaCl pH7.4, 50 Tris, 1% Triton X-100, 1 EDTA

NP-40 Based (in mM): 50 Tris pH7.4, 100 NaCl, 1% NP-40, 10% Glycerol

Gloerich (Gloerich et al. 2010) **(in mM):** 20 Tris pH7.4, 1% Triton X-100, 0.5% Sodium Deoxycholate, 10 EDTA, 150 NaCl

NOTE: All buffers contained protease inhibitors (Sigma) at a 1 in 100 dilution (final concentration one times).

In rat brain tissue RIPA buffer produced the highest amount of Epac1 immunoprecipitation.

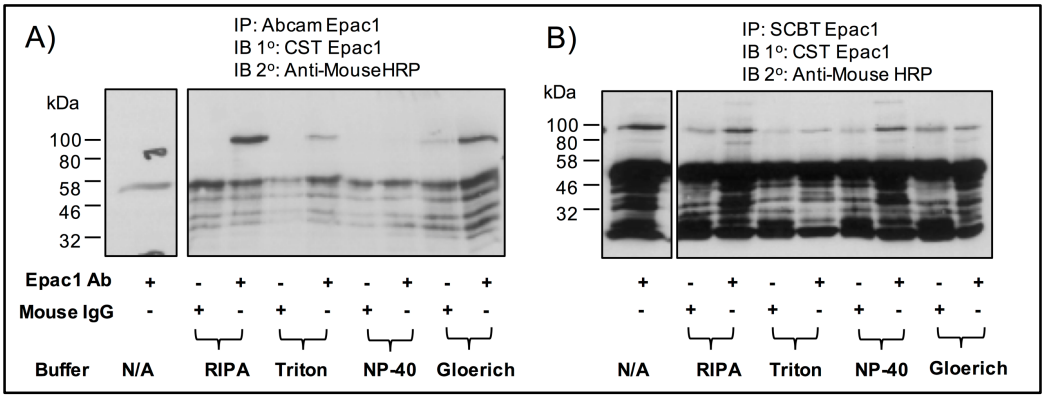


Figure 6.2- Optimisation of Immuoprecipitation buffer. A) Brain lysate (25mg of tissue) was homogenized in various buffers due to poor immunoprecipitation using CHO buffer in tissue. Epac1 was immunoprecipitated by Anti-Epac1 (Abcam) and immunoblotted with Anti-Epac1 (CST). Rabbit IgG at 1ug (same as Anti-Epac1) was included to establish specificity. **B)** As A except using SCBT Epac to immunoprecipitate Epac1.

6.3.3 Considerations: Dynamic recruitment to a signalosome

Epac is a dynamic signalling molecule that undergoes possible translocation to the membrane to activate Rap1 (Ponsioen et al. 2009). To counter the possibility that Epac1 may only being present in a signalosome when under activation we utilised 8-AHA-2'-Me-O-cAMP agarose beads. This allows pull-downs of activated Epac since 8-AHA-2'-Me-O-cAMP (8-AHA) is an Epac agonist similar to 8-pCPT. In brain homogenates, used for optimisation due to high protein content and known Epac1 expression, the Triton X-100 based buffer produced the strongest Epac1 band as detected by immunoblotting. Harsher buffers such as RIPA (used for IP) were not used, due to possible denaturation of Epac under such conditions leading to a lack of Epac activation by 8-AHA-cAMP beads. EtOH beads were used as negative controls with them possessing no bound cAMP analogue. In aortic homogenates CaMKII was pulled down by 8-AHA-cAMP

beads, however confirmation of Epac1 presence in agarose pull down was not possible **(Figure 6.3)**.

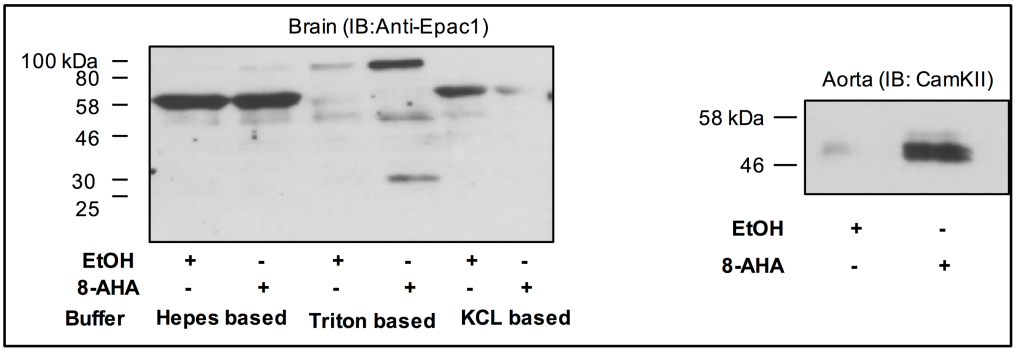


Figure 6.3- Optimisation of Active Epac Pull Downs Using 8-AHA-2´-O-Me-cAMP agarose beads. Aortic or Brain homogenates in appropriate lysis buffer were incubated with 8-AHA-2'-O-Me-cAMP agarose beads to pull down active Epac, which may be recruited to complexes. Pellets (pulldown) were eluted and immunoblotted with anti-Epac1 (CST) or anti-CaMKII.

In a different technical approach to explore active Epac1 involving signalosomes we utilised post activation cross-linking experiments. After addition of 8-pCPT-AM for 5 minutes, tissues were placed in 1% paraformaldehyde (in PBS) to fix protein to protein interactions. Epac1 was shown to immunoprecipitate from mesenteric artery smooth muscle cells using anti-Epac1 (Abcam) (**Figure 6.4**).

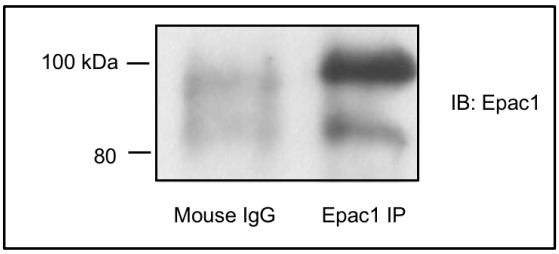


Figure 6.4- Epac1 Immunoprecipitation after PFA (1%) fixation. Mesenteric arteries were fixed post 8-pCPT-AM application followed by RIPA buffer lysis and IP with anti-Epac1 or Mouse IgG. Pellets were immunoblotted with anti-Epac1.

6.3.4 Possible Involvement of AKAPs in Epac1 containing Signalosomes

cAMP/PKA/Epac signalling involves a high degree of localisation, with much of cAMP localisation involving A kinase anchoring proteins (AKAPs). To assess AKAP expression we undertook a far western, also called PKA RII overlay (Hausken et al. 1998). Recombinant PKA RII was expressed in *E.coli* followed by purification using nickel agarose beads (**Figure 6.5A)**. Tissue lysates were run as per a normal immunoblot and transferred to nitrocellulose, followed by blocking. AKAPs present in lysates, specifically their amphipathic helix, are resistant to denaturation and therefore maintain the ability to interact with PKA RI/II (Hausken et al. 1998). Recombinant PKA RII was then incubated with the membrane overnight, followed by washing. Anti-PKA RII (Abcam) was then incubated at room temperature for two hours, followed by standard secondary antibody protocols. Heart SR fraction produced clear bands at both the predicted size of PKA, as expected, but also a clear band at ~18 kDa possibly indicating evidence of AKAP7, known to be expressed in cardiac SR membranes. However, recognition of AKAPs based on size cannot be used as a true evaluation, due to AKAP7γ (AKAP18γ) being approx. 40 kDa (Lygren et al. 2007). A positive control in the form of AKAP79 (truncated mutant) which binds to PKA RII was used to show an interaction between AKAP and PKA RII. A strong band (at the AKAP truncant MW) can be seen, corresponding to clear evidence that our recombinant PKA RII binds to AKAPs via this far-western method (**Figure 6.5B)**. Aortic lysates produced numerous potential bands at 60,100,150 kDa (**Figure 6.5B/C)**.

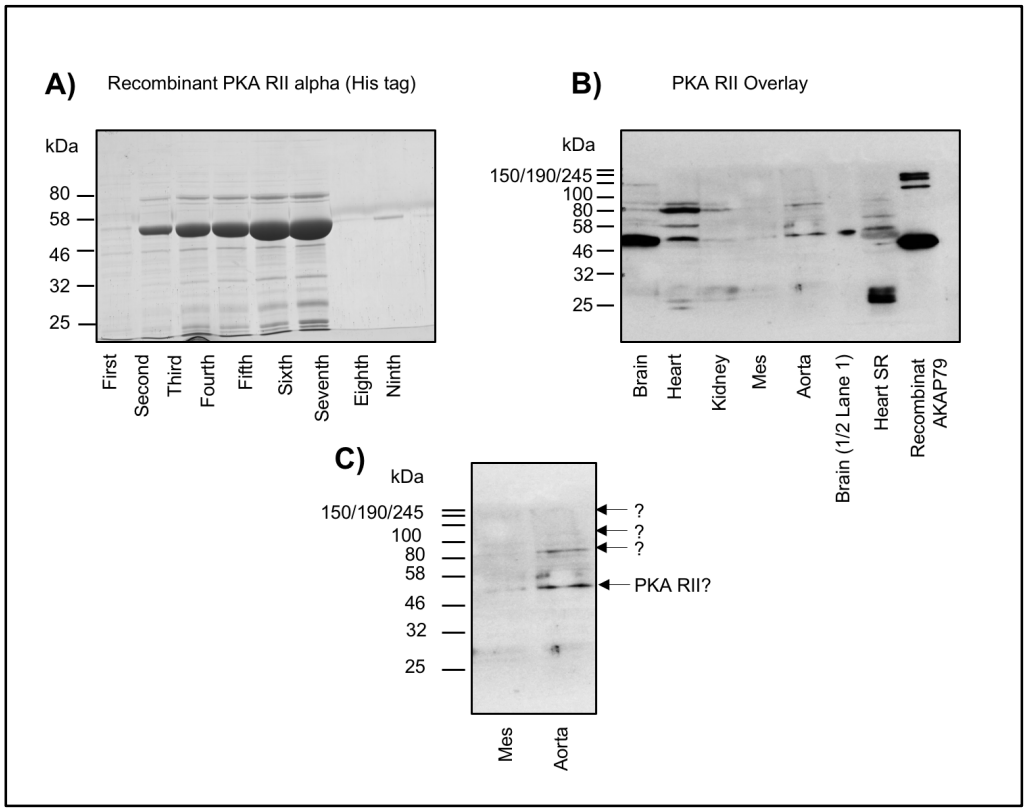


Figure 6.5- Optimisation of PKA Reg II far western. A) Recombinant PKA RII was expressed in *E.coli* followed by lysis and purification. Coomassie staining showed overexpressed of a 50kDa protein as expected for PKA RII **B)** Brain, Heart and recombinant AKAP79 show clear strong staining, whereas vascular lysates appear fainter. Due to possible overloading Lane 6 was ½ the amount loaded in Lane 1 **C)** Inset from B) Mesenteric lysate produced no clear bands, however faint similar banding to Aortic lysate can be seen. Banding in the Aorta lane is at 50 – 100 kDa, with a faint band at 150 kDa.

6.4 Discussion

Endogenous Epac1 has proved incredibly difficult to immunoprecipitate in a number of studies by other authors, either due to the availability of suitable antibodies, problems with extracting Epac from cells and/or the low copy number of Epac proteins (Gupta & Yarwood 2005; Zhao 2006; Gloerich et al. 2010). We show here that, after optimisation, endogenous Epac1 can be immunoprecipitated from arterial lysates. However, the conditions required for immunoprecipitation are harsh and could possibly cause dissociation of transient complexes, while they were also snapshots of complexes at varying Epac activation states, due to currently undetermined levels of Epac activity under rest

in specific arteries (Vasilescu et al. 2004). The lack of known complex partners for Epac1 in the vascular smooth muscle makes it difficult to confirm that complexes are difficult. Potential complex partners, as identified from the signalling pathway elucidated in previous chapters of this study, are CaMKII, phospholamban, IP_3R, PLCε and RyR. Using these optimised (i.e. using RIPA buffer) conditions, we could not identify phospholamban or RyR in Epac-precipitated lysates (data not shown). Interestingly, immunoprecipitation with RyR2 antibodies (validated in **Chapter 5**) produced a faint doublet at 100 kDa using anti-Epac1 (CST) for immunoblotting, potentially consistent with the co-precipitation of Epac (**Appendix 2**). Detection of CaMKII via IP is difficult due to CaMKII being similar weight to IgG heavy chain. Also, only active CaMKII (autophosphorylated) may be recruited to a signalosome requiring prolonged IP of phosphorylated CaMKII.

Epac translocates from the cytoplasm to the plasma membrane under activation and therefore may only form functional complexes when active (Consonni et al. 2012). To assess this we utilised two methods. Firstly, we utilised 8-AHA-2'-O-Me-cAMP agarose beads, allowing pull-down of active Epac1 in complexes. However, 8-AHA-2'-O-Me-cAMP also binds PKARI, therefore PKARI is pulled-down, potentially limiting the use of this technique for investigating exclusively Epac-based signalosomes (Aye et al. 2009).

We recently explored the possible use of paraformaldehyde as an *in-vivo* crosslinker of protein-protein interactions, prior to standard immunoprecipitation. The use of a crosslinker allows "freezing" or fixation of potential dynamic and transient complexes, both of which we expect such signalling cascades to use. This method produces a snapshot of the intact complex, allowing possible

downstream identification of complex partners using immunoblot and mass spectrometry. The possible modification of paraformaldehyde is of concern for mass spectrometry analysis, due to the addition of a 12 Da modifications, however it has been shown that reversal of crosslinking by SDS-PAGE sample buffer is adequate to remove this (Vasilescu et al. 2004; Toews et al. 2008).

AKAPs are commonly involved in cAMP signalling and we provide some evidence of AKAP expression in arterial beds. Previous studies on AKAPs in a number of tissue/cell preparations have been shown (**Table 6.1).** Validation of the exact products are required, however MW can sometimes be used as a possible indicator. In aortic tissue there appears to be bands at 80 kDa, 150 kDa and numerous between 45 and 60 kDa, potentially corresponding to AKAP100, AKAP150 and PKA RII (due to its expression in lysates and the use of anti-PKA RII antibody for detection). Certain AKAPs, such as AKAP100 and AKAP18, have been shown to have sarcoplasmic reticulum localisation (Lygren et al. 2007; McCartney et al. 1995). Further analysis utilising the PKARII/AKAP disruptor peptide HT-31 and its inactive analogue HT-31P, which contains two proline substitutions are needed, as is further AKAP identity validation (Carr et al. 1991).

7 General Discussion

7.1 Overview

A common feature of vasorelaxant pathways is the use of the second messenger cAMP to convey the relaxant signal from cell-surface receptors to downstream effectors. In the vasculature, cAMP has three known effector proteins: PKA, CNGCs and Epac (Morgado et al. 2012). Over the years, the relaxant effects of cAMP have been largely attributed to the activation of PKA and phosphorylation of downstream targets (de Rooij et al. 1998; Gloerich & Bos 2010). More recently, reports show additional roles for Epac as a potent vasodilator and suggest an important vasorelaxant pathway that is distinct from the more traditional cyclic AMP-PKA axis (Stott et al. 2016; Zieba et al. 2011; Roberts et al. 2013). Previous work from our lab has shown that Epac relaxes vascular smooth muscle by activating STOCs that hyperpolarize the cell membrane and reduce voltage-dependent Ca^{2+} influx (Roberts et al. 2013). How Epac activates STOCs is unknown and the central aim of this thesis was to define the signalling pathway by which Epac modulates STOC activity.

Key novel findings discussed in this thesis include (Figure 7.1):

(i) Epac increased STOC frequency and amplitude in rat mesenteric artery smooth muscle cells in a manner dependent upon the activation of CaMKII (**Chapter 3**);

(ii) Epac activation preferentially induced auto-phosphorylation of specific CaMKII γ isoform/s (**Chapter 3**);

(iii) Epac-induced CaMKII activation was likely initiated by IP_3-mobilized Ca^{2+} (**Chapter 4**);

(iv) Epac activation had little effect on intracellular Ca^{2+} store content, but it affected caffeine-induced store release, possibly due to changes in the kinetic behaviour or synchronous opening of RyRs (**Chapter 5**).

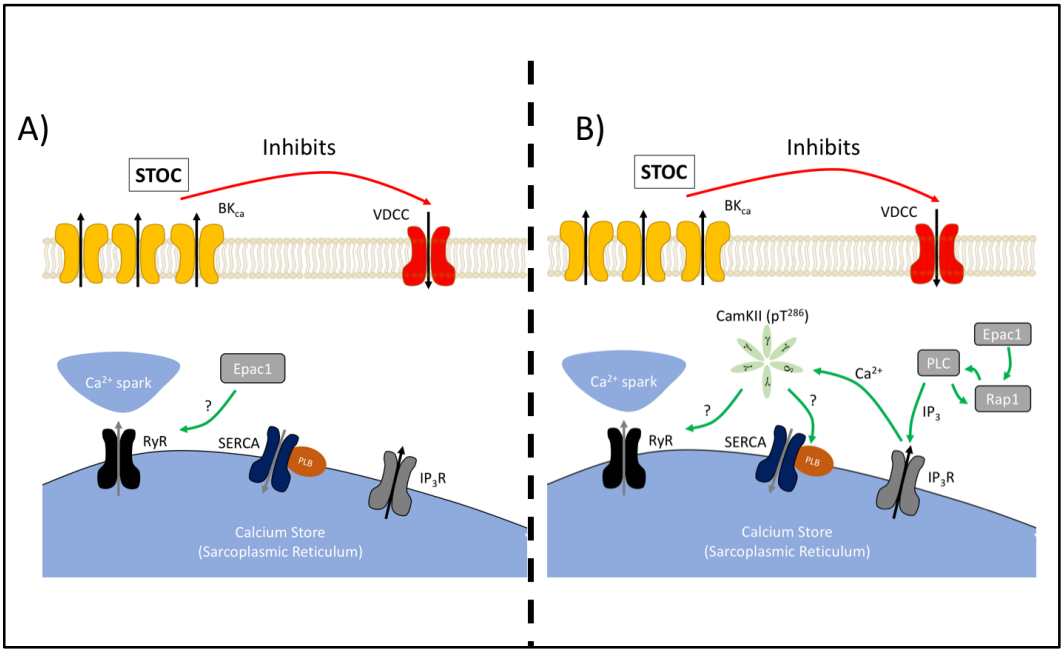


Figure 7.1- Summary of the mechanism by which Epac activates STOCs in RMASMCs: A) Shown is the mechanism proposed by Roberts *et al*. (Roberts et al. 2013), in which Epac1 produces an increase in Ca^{2+} sparks and STOCs. **B)** Proposed mechanism by which Epac induces an increase in STOCs including data shown in this thesis.

7.2 Physiological context & implications

Within cells the major second messenger cAMP produces a number of physiological effects. These cellular effects are produced by the complex integration of 9 adenylyl cyclases, producing cAMP that is tightly controlled by 11 cAMP destroying phosphodiesterases. This control produces cAMP hotspots within the cell, rather than the global cell-wide increase that would be seen due to a diffusion rate of 330 – 970 $\mu m^2/s$ (Agarwal et al. 2016). This compartmentalized cAMP then activates potentially four different effectors, each giving rise to varied downstream signalling pathways (Maurice et al. 2014). This

highly complex signalling mechanism allows extracellular messages to give rise to different and highly reproducible physiological events. Within vascular smooth muscle, cAMP regulates 3 cAMP effectors, PKA, Epac and CNGs. Differences between the *in vitro* cAMP concentration for activation of PKA and Epac (1μM vs 50μM) has led some to predict that Epac signalling may represent a negative feedback mechanism that comes into effect at high cAMP levels, while others have shown that PKA and Epac work synergistically (Christensen et al. 2003; Poppe et al. 2008; Purves et al. 2009; Hewer et al. 2011). There is also the possibility that PKA and Epac are present in differing cAMP microdomains within cells, each with different concentrations of cAMP and therefore different levels of Epac/PKA activation. Our pilot data presented in **Appendix 2 (Section 8.2)** suggests that Epac may indeed exist in multiprotein complexes that include RyRs. Future work will hopefully establish other partners and define whether these are distinct from PKA. Epac signalosomes proved difficult to assess. We showed that immunoprecipitation of Epac1 with RIPA lysis buffer produced the largest amount of immunoprecipitated Epac1, possibly due to the increased power to lyse membranes/SR microsomes where Epac1 is present, as seen using crude fractionation by centrifugation (data not shown). However, such harsh lysis buffers, including detergents such as SDS, may easily dissociate transient, dynamic complexes. Further optimisation of cross-linking experiments may be required to explore the highly spatially regulated cAMP signalosome.

The involvement of CaMKII in our pathways is also particularly interesting due to prior experiments showing CaMKII involvement in vascular smooth muscle contraction (Kim et al. 2000; Rokolya & Singer 2000). The ability for CaMKII to mediate both relaxation and contraction either means that this enzyme has

different functional roles in different vascular beds, or that distinct pools of CaMKII exist that are activated by different stimuli. CaMKII autonomous activity, whereby CaMKII activity is maintained in the absence of Ca^{2+}/CaM, can be produced through various translational modifications, such as oxidation, nitrosylation and O-GlcNAcylation (Toussaint et al. 2016; Erickson et al. 2015; Erickson et al. 2013). Most important physiologically, in terms of vasorelaxation, may be regulation of CaMKII by NO. NO is a common mediator of relaxation in the vasculature, with it mediating the classical endothelium-dependent relaxation method. In cardiomyocytes and *in vitro*, CaMKII has been shown to be activated and inhibited by NO. I hypothesise that CaMKII that is activated to induce relaxation in cells may be potentiated by NO, whereas the CaMKII involved in contraction may be inhibited by NO, due to no prior activation of CaMKII.

7.3 Limitations of the work

A limitation of this work is the use of cAMP analogues to specifically activate Epac. This allows identification of downstream Epac signalling, but does not allow identification of endogenous hormones utilising Epac-dependent pathways. Follow on studies to this project should assess STOC activation by endogenous vasodilators in the presence of selective PKA inhibition. This must not be limited to a single vasodilator, as physiological Epac-mediated STOC effects may be due to a specific pool of cAMP, activated by a specific receptor type.

While we do not show the physiological relevance of Epac, other studies have utilised either PKA inhibition or Epac inhibition in response to endogenous vasodilators to show Epac involvement. In different vascular beds, β-adrenergic and prostacyclin-induced elevation of cAMP changes the sensitivity of the

contractile proteins through Rap-initiated alteration of myosin light chain phosphorylation (Roscioni et al. 2011; Zieba et al. 2011; Sukhanova et al. 2006). Epac knockout has been shown to affect vascular permeability, both basally and upon treatment with atrial naturetic peptide (ANP), which increases permeability (Cullere et al. 2005; Kopperud et al. 2017; Birukova et al. 2008). Knockout studies have also shown Epac to be physiologically relevant within the heart, involved in β-adrenergic-mediated hypertrophy (Epac1) and modulation of Ca^{2+} spark /proarrhythmic events (Epac2) (Pereira et al. 2015; Pereira et al. 2013; Oestreich et al. 2009; Ruiz-Hurtado et al. 2013; Pereira et al. 2007).

All of the work presented in this thesis is undertaken in rat or mouse vascular tissue, and therefore further research of this mechanism in human tissue/cells to identify the potential as a therapeutic target is needed. To this end, we initially explored the possible use of human coronary artery smooth muscle cells. However, under normal cell culture conditions no STOCs were present, nor could they be induced by cell culture medium manipulation (such as serum deprivation) that increased contractile markers (Humphries 2013). This suggests that phenotypic and morphological changes associated with placing smooth muscle cells in culture disrupts the intricate intracellular architecture required to maintain spark/STOC coupling.

7.4 Therapeutic Possibilities

With Epac expression being shown in tissues such as the pancreas, heart and vasculature, global activation of Epac will most probably lead to off-target effects in the form of abnormal glucose regulation and sinus rhythm. Recently, targeting the interaction of Epac with complex partners (particularly PDEs) has been

utilised as an alternative strategy (Wilson et al. 2011; Maurice et al. 2014; Rampersad et al. 2010). As seen in many cAMP signalosomes there are commonly AKAPs, cAMP effectors and PDEs, which degrades the cAMP signal; targeting the activity of the PDE is seen as a therapeutic target to maintain or increase activation of a specific cAMP effector within the signalosome. Currently, two PDE-Epac interaction inhibitors have been shown to increase Epac activation, and with it increase endothelial tube formation and increase endothelial barrier integrity (Wilson et al. 2011; Rampersad et al. 2010). Further work is needed to assess the viability of Epac-PDE disruption *in vivo* and in pathophysiological situations, but this project holds real promise. These disruptors both rely on peptide-based disruption, which while adequate in preliminary basic biomedical research, may not translate to the clinic due to poor permeability and poor bioavailability (Maurice et al. 2014). Other cAMP signalosomes have been targeted involving the interaction of PKA to AKAPs, including non-peptide based small molecule disruptors (Christian et al. 2011).

7.5 Remaining Questions and Future work

Important remaining questions that lead on from this project are:

- Does post-translational modifications of CaMKII such as oxidation, s-nitrosylation or glycosylation affect Epac-mediated CaMKII activation?
- Within vascular smooth muscle is Epac within a dynamic signalling complex? If so, what are the protein complex partners?
- Does Epac activation take place under basal (myogenic tone) or under vasodilatory agonists? If so what are the roles in both the endothelial and smooth muscle in regard to each vasodilatory agonist?

- Is the modulation of Epac1 in vascular smooth muscle a potential therapeutic target for arterial diseases such as idiopathic hypertension or secondary hypertension?
- Finally, and perhaps most fundamentally, what is the physiological relationship between Epac and PKA?

This final point highlights the apparent complexity of cAMP signalling whereby two cAMP effectors produce opposing and synergistic effects in numerous cells, including vascular smooth muscle and endothelial cells. Understanding this interaction between the two and elucidating their roles may prove vital for treatment of arterial disease, and numerous other diseases involving cAMP signalling.

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8 Appendices

8.1 Appendix 1 – Smooth Muscle AKAPs

Table 8.1- Smooth Muscle AKAPs

Name	Alias	Localisation	Smooth muscle expression?	Dual AKAP	Action	Refs
AKAP1	AKAP149, AKAP121 (human) AKAP82 (Rat) D-AKAP1	ER Mitochondria	Yes (mRNA) (Horvat et al. 2012)	Yes	Binds PP1, Tubulin, AMY1, PPD1, mRNA, VEGFR, Cardiac hypoxic response by inhibiting dephosphorisation of DRP1. Knockdown produced cardiac hypertrophy	(Ma & Taylor 2008; Horvat et al. 2012; Kim et al. 2011)
AKAP10	D-AKAP2	Mitochondria	No	Yes	Endocytosis via Rab4 and Rab11.	(Eggers et al. 2009)
AKAP2	AKAP220	Cancers	Yes (Horvat et al. 2012)	Yes	Calcitonin mediated cancer cell tumourisity and metastatic ability. Involved in developmental processes	(Li et al. 2016)(Thakkar et al. 2016)
AKAP3	AKAP110	Testis Cancers	Yes (Horvat et al. 2012)	Yes	Expressed in normal testis only Biomarker for triple negative breast cancer/ovarian cancer. Sperm mobility through tyrosine phosphorylation of AKAP3.	(Esmaeili et al. 2015; Sharma et al. 2016; Xu & Qi 2014; Luconi 2005; Horvat et al. 2012)
AKAP4	AKAP82	Testis Cancers	No	No	Colon cancer cell line proliferation, migration, invasion & apoptosis. Biomarker for breast cancer Sperm motility	(Saini et al. 2013; Jagadish et al. 2015; Miki et al. 2002)

AKAP5	AKAP75 AKAP79 (humans) AKAP150 (rodents)	Neurones (dendritic compartments) Smooth muscle cells Cardiomyocytes	Yes (Horvat et al. 2012)	No	Neuronal electrophysiology causing behavioural changes. Interacts:PP2A,PSD95,AC5/6,LTCC,β-adrenergic receptors & Cav-3. β-adrenoceptor Ca^{2+} handling & ECC KO mice caused cardiac hypertrophy. AKAP150/TRPV4 involvement in endothelial dependent relaxation. Hyperglycaemic BK_{Ca} channel suppression via calcineruin. Form a complex with Cav1.2 (LTCC) and BK_{ca}. PKB/Akt regulation in neurones Diabetes/Glucose regulation by binding of calcineurin.	(Nichols et al. 2010; Weisenhaus et al. 2010; Li et al. 2014; Sonkusare et al. 2014; Nystoriak et al. 2013; Nijholt et al. 2008; Liu et al. 2004)
AKAP6	mAKAP AKAP100	Heart Skeletal Muscle Brain	No	No	Skeletal muscle differentiation and repair. Involved in cardiac hypertrophy via a Epac1,PKA, PDE4D3 containing complex. Involved in the hypoxic response mediated by HIF-1α	(Dodge-Kafka et al. 2005; Lee et al. 2015; Wong et al. 2008)

8.2 Appendix 2 – Possible interaction between RyR and Epac1

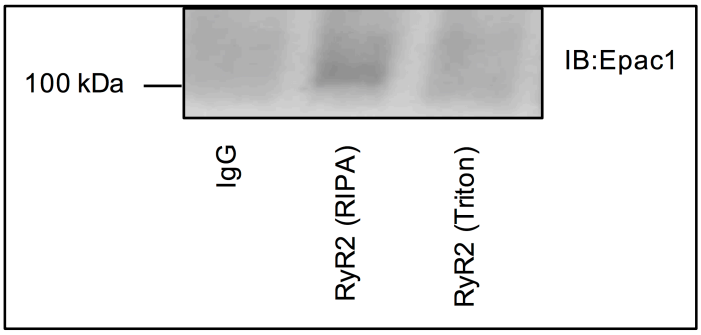

Figure 8.1- Epac1 possibly co-immunoprecipitates with RyR in mesenteric arteries. RyR immunoprecipitation using RyR (34C) was used as in cardiac tissue to immunoprecipitate RyR. Immunoblots using Epac1 (CST) showed a possible co-immunoprecipitation of RyR and Epac.